Examining the response of melanoma cells to immune activity and immunotherapy

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Declaration of originality

I hereby declare that the work presented in this thesis has not been submitted for a higher degree to any other university or institution. To the best of my knowledge this submission contains no material previously published or written by another person, except where due reference is stated otherwise. Any contribution made to the research by others is explicitly acknowledged.

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Abstract

Immunotherapy in the form of immune checkpoint inhibitors has significantly improved the survival of patients with advanced melanoma. However, immune checkpoint blockade is only effective in 15–40% of melanoma patients and failure to respond to immune checkpoint blockade may occur via several tumour intrinsic or extrinsic mechanisms, including overexpression of immune inhibitory ligands and receptors, alterations in antigen processing and presentation, and defects in the interferon gamma (IFNγ) signalling pathway.

In this PhD project, we first examined IFNγ responses in a large panel of immunotherapy-naïve melanoma cell lines with defined genetic drivers; BRAF-mutant, NRAS-mutant, BRAF/NRAS wild type cutaneous melanoma, and GNAQ/GNA11-mutant uveal melanomas (Chapter 2). We investigated the basal and IFNγ-induced expression of immune inhibitory ligands PD-L1 and PD-L2, antigen presenting molecules HLA-ABC and HLA-DR, and nerve growth factor receptor (NGFR) on the surface of these immunotherapy-naïve cell lines to determine the influence of melanoma driver oncogenes on IFNγ signalling. In Chapter 3, we compared tumour necrosis factor (TNFα) response in the same panel of cell lines. We found that melanoma response to IFNγ and TNFα are heterogeneous, and that IFNγ induced PD-L1, PD-L2, HLA-DR and HLA-ABC expression more potently, whereas TNFα preferentially up regulated NGFR expression. We further identified two well-recognised mechanisms of immunotherapy resistance, including the loss of β-2-microglobulin (β2M) and interferon gamma receptor 1 (IFNGR1) expression in these immunotherapy-naïve cells.

In Chapter 4, we extended our characterization of resistance mechanisms to a panel of 16 short-term melanoma cell lines (PD-1 PROG cell lines) derived from patients who progressed on anti-PD-1 or combination of anti-PD-1 and anti-CTLA-4 based immunotherapy. We assessed expression of IFNGR1, PD-L1, PD-L2, HLA-ABC, HLA-DR and B2M in these cells. We additionally analysed expression of transcription factors, melanoma pigment antigens and markers of de-differentiation, including SOX10, MLANA, AXL, MITF and NGFR. We identified several potential mechanisms of immunotherapy resistance in this panel of PD-1 PROG cell lines including loss of β2M expression, increased expression of immune inhibitory molecules, diminished response to IFNγ stimulation and melanoma de-differentiation.

Finally, in Chapter 5, we examined the regulation of PD-L1 and PD-L2 in the melanoma cell lines. Specifically, we assessed the temporal accumulation and stability of total and cell surface-specific expression of PD-L1 and PD-L2.

Our results demonstrate that melanoma responses to IFNγ or TNFα are heterogeneous, frequently downregulated in immunotherapy-naïve melanoma and potentially predictive of response to immunotherapy. Our results also confirmed that loss of antigen presentation molecules, upregulation of immune inhibitory checkpoints, loss of melanoma response to IFNγ and reduced expression of melanoma differentiation antigens, are associated with resistance to PD1-based immunotherapies. . Translation of these findings to the clinic may provide clinicians with the rational design and clinical development of personalized treatment strategies for each patient, which could result in better treatment response and increased long-term survival rates for patients with metastatic melanoma.

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List of Abbreviations

ACT	adoptive cell therapy
ALM	acral lentiginous melanoma
APC	allophycocyanin
B2M	β-2-microglobulin
BAP1	BRCA-1 associated protein
BET	bromodomain extra terminal
BRegs	regulatory B cells
BSA	bovine serum albumin
BTLA	B and T lymphocyte attenuator
BV421	brilliant violet 421
CAFs	cancer associated fibroblasts
CAR	chimeric antigen receptor
CCND1	cyclin D1
CDK	cyclin-dependent kinases
CM	cutaneous melanoma
CMTM6	CKLF-like MARVEL transmembrane domain-containing protein 6
CSN5	COP9 signalosome 5
CTLA-4	cytotoxic T-lymphocyte-associated protein-4
CTLs	cytotoxic T lymphocytes
CYSLTR2	cysteinyl leukotriene receptor 2

DC	dendritic cell
ECM	extracellular matrix
EGF	epidermal growth factor
EIF1AX	eukaryotic translation initiation factor 1A X-linked
EMT	epithelial to mesenchymal transition
ERK	extracellular signal regulated kinase
EZH2	histone methyltransferase
FADD	Fas associated death domain
FAP- α	fibroblast activation protein- α
FasL	Fas ligand
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
GAS	gamma activated sequence
GC	Germinal centre
GITR	glucocorticoid-induced TNFR family related gene
GM-CSF	granulocyte-macrophage colony-stimulating factor
GNA11	G protein subunit Alpha 11
GNAQ	G protein subunit Alpha Q
GSK3β	glycogen synthase kinase 3beta
HDACs	histone deacetylases
HLA	human leukocyte antigen
IDO	indoleamine 2,3-dioxygenase

IFNAR	interferon alpha receptor 1
IFNGR	interferon gamma receptor
IFNγ	interferon gamma
IL	interleukin
IRF1	interferon response factor 1
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-Sensitive Response Element
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
JAK	Janus kinases
LAG-3	lymphocyte activation gene 3
LDH	lactate dehydrogenase
LMM	lentigo maligna melanoma
MAPK	mitogen activated protein kinase
MC1R	melanocortin 1 receptor
MDA	melanocyte differentiation antigens
MDSCs	myeloid-derived suppressor cells
MEK	mitogen-activated protein kinase kinase
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIA	melanoma inhibiting activity
MIF	migration inhibitory factor

MITF	microphthalmia associated transcription factor
MART-1	melanoma antigen recognised by T cells 1
MM	mucosal melanoma
MMPs	matrix metalloproteinases
mTOR	mammalian target of rapamycin
MTORC1	mammalian target of rapamycin complex 1
NF1	neurofibromin 1
NF-κB	nuclear factor-kappa B
NGFR	nerve growth factor receptor
NK cells	natural killer cells
NM	nodular melanoma
NSCLC	non-small cell lung cancer
ORR	overall response rate
OS	overall survival
PD	progressive disease
PD-1	programmed cell death protein 1
PDGF	platelet-derived growth factor
PD-L1	programmed death-ligand 1
PD-L2	programmed death-ligand 2
PE	phycoerythrin
PE-Cy7	phycoerythrin-cyanine
PFS	progression free survival

PGE	prostaglandin-E
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3K/AKT	phosphoinositide 3-kinase/AKT
PIAS4	protein inhibitor of activated STAT 4
PIP2	phosphatidylinositol (3,4) diphosphate
PIP3	phosphatidylinositol (3,4,5)-triphosphate
PR	partial response
PTEN	phosphatase and tensin homolog
RECIST	Response Evaluation Criteria in Solid Tumours
RGMb	Repulsive Guidance Molecule b
RIP	receptor interacting protein
RTKs	receptor tyrosine kinases
SF3B1	splicing factor 3b subunit 1
SHP-2	SRC Homology 2-Domain-Containing protein Tyrosine Phosphatase 2
siRNA	small interfering RNA
SOCS	suppressor of cytokine signalling
SRSF2	Serine and Arginine rich splicing factor 2
SSM	superficial spreading melanoma
STAT	signal transducer and activator of transcription
TAMs	tumour associated macrophages
TANs	tumour associated neutrophils

TGFβ	transforming growth factor beta
Th	T helper
TIGIT	T cell Ig and ITIM domain
TIL	tumour infiltrating lymphocytes
TIM-3	T cell immunoglobulin and mucin domain containing-3
TLR	toll like receptor
TME	tumour microenvironment
TNFR	TNF receptor
TNFα	tumour necrosis factor alpha
TRADD	TNFR associated death domain
TRegs	regulatory T cells
T-VEC	talimogene laherparepvec
UVM	uveal melanoma
UVR	ultraviolet radiation
VEGF	vascular endothelial cell growth factor
WT	wild type

Chapter 1
Introduction

1.1 Melanoma

Cutaneous melanoma is the most aggressive form of skin cancer and its incidence continues to rise around the world (1). Melanoma represents less than 5% of all cutaneous malignancies, but accounts for over 75% of skin cancer-related deaths (1-4). Patients with primary cutaneous melanoma are generally cured by surgery, with a 98% five-year survival rate following removal of the tumour (5). In contrast, survival rates for melanoma that has spread to nearby lymph nodes or distant sites is 60% and 16%, respectively (6).

Melanoma arises from melanocytes, the pigment containing cells of the skin. Melanocytes are located primarily in the basal layer of the epidermis, the most superficial layer of the skin, and can also be found in the hair bulbs, eyes, ears, and meninges - membranes that envelop the brain and spinal cord (7). Melanocytes synthesise the pigment melanin, which provides protection against the damaging effects of ultraviolet radiation (UVR) (8). Melanocytes originate from the neural crest and expression of the tyrosine kinase receptor KIT is critically required for melanocyte function and survival (4). Two other factors regulating melanocyte development are melanocyte-stimulating hormone and melatonin. Melanocyte-stimulating hormone supports melanocyte growth and melanin production, whereas melatonin suppresses melanocyte function under conditions of decreased light while increasing melanocyte proliferation (8, 9).

Exposure to UVR is a major contributor to the transformation of melanocytes. UVR is a strong mutagen and repeated exposure results in the accumulation of mutations, (10), some of which promote tumorigenesis. Common “driver mutations” in melanoma include activating mutations in BRAF and NRAS, such as $BRAF^{V600E}$, or $NRAS^{Q61R}$ (11).

1.1.1 Melanoma subtypes

Metastatic melanoma is classified into several histopathologic subtypes, based on location, the amount of sun exposure on the affected site and morphology (12) (Table 1.1). Superficial spreading melanoma (SSM) is the most common subtype, accounting for approximately 70% of all melanomas. It typically arises in middle aged patients (mean age, 40 years) on intermittently sun-exposed skin such as chest, abdomen, back, upper arms and buttocks, and shows extensive upward spreading of abnormal epithelial cells in the epidermis (12, 13). Activating mutations in the BRAF kinase are common in SSM (14). Lentigo maligna melanoma (LMM) is associated with cumulative UV-induced DNA damage and often occurs on chronic sun exposed skin, face, scalp and neck of elderly patients (15, 16). LMM is associated with mutations in the NRAS and KIT genes and is characterized by proliferation of malignant melanocytes along the basal layer of the epidermis and hair follicles (15). Nodular melanoma (NM) occurs frequently on sun-exposed sites, including the head and neck and is more commonly found in older adults. It has no clear genetic signature, displays a nodular vertical growth pattern into the dermis and has a poor prognosis (12, 13).

In contrast, acral lentiginous melanoma (ALM) and mucosal melanoma (MM) are not associated with UV exposure, develop on the palms of the hands, soles of the feet, on the nails or on mucosal surfaces. ALM is the most common form of melanoma in

people with dark skin, while MM has similar incidence rates across races and occurs in older adults (>70 years) (12, 16). ALM and MM are characterized by increased genomic instability and chromosomal aberrations including chromosomal copy number changes (reviewed in (17). Acral and mucosal melanomas carry nearly 18-fold fewer mutations than cutaneous melanoma but more structural alterations such as copy number variations and aneuploid genomes (18). For example, most ALM and MM show structural variants and high-level amplifications on the long arm of chromosome 11, often involving the Cyclin D1 (CCND1) gene (17). Nearly 40% of ALM and MM have copy number gains and activating mutations affecting the receptor tyrosine kinase KIT (17).

Desmoplastic melanoma is a rare histological subtype, accounting for approximately 1-4% of melanomas. It occurs most frequently on sun exposed sites of older adults, including head and neck, and mutations in the NF1 tumour suppressor gene are common (19). Uveal melanoma is another rare form of melanoma (5% of all melanomas) that develops from melanocytes residing in the uveal tract, including the choroid, ciliary body and iris of the eye. Uveal melanoma (also known as ocular melanoma) is the most common malignant eye tumour in adults (20) and approximately 50% of patients with uveal melanoma will develop metastases, often in the liver, which are almost always fatal (17, 20). Uveal melanoma shares some risk factors with cutaneous melanoma, including fair skin and red/blonde hair and is characterized by specific driver mutations (BRCA-1 associated protein (BAP1), cysteinyl leukotriene receptor 2 (CYSLTR2), eukaryotic translation initiation factor 1A X-linked (EIF1AX), G protein subunit Alpha 11(GNA11), G protein subunit Alpha Q (GNAQ), splicing factor 3b subunit 1 (SF3B1) and Serine and Arginine rich splicing factor 2 (SRSF2)). Some of the genetic alterations are prognostic, for example BAP1 alterations are associated with chromosome 3 monosomy and are the best predictors

of metastases and poor prognosis, whereas EIF1AX and SF3B1 mutations are associated with good prognosis and are inversely associated with metastasis (21) reviewed in Park et al (22)).

Table 1.1 Summary of melanoma subtypes

Subtype	Proportion of melanomas	Location	Etiology	Genetics	Reference
Superficial spreading melanoma	60-70%	Chest and back in men, legs in women	UVR	*BRAF* mutations, chromosomal abnormalities, high tumour burden	(14, 23)
Lentigo maligna melanoma	4-15%	Head and neck	UVR	*NRAS* mutations, *KIT* mutations	(24, 25)
Nodular melanoma	10-15%	Head, neck, chest, back, abdomen and legs	UVR	*BRAF* and *NRAS* mutations, *PTEN* loss	(13, 14, 26)
Acral lentiginous melanoma	2-3%	Palms of the hands, soles of the feet, the nails	Unknown	*BRAF, NRAS* and *KIT* mutations	(27, 28)
Mucosal melanoma	1.4%	Head and neck, anorectal region, female genital tract	Unknown	*KIT* mutations	(29, 30)
Desmoplastic melanoma	1-4%	Head and neck	UVR	*NF1* mutations	(19, 31)
Uveal melanoma	5%	Choroid, iris and ciliary body of the eye	Unknown	*GNA11, GNAQ, CYSLTR2, EIF1AX, SF3B1* and *BAP1* mutations	(32)

UVR, ultraviolet radiation

1.1.2 Melanoma risk factors

The major environmental risk factor for cutaneous melanoma is exposure to UVR, including UV-A, UV-B and UV-C from sunlight or artificial sources (33). Artificial sources of UVR, including tanning beds/solaria, increase melanoma risk and sunbeds are now banned in many countries, including Australia (34). Ultraviolet exposure promotes melanoma development by inducing DNA damage that leads to the accumulation of mutations and eventually, malignant transformation that can directly suppress host immune system (35). In Australia, 63% of all melanomas can be attributed to the high levels of UVR exposure (33).

UVA (320-400 nm) causes direct DNA damage via photosensitized reactions that result in the production of oxygen radical species. It also has a direct immunosuppressive effect in laboratory animals and in humans (36). Sunburns are mainly caused by UVB (280-320 nm) radiation, which is a major contributor to melanomagenesis. UVB is strongly absorbed by DNA, leading to chromosomal damage and mutations that initiate a series of events leading to melanoma. UVC (200- 280 nm) is highly mutagenic and interferes with cell death (necrosis and apoptosis pathways) in melanoma cells, likely due to induced mutations in these pathways (37-39).

Melanoma incidence increases with age, and melanoma is more likely to develop in individuals with red/blond hair and fair skin, and with many common or atypical naevi (moles) (35). These risk factors can cluster in families, as natural genetic variants in the melanocortin 1 receptor (MC1R), for instance, cause red hair and fair skin (40). In individuals with a family history of melanoma, risk of melanoma increases 30-70 times over that of the general population (41). Familial melanoma is associated with

loss of function mutations in the INK4a/ARF locus on chromosome 9p21 (42). This locus encodes two different tumour suppressor proteins; p16INK4a and p14ARF (42). p16INK4a acts as a tumour suppressor via inhibition of cyclin-dependent kinases 4 and 6 (CDK4 and CDK6). The inhibition of CDK4/6 prevents cells from entering S-phase by maintaining retinoblastoma protein in an hypo-phosphorylated state (42). The alternate INK4a/ARF protein, p14ARF, act as a p53 regulator by binding to and sequestering the p53 ubiquitin ligase and negative regulator, hdm2 (43, 44). Highly penetrant germline mutations in the CDK4 also predispose individuals to melanoma (44).

1.1.3 Genomic landscape of melanoma

Cutaneous melanomas are classified into four genetic subgroups based on their driver mutation status: BRAF mutant, NRAS mutant, NF1 mutant and triple wildtype (WT) (45, 46). GNAQ/GNA11 mutations predominate in uveal melanoma (47).

1.1.3.1 BRAF-mutant melanoma

BRAF-mutant melanoma is the most common genetic subtype, and 40-60% of cutaneous melanomas carry mutations in BRAF (48, 49), and these mutations predominantly affect the valine at codon 600 within exon 15 (approximately 90% $BRAF^{V600E}$, $BRAF^{V600E/K/R}$) (Cancer Genome Atlas, 2015). Approximately 90% of BRAF melanoma-associated mutations lead to a substitution of valine (V) for glutamic acid (E) (V600E) at position 600, another 8%-20% change valine (V) for lysine (K) (V600K) and 1% lead to the substitution of valine (V) for arginine (R) (V600R) (48). Mutant $BRAF^{V600}$ is constitutively activated, with markedly increase kinase activity that promotes the uncontrolled proliferation of melanoma cells (50-52). $BRAF^{V600}$-mutant melanoma has a more aggressive phenotype compared to BRAF WT melanomas (48) and in patients with stage IV melanoma, $BRAF^{V600}$ mutations is

linked to shorter overall survival compared to patients with BRAF WT melanoma (26, 53, 54).

$BRAF^{V600}$ mutations tend to occur in younger patients with no history of sun damage and is associated with superficial spreading and nodular histology in primary cutaneous melanoma (51, 54), and brain metastases in advanced disease (53). $BRAF^{V600}$ mutations are rare in acral and mucosal melanoma and do not occur in uveal melanoma (55). $BRAF^{V600E}$ and $BRAF^{V600K}$-mutant melanomas display different features, suggesting distinct etiology and behavior. In particular, V600K melanoma show a higher degree of cumulative sun-induced damage, the disease-free interval from the primary melanoma to first distant metastasis was shorter in patients with V600K melanoma compared with V600E melanoma (17.4 vs. 39.2 months) (56); these differences could be due to the fact that V600K-driven melanoma occur in older patients compared to V600E-driven melanoma (56).

The selective inhibitors of $BRAF^{V600E/K}$, dabrafenib, vemurafenib and encorafenib, are approved for the treatment of patients with advance $BRAF^{V600E/K}$ mutant melanoma. These potent and selective small molecular inhibitors specifically target $BRAF^{V600E/K}$ mutant proteins (51). Resistance to BRAF inhibition is mainly associated with MAPK reactivation through MEK (57, 58). In order to overcome therapeutic resistance, BRAF inhibitors are often combined with MEK inhibitors like trametinib (50). Combination treatment with BRAF and MEK inhibitors has been shown to significantly increase objective response rates and significantly decreases the development of acquired resistance (52, 59-62). Moreover, combination treatment also produced fewer cutaneous adverse events and longer cutaneous adverse event-free intervals in comparison to BRAF inhibitor monotherapy (63). Consequently, combination therapy (dabrafenib plus trametinib or vemurafenib plus cobimetinib) is

now considered standard of care for patients with $BRAF^{V600E/K}$ mutated advanced melanoma (48). The combination of BRAF and/or MEK inhibitors with immunotherapy is currently under investigation (48).

1.1.3.2 RAS-mutant melanoma

RAS-mutant melanoma (Figure 1.1) is the second most frequent genetic subtype of cutaneous melanoma, accounting for 15-30% of cases including sun-exposed and non-sun-exposed melanoma, as well as mucosal and acral melanoma. Mutations are most common in NRAS but are also found in the homologue GTPases KRAS and HRAS (64) (Cancer Genome Atlas, 2015). RAS mutations constitutively activate many signalling cascades, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathways, to promote aberrant cell growth and proliferation (65). NRAS mutations are found in 20% of cutaneous melanoma, 10% of acral melanoma, 5-16% of mucosal melanoma (66-69) but not in uveal melanoma (51). RAS mutations most commonly affect codon 61 and also impact codons 12 and 13. These mutations are often seen in older patients (>55 years at diagnosis) with a chronic pattern of UV exposure (70-72). Histopathologically, NRAS mutations are associated with lower rates of ulceration and thicker primary tumours (73). Clinically, NRAS mutations are associated with more aggressive disease and a poor prognosis in advanced stage disease (66, 70, 74).

Preclinical data demonstrated that melanomas with NRAS mutations were effectively targeted with a combination of MEK inhibitors and CDK4 inhibitors (51). In a phase 2 study the combination of the MEK inhibitor binimetinib and the CDK4 inhibitor LEE011 produced a response rate of 41%, with partial responses seen in 9/22 patients with NRAS-mutant melanoma (75). One study found that melanomas with

NRAS mutations respond to anti-PD-1 therapy (76), suggesting that the combination of MEK inhibitors and immunotherapy could be used in NRAS-mutant melanoma.

1.1.3.3 Neurofibromin 1 (NF1)-mutant melanoma

Neurofibromin 1 (NF1) is the third most frequently mutated gene in melanoma after BRAF and NRAS (77). Neurofibromin is a tumour suppressor that negatively regulates RAS by stimulating RAS GTPase activity (78). NF1-mutant melanomas have the highest mutation rate compared to other genetic subtypes (11, 51). Mutations in NF1 represent a common genetic mechanism of activating the MAPK pathway in the absence of $BRAF^{V600}$ or NRAS mutations (11, 79). NF1 mutations are detected in 12% of melanomas with unaltered BRAF and NRAS genes (80). NF1 is a large gene (350kb, 62 exons including 58 constitutive and 4 alternatively spliced exons) and as such exhibits a high incidence of somatic mutations, with a characteristic UV signature (81). NF1-mutant melanomas commonly occur on chronically sun-exposed skin in older males (82) and NF1 alterations are especially common in desmoplastic melanoma (81).

1.1.3.4 Triple wild type (WT) melanoma

Triple wild type (WT) melanoma makes up the last genetic subgroup of cutaneous melanoma comprising approximately 15% of cutaneous melanomas. This melanoma subtype is defined by the absence of BRAF hotspot mutations, RAS mutations, or NF1 mutations. Several mutations, including known genetic drivers of uveal melanoma GNAQ and GNA11, and KIT mutations are found in some triple WT melanoma (79). Important features of the triple WT subtype include enrichment in recurrent KIT mutations, focal amplifications, copy number changes and complex structural rearrangements (79). Only 30% of triple WT samples harbour a UV signature, compared with over 90% for the other three subtypes. The prevalence of

triple WT is highest in mucosal melanoma (70.7%), compared to other melanoma subgroups (83), but is not associated with mutational spectrum, tumour mutation burden, or prognosis (83).

1.1.3.5 GNAQ and GNA11 mutations in uveal melanoma

Mutations in G-protein-coupled receptors alpha subunits, GNAQ encoding Gq protein and GNA11 encoding G11 have been found in approximately 85% of uveal melanocytic tumours (84, 85). These mutations result in a substitution of one amino acid at Q209 or R183 residues leading to constitutive activation of oncogenic Gq/11 subunits. Gq/11 mutants found in uveal melanoma promote tumorigenesis by activating many signalling cascades, including the MAPK, PI3K, protein kinase C, ß-catenin and YAP pathways (reviewed in (22);(84).

1.1.4 Important signaling pathways in melanoma

The common melanoma driver mutations detailed above lead to aberrant activation of several cell signalling pathways (Figure 1.1), and the following section discusses four key signalling pathways that are frequently altered in melanoma.

1.1.4.1 The mitogen-activated protein kinase (MAPK) pathway

Due to the predominance of NRAS (mutated in 10-30% of melanomas) and BRAF (mutated in 40-60% of melanomas) mutations in melanoma, the MAPK pathway is constitutively activated in nearly 80% of cutaneous melanomas (86). This signalling pathway controls many intracellular processes, including cellular differentiation, proliferation and apoptosis (87) (Figure 1.1). The MAPK pathway transmits signals generated from growth factor-bound receptor tyrosine kinases (RTKs), other (non-tyrosine kinase) cytokine receptors, heterotrimeric G-protein-coupled receptors or growth factor independent signals like cellular stress (64). Interactions between RTKs

(e.g. EGFR and KIT) and their ligands (epidermal growth factor and stem cell factor, respectively) activate RAS proteins leading to the phosphorylation and activation of the MAP kinase kinase kinase proteins (ARAF, BRAF and CRAF). Mutations activating NRAS directly activate the RAF proteins (88). Activated RAF interacts and phosphorylates the MAP kinase kinase proteins MEK1 and MEK 2, which subsequently phosphorylate and activate the serine/threonine MAP kinases ERK1 and ERK2 (89, 90). The ERK proteins transcriptionally activate 100s of gene targets to influence cell proliferation, differentiation and survival (50).

1.1.4.2 The phosphoinositide 3-kinase/AKT (PI3K/AKT) pathway

In addition to MAPK pathway activation, NRAS mutations also lead to activation of the phosphoinositide 3-kinase/AKT (PI3K/AKT) pathway (Figure 1.1). This pathway is important in regulating cellular proliferation, survival, growth, protein synthesis, metabolism, and motility in multiple cell types (91, 92). Similar to the MAPK pathway, PI3K can be activated by RTKs and activated RAS proteins, leading to the phosphorylation and conversion of phosphatidylinositol (3,4) diphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-triphosphate (PIP3). PIP3 serves as a high-affinity binding ligand for PH-domain-containing AKT; AKT is recruited to the cell membrane for phosphorylation at residues Ser473 and Thr308 by the mammalian target of rapamycin complex 2 (mTORC2) complex and PDK1, respectively (92-94). Activated AKT promotes phosphorylation of a host of proteins, including the serine threonine kinase mTOR, to affect cell growth, survival, and cell cycle entry (91).

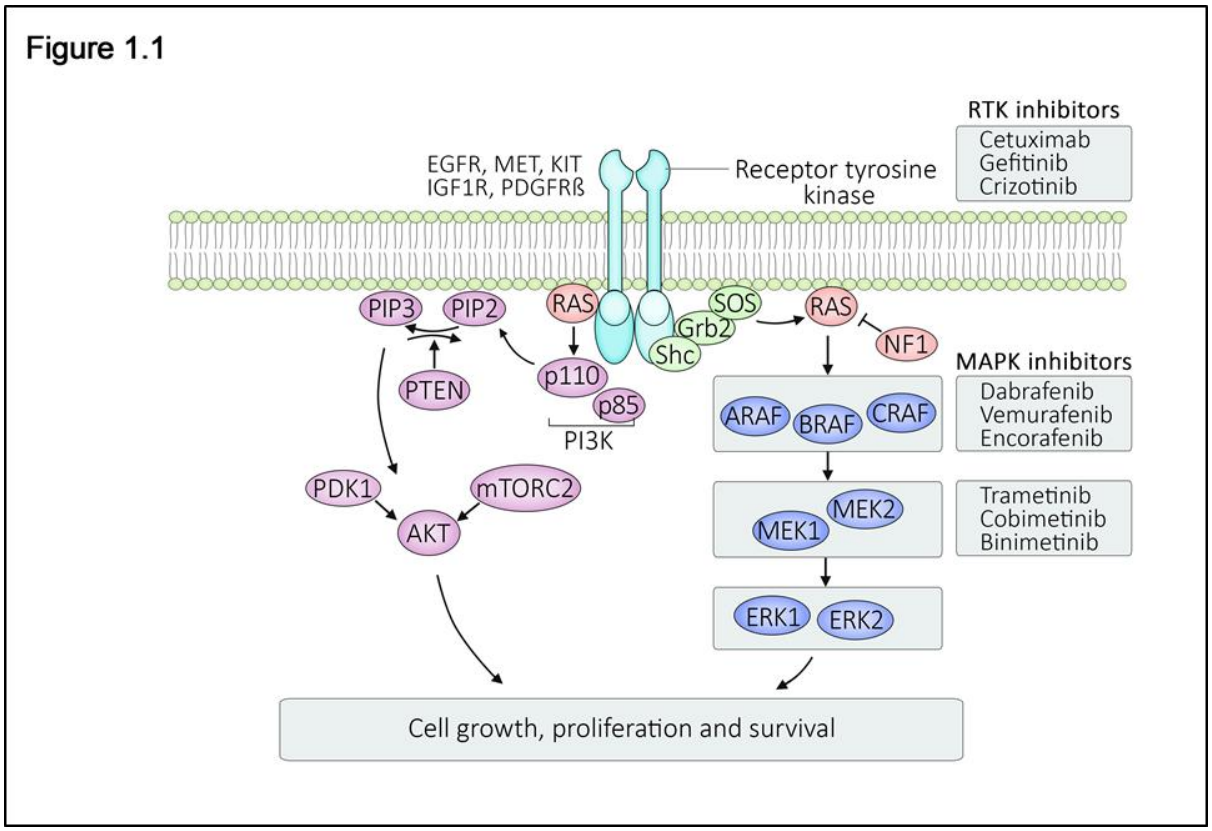

Figure 1.1 MAPK and PI3K/AKT signalling pathways

Binding of receptor tyrosine kinases (RTKs) to their ligands activates the RAS GTPase proteins which initiate signalling via two key cascades, (i) the mitogen activated protein kinase (MAPK; RAF-MEK-ERK) and (ii) the phosphoinositide 3-kinase/AKT (PI3K/AKT) pathways. Activated PI3K (complex of p110 and p85 subunits). phosphorylates the phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2) forming phosphatidyl-3,4,5-trisphosphate (PIP3) at the plasma membrane. AKT is one of the major downstream effectors of PI3K and it undergoes complete activation upon phosphorylation by PDK1 and by the mammalian target of rapamycin (mTOR) complex 2 (mTORC2). Loss of phosphatase and tensin homolog (PTEN) favours the accumulation of PIP3 from PIP2, resulting in AKT/mTOR activation and the neurofibromin (NF1) tumour suppressor stimulates RAS GTPase activity to inhibit RAS signalling. Both the MAPK and PI3K/AKT cascades activate multiple proteins involved in cell growth, survival, and proliferation. The most frequent mutations affecting these pathways are NRAS (mutated in 10-30% of melanomas), BRAF (mutated in 40-60% of melanomas), NF1 (mutated in around 12% of melanomas), and PTEN (mutated in 15-20% of melanomas). (Adapted from Pinho et al. 2019. Emerging Novel Therapies in Overcoming Resistance to Targeted Therapy, manuscript in preparation).

The tumour suppressor PTEN as an important negative regulatory phosphatase in the PI3K/AKT pathway, and functions to dephosphorylate PIP3. In melanoma, activation of the PI3K/AKT pathway is mainly due to PTEN loss-of-function mutations, such as missense mutations, frameshift mutations and/or deletions in the PTEN gene (93, 95) (Figure 1.1). Loss of PTEN function is correlated with increased phosphorylation and activation of AKT in metastatic melanoma (49). Apart from NRAS mutations, other genetic and epigenetic events such as specific point mutations in PIK3CA, AKT1 and AKT3 genes, detected in 3%, 1% and 1.5% of melanomas, respectively, (93) also contribute to the activation of the PI3K/AKT pathway in melanoma. It is also important to mention that in addition to activation of upstream receptor tyrosine kinases (e.g., KIT, EGFR, HER2/neu, etc.), NF1 mutations can result in enhanced NRAS activity, leading to activation of downstream PI3K/AKT and MAPK pathways (80).

1.1.4.3 The WNT/ß-catenin pathway

The wingless/Integrated WNT/β-catenin pathway is also deregulated in melanoma and its deregulation leads to multiple abnormalities in cell homeostasis, migration, growth and development. Several components of the Wnt/ß-catenin pathway, such as APC, ICAT, LEF1 and ß-catenin are modified in melanoma tumours, leading to activation of this signalling pathway (96). Activated WNT/β-catenin signalling is associated with aggressive tumour growth (97), and results in a phenotypic switch from a highly proliferative/non-invasive state to a slow proliferating/metastatic and invasive phenotype (97).

In melanoma, the WNT/ß-catenin pathway is also responsible for regulating function of the microphthalmia-associated transcription factor (MITF), a master transcription factor that regulates melanocyte development and progression (97, 98). Specifically,

MITF regulates genes involved in the development of the melanocytic lineage (99), including differentiation, proliferation and survival of melanocytes (100). MITF expression is also important for melanocyte transformation (101) and tumour survival (100),as MITF confers resistance to apoptosis through expression of anti-apoptotic proteins BCL2, BLC2A1 and BIRC7 (100). MITF expression is often lost as the melanoma progresses. Low MITF expression is associated with increased melanoma invasiveness and metastasis, and amplification of MITF is associated with poor clinical outcome.

1.1.4.4 The p53 pathway

The p53 signalling pathway is also important in melanoma progression although mutations in this pathway are less common in melanoma and occur in only 1-5% of primary tumours and 11-25% metastatic melanomas (102, 103). p53 is a tumour suppressor that is frequently mutated in most cancers. p53 regulates expression of genes and pathways controlling cell cycle, apoptosis, senescence, autophagy and DNA repair in response to cellular stress.

1.1.5 Biomarkers in melanoma

Several diagnostic, prognostic and predictive biological markers have been reported for melanoma and these biomarkers are used to inform on the progression, severity and best treatment options for this disease (104).

Diagnostic biomarkers help to confirm the presence of melanoma which is useful in the early detection of the disease. Prognostic biomarkers evaluate the outcome of the disease (104) and can be used in the clinic to determine best treatment strategies (105), whereas predictive biomarkers evaluate the efficacy and response to specific treatments.

Examples of **prognostic biomarkers** in primary melanoma include clinical features of the disease (melanoma thickness, presence or absence of ulceration, lymph node involvement, patient age, uncommon melanoma subtype e.g. nodular or acral, tumour localization to trunk, head or neck), pathological characteristics (presence or absence of mitosis, mutations in the BRAF or NRAS genes, the degree of lymph nodes involvement, presence or absence of extracapsular extension) (reviewed in (104)). Prognostic biomarkers associated with poor outcomes of metastatic melanoma include visceral metastases, elevation of serum lactate dehydrogenase (LDH), decrease in serum albumin, presence of abnormal platelets and poor performance status (equal or more than 1, according to the Eastern Cooperative Oncology Group (ECOG) classification) (reviewed in (104). Other negative prognostic factors in melanoma include elevated serum levels of S100B, C-reactive protein and melanoma inhibiting activity (MIA) protein (106).

Predictive biomarkers evaluate the efficacy and response of specific treatments. For example, absence of the $BRAF^{V600}$ mutation, elevated serum LDH, poor ECOG performance status, having three or more solid organ metastases, stage IV baseline disease and lesion diameter of more than 58 mm are predictive of poor response to MAPK inhibitor therapy. Good response to PD-1 blockade is generally associated with tumour PD-L1 expression (107-112), whereas biomarkers of poor response to immune checkpoint blockade include elevated serum LDH, large tumour size (> 102 mm) and advanced disease stage IVM1a or IVM1c (reviewed in (104, 113, 114).

1.1.6. Melanoma staging

The updated American Joint Committee on Cancer (AJCC) staging system for cutaneous melanoma (8^{th} edition) is used to guide prognostication of the disease, as well as better inform risk management and stratification, and guide treatment

selection for melanoma patients. Using this system, melanoma can be broadly classified into four stages (Stage 0-IV) based on the extent and spread of the disease.

- Stage 0 describes melanoma restricted to the top and outer layer of the skin (115, 116).

- Stage I and Stage II are indicative of localized melanoma that has not moved beyond the primary site with 2 mm or more in thickness with or without ulceration (116).

- Stage III describes regional metastases, characterized by spreading to lymph nodes near the primary site, nearby skin or subcutaneous. The major factor for classification of this stage is the presence of metastatic lymph nodes that categorizes such patients into stage IIIA (single metastatic node), stage IIIB (2 to 3 metastatic nodes) or stage IIIC (multiple metastatic nodes) (116)

- Stage IV describes distant metastases, in which spread to distant skin and/or other parts of the body has occurred. The M1a, M1b, M1c or M1d categorization is based on the location of different metastases, number and size of tumors. In M1a, the tumor has spread to distant skin, the subcutaneous layer or to distant lymph nodes. In M1b, it has metastasized to the lung. M1c is the stage that the tumor metastasized to vital organs other than the lungs and in M1d, it has metastasized to the central nervous system (116).

Other categorization of melanoma staging is based on based on tumor, lymph node, and metastasis characteristics (TNM staging), Tumour (T) that describes the thickness of the melanoma categorised in 5 main stages; Tis means the melanoma cells are only in the very top layer of the skin surface, T1 means the melanoma is

less than 1 mm thick, T2 means the melanoma is between 1 mm and 2 mm thick, T3 means the melanoma is between 2 mm and 4 mm thick and T4 means the melanoma is more than 4 mm thick (117).

Node (N) divided to 4 stages describing whether cancer cells are in the nearby lymph nodes or lymphatic ducts; N0 means that the nearby lymph nodes don't contain melanoma cells, N1 means there are melanoma cells in one lymph node, N2 means there are melanoma cells in 2 or 3 lymph nodes, N3 means there are melanoma cells in 4 or more lymph nodes (118).

Another classification is Metastasis (M) which describes whether the cancer has spread to a different part of the body; M0 means the cancer hasn't spread to another part of the body.

M1 means the cancer has spread to another part of the body which divided to three other stages; M1a, M1b and M1c which describes above.

1.2 Cancer immunity

1.2.1 Immune surveillance

The notion that the immune system can recognize and eliminate tumours was proposed in the 19th century. In the 1890s, William Coley pioneered the development of cancer immunotherapy, by treating sarcoma patients with microbial cultures after observing disappearance of a patient's tumour following an infection episode (119). Over 1000 cancer patients with inoperable bone and soft tissue sarcomas were treated with "Coley's toxins" in the following 40 years, with an overall success rate of 10% (120). Despite these promising results, many physicians remained skeptical. In 1970, Burnet and Thomas proposed the concept of immune surveillance, in which the host maintains immunological resistance to cancer development (121).

The concept of cancer immunosurveillance suggests that cancer development follows the phases of elimination, equilibrium and escape, also termed "The three E's" (122). When neoplastic cells develop due to accumulation of mutations, protective immune mechanisms are mobilized to identify and eliminate the transformed cells. Should elimination fail, chronic inflammation develops in an attempt to contain the growing tumour while reducing damage to the host tissue. At this stage, equilibrium is reached between the immune system and the tumour. Tumours eventually take advantage of the anti-inflammatory mechanisms and escape the immune response.

Evidence of immune surveillance comes from both animal models and clinical observations:

1. Immunodeficient mice have higher incidence of both spontaneous and chemically induced tumours (123, 124).

2. Immunocompromised patients (such as those receiving immunosuppression after solid organ transplantation to prevent rejection) have a higher frequency of certain cancers, including skin cancers and lymphomas (125).

Further clinical observations support the concept of tumour immune surveillance in humans. The clonal expansion of T cells in melanoma is accompanied by tumour regression (126, 127) and the degree of lymphocyte infiltration is associated with good clinical outcomes in multiple malignancies including basal cell carcinoma, malignant lymphoma, Merkel cell carcinoma, mesothelioma, lung carcinoma, and congenital fibrosarcoma (128-130). In colorectal cancer staging, the immunoscore, derived from the density of intratumoral and peritumoral T cells, was shown to be a better prognostic marker compared to the tumour, node and metastasis (TNM) system (129, 131). It's been shown that the immunoscore also has prognostic value in other cancer types including breast, prostate, kidney, lung and melanoma (reviewed in (131-133)) and its effects in predicting treatment response in other cancer types is under investigation (133).

1.2.2 Overview of cancer immunity

The immune system employs two mechanisms, innate immunity and adaptive immunity (134) to recognize and eliminate non-self-products, while also maintaining tolerance to self.

1.2.2.1 Innate immune responses

Innate immune responses are fast and non-specific and are not capable of forming immunological memory. The innate immune response (also known as inflammation) is initiated as the first line of defence; chronic inflammation develops if the acute inflammatory response has failed (135). Dendritic cells, tumour-associated

macrophages (TAMs), tumour-associated monocytes, neutrophils, and Natural killer (NK) cells are the main innate cells in tumours. Chronic inflammation in cancer provides a means for tumour tissue remodelling that supports and nourishes the tumour. Consequently, chronic inflammation and its cellular components represent a potential therapeutic target.

1.2.2.2 Adaptive immune responses

Adaptive immune responses are specific to the injurious agent, develop over a period of several days and are somewhat delayed compared to innate immunity and are capable of forming immunological memory allowing a rapid response upon subsequent re-challenge with the agent. In the adaptive immune response to cancer, cancer-specific antigens (such as products of mutated proteins) are recognised as non-self and targeted by T lymphocytes and B lymphocytes. Recognition occurs via antigen receptors (T cell receptors and B cell receptors, respectively) and for T cells, requires the antigenic peptide to be presented on major histocompatibility complex (MHC) molecules expressed by antigen-presenting cells or tumour cells (134). There are two types of MHC molecules, MHC class I (MHC I) also known as human leukocyte antigen (HLA)-A, B and C (HLA-ABC) molecules, and MHC class II (MHC II) known as HLA-DR (136). The MHC-I/peptide complexes are recognised by the CD8+ T cells that, after differentiation into cytotoxic T cells, travel to the tumour and kill tumour cells. The MHC-II/peptide complexes are recognised by CD4+ T cells that differentiate into cytokine-producing T helper (Th) cells. Distinct types of Th cells include the T helper 1 (Th1) cells that are important for anti-cancer responses, Th2 cells that interact with B cells to promote antibody production, and Th17 cells associated with immunopathology (137). Regulatory T cells (TRegs) are a separate lineage of CD4+ T cells that maintain self-tolerance and they are associated with

immunosuppression in cancer (138). The roles of distinct T cell subsets in cancer progression and control are described in more detail in section 1.3.1.2.

The adaptive immune response to cancer is best characterized by the Cancer-Immunity Cycle, a series of seven major cyclic steps that proceed to amplify T cell responses, leading to effective killing of cancer cells (139). Cancer cell derived neoantigens are captured and processed by dendritic cells (step 1), and the neoantigen-derived peptides are subsequently presented on MHC class I and MHC class II molecules to T cells (step 2), resulting in the T cell priming and effector T cell responses against the cancer-specific antigens (step 3). In steps 4 and 5, effector T cells traffic to and infiltrate the tumour, leading to recognition of cancer cells by T cells through the interaction between the TCRs and tumour antigens bound to MHCs (step 6). Cancer cells are eliminated (step 7), which causes the additional release of tumour associated antigens and the re-initiation of step 1 (reviewed in (31, 139)). This cyclic order of events is required to establish an appropriate immune response against cancer, and failure in any of these seven steps can lead to tumour immune escape (140).

Many tumours are immunogenic (i.e. capable of inducing an immune response) but the immune response is either too weak to reject a rapidly growing tumour, or the tumour exerts a suppressive effect on the host immune system (141, 142). Although the adaptive immune response can result in tumour eradication, it is subject to corruption and may instead promote tumour development (143, 144).

1.2.3 Mechanisms used by cancer to evade the immune response

Cancer cells have different strategies to evade the adaptive immune response, which result in lack of recognition and elimination of tumour cells by the immune system. These strategies result in tumour progression and metastasis (145, 146) and include:

1. Loss of expression of MHC molecules, or components of the MHC I antigen presentation pathway on tumour cells resulting in the lack of tumour recognition by the host immune system (147, 148).

2. Downregulation or loss of tumour antigens recognised by T cells. For example, melanoma cell de-differentiation results in loss of melanocytic differentiation antigens (MART1, gp100) commonly recognised by T cells (149).

3. Induction of inhibitory checkpoints on T cells or tumour cells. Examples include PD-L1 (on tumour cells), PD-1 and CTLA-4 (on T cells).

4. Secretion of soluble factors that inhibit immune responses, such as IL-6, IL-10 and transforming growth factor beta (TGFβ) (150).

5. Expansion of myeloid-derived suppressor cells (MDSCs) by releasing cytokines that induce MDSC formation (GM-CSF, CSF-1, PGE2, IL-6, or IL-10 (151)). MDSCs suppress T cells by producing arginase-1, reactive oxygen species (ROS) and nitric oxide synthase (NOS) (152-154).

6. Accumulation of TRegs, which suppress the proliferation and cytokine production of Th1 cells and cytotoxic T cells (CTLs), and can suppress function of B lymphocytes, NK cells and dendritic cells (146, 150, 155).

7. Induction of T cell apoptosis. Tumour cells can induce apoptosis in immune cells by releasing exosomes containing Fas ligand (FasL), which binds the Fas receptor on T cells inducing cell death, and by producing immunosuppressive cytokines like TGFβ and IL-10 (156). At the same time, tumour cells themselves evade apoptosis by overexpressing Bcl-2 or other anti-apoptotic proteins (150).

8. Immune exclusion. The inability of T cell to penetrate into the tumour and come into contact with cancer cells is an important mechanism of immune escape in multiple cancer types, including melanoma (157). Tumour microenvironment, including cancer associated fibroblasts and myeloid suppressive cells, appear to play a major role in maintain this phenotype via TGF-β production and WNT signalling (158, 159). Oncogenic signalling pathways such MAPK, contribute to immune exclusion (160, 161).

9. Loss of responsiveness to IFNγ. Mutations in the components of the interferon gamma signalling pathway, including IFN gamma receptor (IFNGR), JAK1 and JAK2, and STAT1,, can lead to loss of tumour cell response to the T cell-derived IFNγ (162, 163).

1.3 Tumour microenvironment

The tumour microenvironment (TME) is composed of cancer cells and other cellular components that play a key role in tumour development and progression. Tumour stroma contains different types of cells and structures such as vascular and lymphatic endothelial cells, fibroblasts and multiple types of immune cells surrounded by the extracellular matrix (ECM) (164). While tumour stromal cells are not malignant, they generally acquire pro-tumorigenic properties due to direct or indirect interactions with cancerous cells (165) (Figure 1.2). Composition of the TME can dictate disease progression. For example, upregulation of genes associated with ECM remodelling, like laminin-5γ2 and urokinase, support melanocyte trans-differentiation and increase migratory and invasive ability of melanoma cells (166).

Figure 1.2

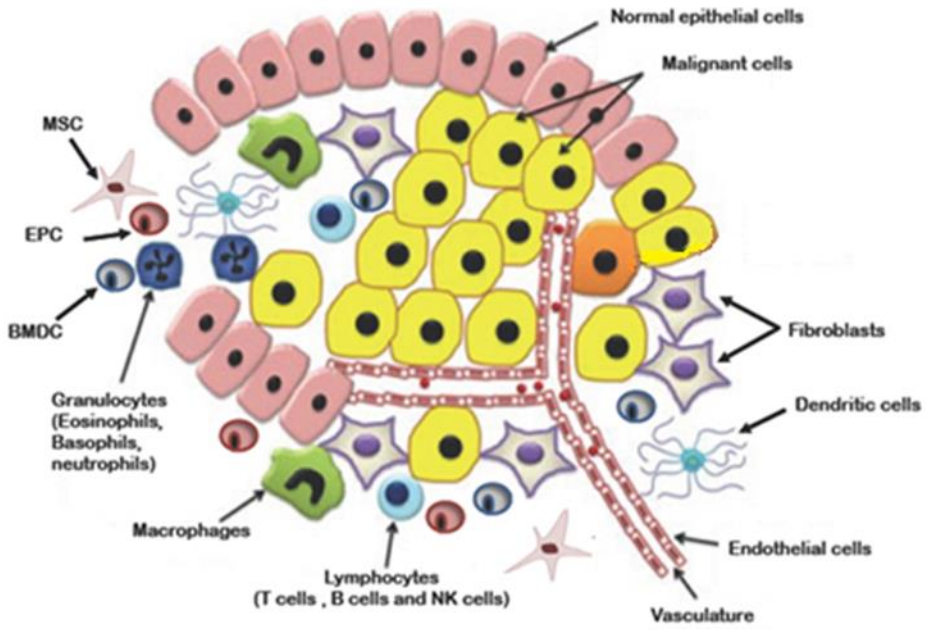

Figure 1.2 Major components of the tumour microenvironment

The tumour microenvironment consists of different immune and non-immune cell types and structures. Presence of heterogeneous cells and their secreted soluble factors, cytokines, chemokines and signalling molecules in tumour microenvironment promote neoplastic transformation, support tumour growth and invasion. Figure derived from (167).

1.3.1 Stromal cell types in the tumour microenvironment

The stromal component of the TME is comprise of three major groups - cells of mesenchymal origin, immune cells, and non-cellular components (168). Cells of mesenchymal origin include fibroblasts, myofibroblasts, mesenchymal stem cells (MSCs), adipocytes and endothelial cells (168). Cancer associated fibroblasts (CAFs) are the predominant cell type in this group and they are responsible for the structural architecture of the extracellular matrix (169). Activated CAFs are the predominant source of secreted growth factors and cytokines that support

tumourigenesis (169, 170). For example, activated CAFs secrete vascular endothelial growth factor (VEGF), which promotes angiogenesis and increases vascular permeability, supplying nutrition and supporting tumour metabolism (170).

The second group of cells in the TME includes immune cells. These are a diverse array of cell subsets including antigen-presenting cells such as macrophages and dendritic cells and lymphocytes such as T-lymphocytes, B-lymphocytes, NK T cells and NK cells (169, 171). Each cell type can have a positive or negative effect on tumour progression, depending of their interactions with cancer cells and other cells within the TME (168).

The ECM forms a large non-cellular component of the TME. The ECM scaffold provides structural support to tumour cells, stromal cells and the vasculature (169), and consists of fibrillar collagens, proteoglycans and glycoproteins (172).

Given their key role in promoting tumour progression, targeting specific groups of stromal cells may have beneficial effects in the treatment of cancer (173). Stromal cells in the TME can also influence response to therapies. Understanding how stromal cells interact with cancer cells may reveal novel targets for therapeutic intervention.

1.3.1.1 Non-immune cell types in the tumour microenvironment

Tumour vasculature

Tumour angiogenesis, the formation of new blood vessels within tumours, is essential for tumour progression, as it provides oxygen and nutrients, removes waste products and enables tumours to metastasise. Tumour blood vessels consist of tumour endothelial cells and perivascular cells. Tumour endothelial cells line the blood vessels and play an important role in angiogenesis, maintaining the direction of

vessel growth, and perfusion and oxygenation of the newly formed blood vessels. Tumour endothelial cells in metastatic tumours have highly proangiogenic phenotype with upregulation of several angiogenesis-related genes, such as VEGFR-1, VEGFR-2, and VEGF (174, 175). They produce matrix metalloproteinases to break down vascular basement membrane and degrade the extracellular matrix, enabling haematogenic dissemination of cancer cells. Endothelial cell targeting is a promising treatment strategy and identification of several markers preferentially expressed on human tumour endothelium could lead to the development of new therapies, as well as imaging and diagnostic agents for cancer (176). PlGF, VEGF-A, VEGF-B, VEGF-C, VEGF-D, mVEGRF3, LYVE-1, CD34, CD31 are some examples of such markers (177).

Perivascular cells such as pericytes for micro vessels, and smooth muscle cells for arteries and veins, envelope the outer surface of blood vessels and play a role in vessel contraction and relaxation (178). Perivascular cells also stabilize blood vessels and participate in angiogenesis. Within the vasculature system of the tumour microenvironment, pericyte coverage and morphology are dependent on the type of tumour, i.e. benign versus malignant, primary versus metastatic (179). Because of the hypoxic nature of metastatic tumours, their blood vessels have fewer pericytes compared with non-metastatic tumours (178).

Adipocytes

Adipocytes are responsible for the synthesis and storage of fat. They originate from mesenchymal stem cells, store energy in the form of lipids and release energy in response to hormonal stimulation as fatty acids, used for fuel when glucose supply is limiting (180, 181).

Adipocytes support tumour neovascularization and tumour cell growth by producing cytokines and growth factors. In cancer, adipocytes produce inflammatory cytokines, such as TNFα, IL-6, IL-1β, and CCL2, which recruit immune and inflammatory cells such as lymphocytes and macrophages and enhance chronic inflammation (182). These cytokines are associated with poor clinical outcomes as they contribute to local tumour growth and cancer de-differentiation (182, 183). In the TME, adipocytes express leptin, HOXC8 and HOXC9, Ucp1 (184).

Cancer associated fibroblasts (CAFs)

Cancer associated fibroblasts (CAFs) is a major stromal cell types in the tumour microenvironment. The majority of CAFs is derived from normal fibroblasts following activation by growth factors, adhesion molecules, reactive oxygen species and microRNAs (177, 185). CAFs can also be derived from mesenchymal stem cells, epithelial cells, pericytes, adipocytes and endothelial cells (186). CAFs contribute to neoplasm initiation (177, 185) and help cancer progression though ECM remodelling, induction of angiogenesis, recruitment of inflammatory cells, and direct stimulation of cancer cell proliferation via the secretion of growth factors and immunosuppressive cytokines (177). CAFs also play an essential role in the metastasis of cancer (187), by promoting tumour neovascularization through the secretion of fibroblast growth factor 2 (FGF2) and VEGF (177), as well as expressing other pro-tumorigenic factors including Galectin-1, Chemokine (C-X-C motif) ligand 12 (CXCL12), MMP-2, Interleukin-22 (IL-22), HGF, TGFβ and platelet-derived growth factor (PDGF) (177). CAFs express variety of molecules that could act as targets for immunotherapies like fibroblast activation protein- α (FAP-α). Mouse model experiments of colon carcinoma showed, by targeting FAP-α the accumulation of CAFs was reduced suggesting FAP-α as important marker of CAF-targeted therapy (188, 189).

1.3.1.2 Immune cell types in the tumour microenvironment

T cells

Adaptive immune responses to cancer are mediated by T cells, a defined subset of lymphocytes that develop in the thymus and continuously circulate through blood and lymphatic systems until their activation in secondary lymphoid organs (such as lymph nodes) by antigen-presenting dendritic cells. Following activation, T cells differentiate into effector and memory subsets capable of gaining access to peripheral tissues, such as tumours (190).

Major subsets of T cells in the tumour microenvironment include effector CD8+ T cells, a tissue resident memory CD8 T cell subset (T_{RM}), conventional CD4+ T cells and TRegs. T cell receptors (TCRs), unique to each T cell, recognise tumour-derived peptides bound to MHC molecules on tumour cells and/or antigen-presenting cells in the TME. Tumour-T cell interactions may result in direct or indirect tumour killing but may also promote tumour growth.

CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs), are capable of eliminating tumour cells by direct killing, and this process requires cell-to-cell contact (191). CD8+ T cells (named after the CD8 co-receptor that they commonly express) recognise tumour antigens in the form of peptides bound to MHC class I molecules. Tumour antigens recognised by CD8+ T cells are commonly derived from mutated or overexpressed tumour proteins, many of which are proteins of the melanocytic differentiation pathway. For example, cancer-testis antigens such as NY-ESO-1 and LAGE-1 are commonly expressed by different types of cancers including melanoma but are not found in normal adult tissues. As such, they are ideal targets for antigen-specific immunotherapy (192). Loss of tumour antigens, such as through melanoma de-differentiation, or loss of MHC class I expression by tumour cells, will inevitably

result in loss of CD8+ T cell recognition, loss of tumour killing, and cancer progression (193, 194). Presence of CD8+ T cells in melanoma tumour biopsies is generally associated with good prognosis (195, 196) and at least some of the beneficial effects have been attributed to the unique non-recirculating subset of CD8+ memory T cells, known as tissue resident memory T cells (TRM) (197, 198). Tissue-resident memory T cells (T_{RM} cells) control tumour growth more effectively than circulating memory T cells, by virtue of faster response to antigens, higher expression of cytotoxic molecules and closer contact with tumour cells. The presence of T_{RM} cells in melanoma lesions was associated with good clinical outcomes (199), and correlated with increased efficacy of cancer vaccines (199), better response to adoptive cell therapy (199), and better response to anti-PD-1 immunotherapy (200).

Conventional CD4+ T cells (named after the CD4 co-receptor that they commonly express) recognise tumour antigens in the form of peptides bound to MHC class II molecules, also known as HLA-DR molecules. The majority of cancer cells do not express MHC class II molecules and therefore cannot be directly targeted by CD4+ T cells. Melanoma is a notable exception, as many primary and metastatic tumours are HLA-DR positive (201, 202) and therefore capable of directly interacting with CD4+ T cells. Upon activation, CD4+ T cells differentiate into distinct effector subtypes that secrete specific cytokines. T helper 1 (Th1) cells that secrete IL-2, interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) which are associated with good prognosis in advanced melanoma. IFNγ is essential for the activation of mononuclear phagocytes, such as macrophages, and polarisation towards the M1 (inflammatory) phenotype, a macrophage phenotype associated with enhanced phagocytic activity and anti-cancer properties. IFNγ has both direct and indirect anti-tumour effects, such as inducing apoptosis in cancer cells and killing tumour vasculature (203, 204) (described in further detail in section 1.4). T helper 2 (Th2) cells produce IL-4, IL-5,

IL-6 and IL-13, cytokines associated with B cell proliferation and antibody production. They are uncommon in melanoma TME. Th2 cells are involved in responses against extracellular pathogens and parasites. They are also responsible for coordinating humoral immunity and allergic inflammatory responses (205). The role of Th2 cells in anti-tumour immune response in not clear, although several studies have reported association of this cell subset with carcinogenesis and tumour progression (205-207). A subpopulation of Th2 cells produce IL-24 which suppress human ovarian cancer cell growth both in vitro and in vivo, and induce substantial “bystander antitumor” immunity in patients (208-210).

Th17 cells producing IL-17 are associated with immunopathology. In cancer, CD4+ T cells often co-produce IL-17 and IFNγ and are referred to as Th17.1 cells (211). IL-17 produced by Th17 cells has an important role in inflammation responsible for immunopathology in both cancer and several autoimmune disorders (205). Association of Th17 cells with tumour initiation and growth in the context of chronic inflammation has been shown in murine models (212, 213). On the other hand, Th17 cells may contribute to protective antitumor responses (205). It’s been shown that the presence of Th17 is negatively correlated with TReg cells and positively correlated with effector immune cells including IFN-γ-secreting Th1 cells, cytotoxic CD8 T cells, and NK cells within the tumor microenvironment (205, 214). Th17 cells produce several cytokines including IL-21, IL-22, and IL-26 that facilitate their recruitment to sites of inflammation, including tumour growth (205, 215).

TRegs develop in the thymus as a defined lineage of CD4+ T cells (thymic TRegs) or differentiate in situ from conventional CD4+ T cells under conditions of chronic antigen persistence, such as in chronic virus infections and cancer (induced or peripheral TRegs). Both types of TRegs express the transcription factor FOXP3

(216), and both subsets are increased in cancer (217). Increased TReg numbers in blood and tumour biopsies correlate with poor prognosis, whereas depletion of TRegs combined with vaccination and chemotherapy can induce effective anti-tumour responses (218, 219). TRegs inhibit anti-cancer immune responses through multiple mechanisms, including direct suppression of effector T cells (220), competition for IL-2 (221), secretion of the immunosuppressive cytokine TGFβ (222), and intercellular transfer of cyclic AMP (223). Direct killing of CD8+ T cells and DCs by TRegs has also been reported (224).

B cells

B cells are antibody-producing cells that can have both pro- and anti-tumour effects. There is increasing evidence that B cells are involved in immune responses to melanoma, as antibodies with anti-melanoma reactivity could be found in both immunotherapy-naïve and immunotherapy-treated patients (225). The exact role of B cells in melanoma is not yet clear (225). A subset of B cells known as B regulatory cells (BRegs), can suppress T cell responses though their production of IL-10, a cytokine with strong anti-inflammatory effects and/or TGFβ (226).

Natural killer (NK) cells

NK cells are capable of directly killing tumour cells that have lost MHC class I expression (227). NK cells have two types of receptors, (i) natural cytotoxicity receptors (NCRs) that detect the altered expression of ligands on the surface of tumour cells resulting in NK cell activation (228), and (ii) killer immunoglobulin-like receptors (KIRs), which recognise MHC class I molecules resulting in NK cell inhibition. In the steady-state, KIR-mediated inhibition overrides NCR-mediated activation signals. In cancer, triggering of the NCR while also failing to engage KIRs

due to MHC class I loss, delivers an activation signal to the NK cells, leading to NK cell activation and tumour cell lysis (228).

NK cell-mediated killing depends on perforin and granzyme and also FasL, and TNF related Apoptosis-Inducing Ligand (TRAIL) (227) NK cells also secrete pro-inflammatory cytokines and chemokines such as IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF), CCL5, XCL1 and IFNγ (227). In mouse models, CCL5 and XCL1 induced accumulation of conventional DCs (cDCs) whereas in humans, intratumoral levels of CCL5, XCL1, and XCL2 transcripts correlated with gene signatures of NK cells and cDCs (229). Accumulation of cDCs following NK cell activation can attract T cells (230), re-stimulate and expand tumour-specific CD8+ T cells (231), and increase T cell effector function via DC secretion of IL-12 (229, 232). Moreover, it's been shown that NK cells demonstrate innate and potent anti-metastatic activity and NK cell immunotherapy has great impact in the treatment of metastatic patients with promising clinical outcomes (233).

TRegs and MDSCs inhibit activation and function of NK cells via different mechanisms including secretion of cytokines (TGFβ) and metabolites (adenosine, Prostaglandin-E (PGE) and IDO) that suppress maturation, proliferation and functional activities of NK cells. Restoring NK cell function by overcoming the immunosuppressive nature of tumour microenvironment provides an attractive therapeutic option (234).

Tumour associated macrophages (TAMs)

Macrophages are antigen-presenting cells resident in tissues. TAMs differentiate in the TME from peripheral blood monocytes recruited to the tumour and represent a large population of tumour-resident myeloid cell (235). TAMs promote tumour

progression by multiple mechanisms including enhancement of cancer cell proliferation, invasion, and metastasis, stimulation of tumour angiogenesis, and inhibition of anti-tumour immune responses mediated by T cells (236). There are two types of TAMs, M1-polarised and M2-polarised macrophages. M1-polarized macrophages are pro-inflammatory, develop in the presence of GM-CSF and immunostimulatory cytokines such as IFN-γ, IL-12 and IL-23 and contribute to tumour rejection. Alternatively activated (M2-polarized) macrophages are pro-tumorigenic, develop in the presence of M-CSF and Th2 cytokines like IL-4, IL-10, and IL-13, and contribute to chronic inflammation, tumour angiogenesis, tumour metastasis and tumour growth (235). M2-polarized pro-tumorigenic macrophages release immunosuppressive products into the tumour microenvironment (e.g. arginase, IL-10 and TGFβ) and drive tumour tissue remodelling through production of extracellular matrix proteins and cytokines supporting formation of new blood vessels (e.g. VEGF-A). Programming TAMs from M2-polarized to M1-polarized (classically activated) is an attractive therapeutic option. TAMs inhibit cytotoxic T lymphocyte (CTL) responses through production of IL-10 (237), indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE) whereas TAM-derived CCL17, CCL18 and CCL22 can attract TRegs (238), thus enhancing immune suppression in the TME (235).

Myeloid-derived suppressor cells (MDSCs)

MDSCs are a heterogeneous population of myeloid cells, including early myeloid progenitors, immature granulocytes, macrophages, and dendritic cells at early stages of differentiation (239). MDSCs are characterised by their ability to potently suppress T cell responses. These cells use a broad range of suppressive molecules to silence immune responses and inhibit anti-tumour effects of the immune system, leading to tumour growth and metastasis (240). MDSCs suppress immune responses through

their production of reactive oxygen and nitrogen species, arginases 1 and 2, enzymes that metabolise L-arginine, IDO and immunosuppressive cytokines (239, 240). Degradation of arginine by arginase 1 causes a translational blockade in tumour-infiltrating T cells leading to cell cycle arrest (241). IDO breaks down tryptophan, and a reduction in tryptophan levels leads to T cell cycle arrest and T cell anergy (242). MDSCs also promote differentiation of conventional CD4+ T cells into immunosuppressive TRegs (243). Reactive oxygen species produced by MDSCs have the ability to induce T cell apoptosis (153) while immunosuppressive cytokines such as TGFβ and IL-10 inhibit anti-tumour effects of T cells and recruit TRegs (244, 245). Taken together, MDSCs block T cell activation, inhibit T cell proliferation, and promote T cell apoptosis. The efficacy of immunotherapy is inversely correlated with MDSCs activity (246), suggesting that MDSCs are a promising target in cancer immunotherapy.

Dendritic cells (DCs)

DCs are professional antigen-presenting cells critical for the activation of naïve T cells. They uptake, process and present tumour-derived antigens to naive T cells. DCs activated through danger signals (such as microbial products) undergo a process of maturation, whereby their ability of process antigen is decreased and their ability to stimulate T cell responses is dramatically increased. DCs sample tumour antigens in the tumour tissue and migrate to the lymph node where they interact with naïve T cells. Naïve T cells proliferate and differentiate into effector T cells, capable of travelling to tumours and interacting with tumour cells. In the context of cancer, immune tolerance to cancer is attributed to the inability of DCs to mature properly (247).

DC presence in tumours has been associated with both good and poor prognosis (247). For example, in colorectal cancer, DCs were associated with shorter disease-free and overall survival (248), whereas in melanoma tumour DCs were associated with disease regression (249).

Several subsets of DCs with distinct functions, morphology and localization are found in the TME, including classical myeloid DCs, plasmacytoid DCs that produce type I interferons, and occasionally Langerhans cells in skin lesions (250). A rare subset of dendritic cells expressing transcription factor BATF3, and capable of cross-presenting tumour antigens to CD8+ T cells, was critical for activation of naïve CD8 T cells in tumour-draining lymph nodes and attraction of effector CD8 T cells to the tumour site (251).

Neutrophils

Tumour cells that express ligands for neutrophil chemokine receptors CXCR1 and CXCR2, including chemokines CXCL8, CXCL5, CXCL6 can recruit circulating neutrophils to the tumour site (252-254). Tumour associated neutrophils (TANs) can be polarized to an N1 (anti-tumoural) or N2 (pro-tumoural) phenotype in response to the microenvironment (252). N1 neutrophils, such as those induced after TGFβ blockade, produce immune activating cytokines and chemokines and low levels of arginase, and are capable of killing cancer cells (255), and additionally, activate the immune response against tumours (256). In contrast, N2 neutrophils are characterized by high expression of CXCR4, VEGF, and gelatinase B/MMP9, and directly contribute to tumour progression, invasion and angiogenesis (257) or via ECM remodelling (252).

1.3.2 Interactions between cancer cells and immune cells in the tumour microenvironment

Cancer cells interact with non-cancerous cells in the TME occur via direct cell-cell contacts or indirectly via secretion of soluble factors, like cytokines and chemokines (258). Both types of interactions can result in a temporary or permanent alteration of cell phenotype and function, by inducing expression of genes involved in tumour cell survival, proliferation, invasion and metastasis and/or immune evasion (123). Table 1.2 details the role of cytokines known to influence tumour growth and progression in melanoma (259).

Table 1.2 Main cytokines in the melanoma microenvironment

Cytokine	Main Source	Targets	Major functions
IL-10	TAMs, BRegs, TRegs	NK cells, CD8 T cells	Inhibits production of IFNγ by Th1 cells. Inhibits expression of MHC class II
IL-12	Antigen presenting dendritic cells	T cells, NK cells	Promotes Th1 differentiation and IFNγ production; enhances cytotoxic activity of CD8 T cells and NK cells
IL-18	Tumour cells, epithelial cells, TAMs, DCs	NK cells, T cells	Together with IL-12 promotes IFNγ production by Th1, CD8 and NK cells
IL-2	Activated CD4+ T cells and NK cells	Activated CD4+ and CD8+ T cells, TRegs	Limits T cell responses, stimulates T cell proliferation, expands TRegs
IL-6	CD4+ T cells, TAMs, CAFs	Activated B cells, plasma cells	Promotes differentiation of B cells to plasma cells, promotes proliferation, survival and differentiation of malignant cells
IL-8	Tumour cells, TAMs	Neutrophils, MDSCs	Recruits neutrophils and MDSCs to TME, dampens anti-tumour immune responses
TGFβ	T cells, B cells, TAMs, CAFs	CD8+ T cells, NK cells, activated B cells	Suppresses immune activity, inhibits T cell and B cell proliferation, inhibits IFNγ production

TGFβ, transforming growth factor ß; TAMs, tumour-associated macrophages; CAFs, cancer-associated fibroblast; NK cells, natural killer cells; BRegs, regulatory B cells; TRegs, regulatory T cells; MHC, major histocompatibility complex; DCs, Dendritic cells; MDSCs, myeloid-derived suppressor cells; IL, interleukin; Th, T helper; IFNγ, interferone gamma; TME, tumour microenvironment

1.4 IFNγ and TNFα: important cytokines in melanoma microenvironment

T cells secrete multiple cytokines that can have various biological effects on tumour cells, including stimulation or suppression of tumour growth, invasion, angiogenesis and metastasis. The two principal cytokines produced by T cells in the TME are interferon gamma (IFNγ) and tumour necrosis factor α (TNFα).

1.4.1 Interferons (IFNs)

Interferons (IFNs) are multifunctional cytokines that participate in tumour surveillance and defence against viral and bacterial infections (260), by activating innate and adaptive immune responses (260, 261). IFNs can be produced by, and act on, both tumour cells and immune cells (261), with the exception of IFNγ that is produced by T cells and NK cells. IFNs can either enhance or suppress the immune response, depending on the timing and duration of IFN signalling (262).

There are three types of IFNs (Type I, Type II and Type III) that are categorised based on their structure and immunomodulatory characteristics. Interferons are structurally and functionally distinct and each group binds to their specific receptors (260, 263) (Figure 1.3)

Type I interferons include IFNα, IFNβ, IFNε, IFNκ, and IFNω. IFNα and IFNβ are the most abundant, and are ubiquitously expressed (260). All type I interferons bind to a heterodimeric receptor composed of two subunits, IFNα receptor 1 (IFNAR1) and IFNα receptor 2 (IFNAR2) (264), with IFNβ having the highest affinity for the receptor and IFNα, the lowest. IFNα/β receptor lacks intrinsic kinase activity and relies on associated Janus kinases (JAKs) to initiate signalling. Ligand binding leads to the

phosphorylation of receptor-associated TYK2 and JAK1, and subsequent phosphorylation of signal transducers and activators of transcriptions, STATs. Phosphorylated STAT1 and STAT2 dimerise and bind to the DNA-binding protein, p48 to form the IFN-stimulated gene factor 3 (ISGF3) transcription factor. This factor translocates to the nucleus and activates transcription of IFN-responsive genes (264, 265).

Type III interferons include the four members of the IFNλ family (IFN-λ1 (IL-29), IFN-λ2 (IL28A), IFN-λ3 (IL-28B) and IFN-λ4), which bind a distinct heterodimeric receptor composed of the IFNλR1 and IL10R2 chains. Similar to type I IFNs, signal transduction of type III IFNs involves receptor-associated JAK1 and TYK2, downstream STAT proteins and ISGF3 (264, 266). Binding of Type III IFNs to the receptor complex activates JAK1 and TYK2 and the two kinases cross-phosphorylate and thus activate one another (267). Due to their similar signalling cascade, type I and III IFNs share many anti-viral, anti-proliferative, apoptotic, and immunomodulatory effects (268).

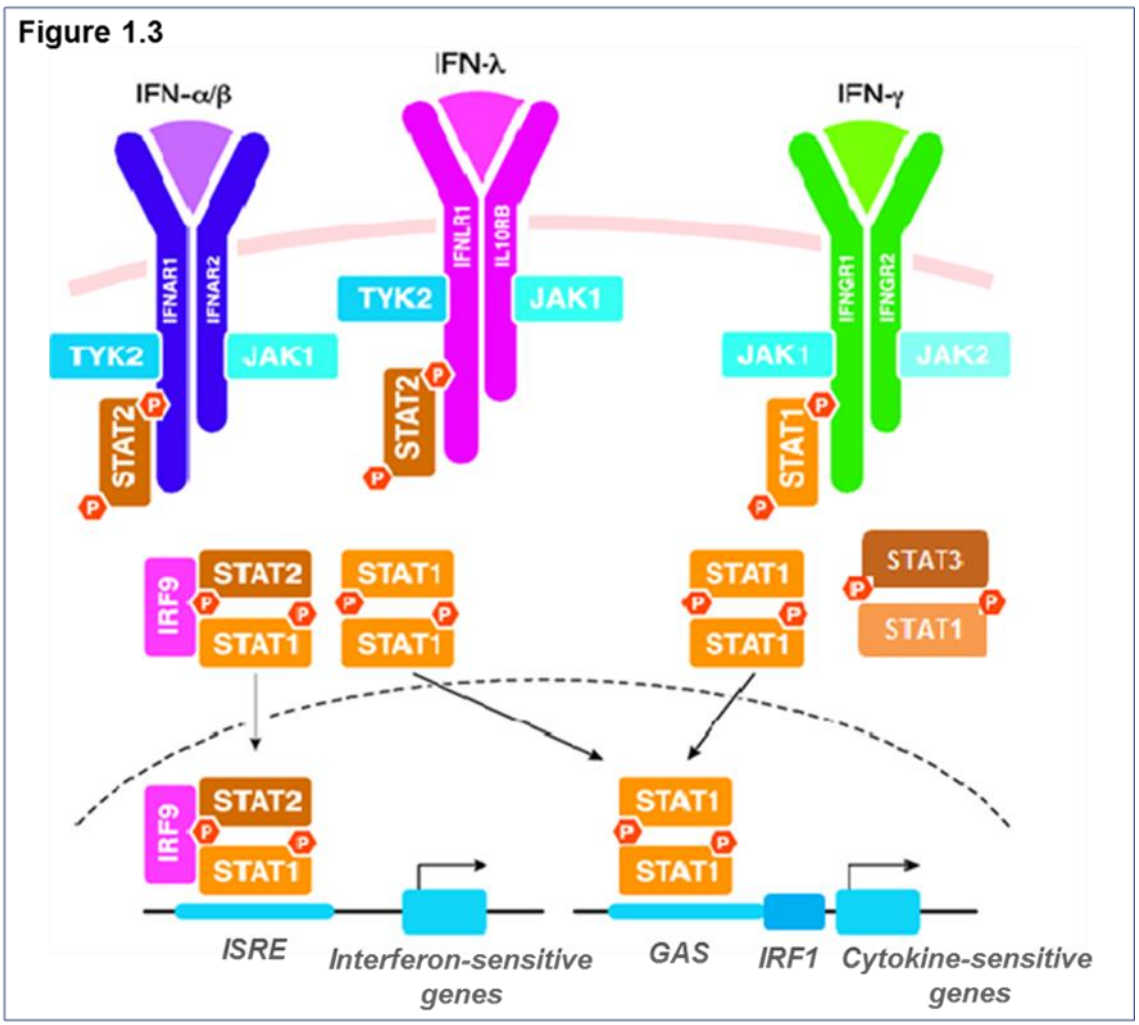

Figure 1.3 Interferon signalling pathways

IFN type I binding to the receptor (IFNAR1/IFNAR2) induces phosphorylation of receptor-associated TYK2 and JAK1, which leads to phosphorylation and activation of STAT1, STAT2 and IRF9. The complex of STAT1/2/IRF9 (known as IFN-stimulated gene factor 3) translocates to the nucleus and binds to the IFN-Sensitive Response Element (ISRE) initiating expression of hundreds of IFN-dependent genes. IFN type III binding to the receptor (IFNλR1/IL10R2) induces phosphorylation of receptor-associated JAK1 and TYK2, and IFN type II binding to the receptor (IFNGR1/IFNGR2) induces phosphorylation of receptor-associated JAK1 and JAK2. All three IFN types induce phosphorylation of STAT1, which then forms homodimers or heterodimer with STAT3, translocate to the nucleus, bind the canonical gamma activated sequence (GAS) elements in the nucleus and promotes expression of IFN-dependent genes. Figure derived from (269).

IFNγ is the only member of type II interferons, and is produced by Th1 cells, CD8+ T cells, NK T cells and NK cells (270). IFNγ signals through the receptor composed of two chains, IFNγR1 and IFNγR2 (163, 260). Binding of IFNγ and receptor assembly results in the cross-phosphorylation of receptor-associated JAK1 and JAK2, which in turn triggers dimerization and phosphorylation of the transcription factor STAT1, or occasionally, STAT3 (Figure 1.3) (265, 270, 271). Phosphorylated STAT1 translocates to the nucleus and binds to gamma-activated sequence in the promoters of target genes, including interferon response factor 1 (IRF1). IRF1 transcription activates the expression of many IFNγ response genes and secondary response genes, like Class II Trans Activator (260, 272). Negative regulators of IFNγ signalling, like the Suppressor Of Cytokine Signalling proteins (SOCS) 1 and 3, bind to and directly inhibit JAK activity (265, 273). Although IFNγ mainly signals via the JAK-STAT pathway, IFNγ can also act through alternative signalling pathway independent of STAT1 recruitment (274). In the absence of STAT1, one third of IFNγ stimulated genes can still be induced (275).

IFNγ plays multiple roles in tumour progression, through MDSCs activation, exacerbation of Th17 associated inflammation (276) and upregulation of the immune checkpoint PD-L1, leading to tumour immune escape (277). MDSCs activation can suppress immune responses by inhibiting CD4+ and CD8+ T cell function, while inflammation induced by Th17 cells help convert conventional CD4+ T cells to immunosuppressive TRegs. On the other hand, anti-tumour effects of IFNγ are well documented and include:

1. Suppression of carcinogen-induced sarcomas and spontaneous carcinomas through IFNγ-dependent immune surveillance mechanisms (124, 276, 278).

2. Enhanced tumour recognition by the immune system via upregulation of MHC class I and class II molecules on tumour cells (203, 279-281).

3. Anti-proliferative and direct cytotoxic effects on tumour cells (276, 282).

4. Anti-angiogenic effects, including endothelial cell killing and inhibition of endothelial cell proliferation (203, 282, 283).

5. Antagonizing the activity of immune suppressive cytokines such as TGFβ or IL-10 (276).

6. Activation of NK T cells and NK cells in the TME (284, 285).

Several reports have demonstrated an increase in IFNγ production by T cells after therapeutic immune checkpoint blockade (286-288). Consequently, mutations in the IFNγ receptor or components of its signalling pathway (i.e loss of function mutation in JAK1 JAK2 and STAT1 or IFRNGR1/2 deletion) are associated with resistance to immune checkpoint inhibitors in melanoma (193, 194, 288-291).

1.4.2 Tumour necrosis factor alpha (TNFα)

Tumour necrosis factor alpha (TNFα) is a proinflammatory cytokine mainly expressed by activated macrophages, T lymphocytes and NK cells (292). TNFα is part of a superfamily composed of 19 ligands and 29 receptors (293). TNFα acts via binding to its receptors, TNFR1 and TNFR2. TNFR1 is ubiquitously expressed in essentially all cell types (293) while TNFR2 is expressed mainly on lymphocytes and endothelial cells (294). TNFR1 possesses a death domain that is absent in TNFR2. TNF binding triggers receptor trimerization that leads to the recruitment and assembly of other intracellular signalling molecules and results in the activation of downstream signalling pathways (295). The proapoptotic pathway is generally triggered by soluble

TNFα binding to TNFR1. The death domain of TNFR1 assembles a death-inducing signalling complex containing adaptor molecules TNFR-associated death domain (TRADD), receptor interacting protein (RIP) and Fas-associated death domain (FADD) that recruits caspase 8. This death-inducing signalling complex triggers apoptosis through caspase activation, DNA fragmentation and nuclear collapse (Figure 1.4) (295, 296).

Activation of survival pathways involves recruitment of TNFR-associated factor 2 (TRAF2) to either TNFR1 or TNFR2. TRAF2 activates nuclear factor-kappa B (NF-Kb) and the MAPK pathways, ultimately resulting in the transcription of proinflammatory and immunomodulatory survival genes (297).

The effects of TNFα within the tumour microenvironment may depend on its local concentration and site of expression. High concentration of TNFα can induce anti-tumoural responses while low, sustained levels of TNFα can promote tumour progression (295, 298, 299) via direct and indirect mechanisms:

1. TNFα promoted tumour invasion through NF-kB and JNK-mediated upregulation of migration-inhibitory factor (MIF) in macrophages and enhanced production of matrix metalloproteinases (MMPs) in tumour cells (reviewed in (293).

2. TNFα induced angiogenesis by stimulating production of angiogenic factors, such as vascular endothelial cell growth factor (VEGF) in the TME (298).

3. TNFα helped attract inflammatory cells to the TME, which increased proliferation and survival of tumour cells (295, 298).

4. TNFα suppressed immune responses (300) by facilitating accumulation of TRegs (301), BRegs (302) and MDSCs (303).

5. TNFα exposure promoted the activation-induced cell death in CD8+ T cells (304, 305).

6. TNFα produced by CD4 tumour-infiltrating T cells inhibited cytotoxic CD8+ T cell responses. (202).

7. TNFα induced expression of immune suppressive molecules, PD-L1 and T cell immunoglobulin and mucin domain containing-3 (TIM-3), which inhibited CD8+ T cell activation in the tumour microenvironment (300, 306). Due to its protumourigenic activities, constitutive expression of TNFα in the tumour microenvironment is usually associated with poor prognosis. Inhibition of TNFα may help increase numbers of functional CD8+ T cells in the TME (300).

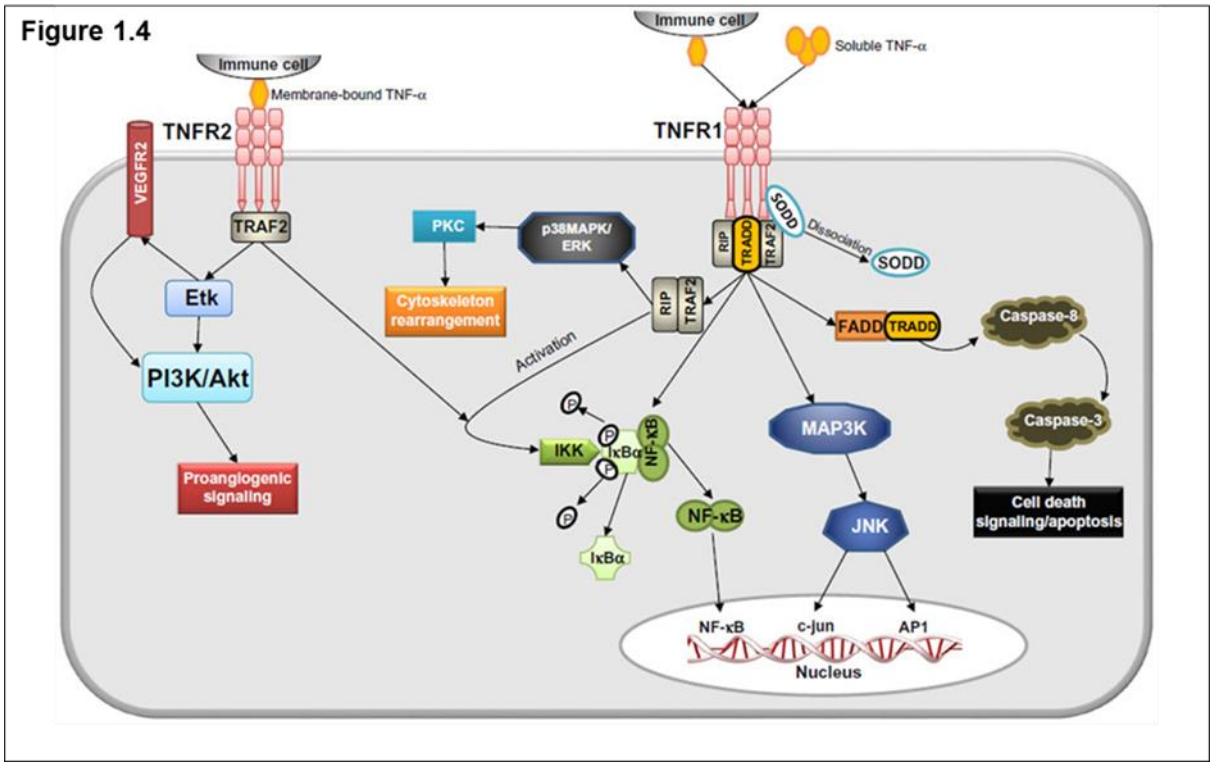

Figure 1.4 TNFα-regulated signalling pathways

TNFα signals through two receptors, TNFR1 and TNFR2, that bind mainly soluble or membrane TNFα, respectively. Unlike TNFR2, TNFR1 possesses a death domain that assembles a death-inducing signalling complex containing adaptor molecules TRADD and FADD and caspase 8. Death-inducing signalling complex triggers apoptosis through caspase activation. Recruitment of the adaptor protein TRAF2 to either TNFR1 or TNFR2 promotes activation of NF-kB and MAPK pathways, and the transcription of genes associated with cell survival and production of inflammatory cytokines. Figure adapted from (307).

1.5 Current therapies for melanoma

Treatment options for melanoma depend on the type of melanoma, the stage and severity of the disease. Table 1.3 summarizes common treatment strategies for melanoma including chemotherapy, targeted therapy and immunotherapy.

1.5.1 Surgery

In patients with stage I-III melanoma, surgery is the primary therapeutic option (308). In advanced disease, metastasectomy (surgical excision of the metastatic lesions) continues to be the standard of care for patients who present with a solitary melanoma metastasis (309). Local recurrence such as in-transit disease is also effectively treated by surgical excision. Moreover, surgery can also be useful for patients with metastatic disease that spread beyond the regional nodes (310).

Surgical resection of melanoma lesions has been shown to increase survival rate. For example, in melanoma patients with subcutaneous and lymph node metastases, surgical resection increases the median survival rate to 35 months, compared to just 6 months for patients without surgery (311). Lung is one of the most common sites of metastasis for melanoma patients and patients with lung metastases, but no prior surgery had a median survival of 13 months compared with 40 months after surgical resection, with a 5-year survival of 8% and 35%, respectively (312, 313). Similarly, the survival rate of melanoma patients with gastrointestinal metastases and no surgery is 4 to 6 months while surgical resection resulted in a 5-year survival in 27% of patients (309).

1.5.2 Chemotherapy

Chemotherapy has been used to treat advanced melanoma for over three decades. Chemotherapeutic agents like dacarbazine, temozolomide, tamoxifen and cisplatin have modest anti-tumour efficacy in metastatic melanoma, when used alone or in combination (308, 314). Single-agent chemotherapy is well tolerated but is associated with overall response rates of only 5% to 20% (314). Combination chemotherapy demonstrated a slight improvement in response rate and progression free survival, but not overall survival or quality of the response (314).

1.5.3 Targeted therapy

The identification of driver mutations in melanoma was a critical step towards the effective treatment of this disease (315). Targeted therapy uses small molecular inhibitors that specifically block the activity of the kinase that drives melanoma cell proliferation and survival.

Treatment of patients with advanced $BRAF^{V600E/K}$ mutant melanoma with selective BRAF inhibitors (vemurafenib, dabrafenib or encorafenib) has dramatically improved patient response rates and overall survival. For instance, treatment with vemurafenib or dabrafenib produced response rates of 48% to 50% with a median progression free survival (PFS) of 5.1 to 5.3 months and a median OS of 13.6 months (316-318). Similarly, $BRAF^{V600}$ mutant melanoma patients treated with encorafenib showed response rates of approximately 70% and a median PFS of 11.3 months (319). Targeting the downstream MEK proteins has also shown significant anti-tumour activity in patients with $BRAF^{V600}$ mutant melanoma (320). Selective inhibitors of the MEK1/2 kinases include trametinib, binimetinib and cobimetinib (315) (Figure 1.1). Treatment of patients with $BRAF^{V600}$ mutant melanoma with trametinib monotherapy

resulted in a response rate of 22% and improved OS comparison to standard chemotherapy (320). Today, patients with $BRAF^{V600}$ mutant melanomas are treated with combination of BRAF and MEK inhibitors (315). Different studies confirmed that combination therapy showed less cutaneous toxicity with high response rates of around 70%, improved median PFS of 12 months, and improved median OS of approximately 24 months (315, 321, 322).

Most patients will develop resistance to BRAF inhibitors, usually via mechanisms that reactivate the MAPK pathway (mutations in NRAS or MEK, overexpression of kinases (CRAF, Osaka thyroid (COT)) or expression of alternate BRAF splice variants). The activation of the PI3K/AKT pathway is also associated with resistance to BRAF inhibitors and occurs predominantly via receptor tyrosine kinase activation (315). Another treatment option for patients who have failed targeted therapy is immune checkpoint blockade (section 1.6).

1.5.4 Immunotherapy

Immune checkpoint inhibitors have dramatically improved the survival of patients with advanced melanoma (323) and several other forms of immunotherapy have also been used in the treatment of melanoma including cytokines, oncolytic viruses, vaccines, nonspecific immune stimulators and adoptive cell therapy (ACT) (323).

Cytokines

A wide range of cytokines, including IL-2, IL-12, IL-15, IL-21, GM-CSF, and IFN-α have shown efficacy in preclinical murine cancer models (324), and IFN-α and IL-2 are used in the treatment of human melanoma. Adjuvant use of IFN-α2 improved disease-free survival, and OS in the majority of studies (325-327), with response rates of 10-20%. The precise mechanism of action is poorly understood but may

include direct anti-proliferative effects on melanoma cells, augmentation of NK cell killing, and upregulation of tumour antigen expression and antigen presentation via increased MHC molecule expression. Patients with non-visceral disease are more likely to respond to cytokine therapy but treatment is associated with severe toxicities (328).

Oncolytic viruses

Oncolytic viruses are non-pathogenic viruses that selectively infect and kill neoplastic cells but don't affect the non-transformed cells (329). Talimogene laherparepvec (T-VEC) is a modified form of the herpes simplex type 1 virus currently in clinical trial for melanoma. T-VEC needs to be injected intralesionally, therefore its use is restricted to patients with visible subcutaneous disease (330). T-VEC was the first oncolytic virus to be combined with the immune checkpoint inhibitor ipilimumab for treatment of advanced melanoma (331). The combination of T-VEC and ipilimumab produced overall response rate (ORR) of 39% and PFS of 8.2 months compared with 18% ORR and PFS of 6.4 months with ipilimumab monotherapy (331).

Vaccines

Melanoma vaccines use synthetic antigenic peptides, or tumour-derived antigens administered in an immunogenic way to trigger an immune response. Experimental clinical trials for "melanoma vaccines" are currently in progress and different types of antigens have been used in vaccine production, including tumour peptides, glycolipids, synthetic tumour-associated antigens like MLANA/MART-1, gp100, tyrosinase, tyrosinase-related protein-1, and tyrosinase-related protein-2. These antigens are administered with dendritic cells, adjuvants, cytokines or oncolytic viruses to boost the immune response (332-334) . In one study, combination of gp-

100 vaccine and IL-2 was shown to be associated with higher response rates and longer PFS than IL-2 alone (reviewed in (333, 335). Cancer-testis antigen NY-ESO-1 is a common target for cancer immunotherapy, due to widespread expression in melanoma and a range of other cancers, restricted expression in normal tissues, and strong immunogenicity. Both peptide and protein-based cellular vaccines and adoptive cell transfers of NY-ESO-1 reactive T cells are currently being trialled in melanoma (http://www.clinicaltrials.gov) (336).

There are two categories of vaccines using tumour cell-derived antigens, autologous and allogeneic vaccines. Autologous vaccines use patient's own tumour cells. In a phase II clinical trial for metastatic melanoma, an autologous vaccine composed of tumour-derived heat shock protein gp96 was shown to induce an MHC class I mediated immune reaction in a proportion of treated patients. However only 2/28 patients enrolled had a complete response, and 3/28 had stable disease at the end of follow up (reviewed in (333, 337).

Allogeneic vaccines use melanoma cells from other patients selected for a variety of shared antigens. In the largest phase II clinical trial, use of Canvaxin polyvalent cancer vaccine showed a prognostic significance for melanoma patients with stage III and IV melanoma, but phase III clinical trial for stage III unresected/stage IV melanoma showed unfavourable results (333, 338).

Toll like receptor (TLR) agonists

Toll like receptors (TLR) recognize pathogen-associated or damage-associated molecular patterns. Activation of TLRs triggers innate immune responses with release of pro-inflammatory cytokines, and activation of local phagocytosis and

antigen presentation (339). TLR7 agonist imiquimod has been used to treat residual disease where wide surgical resection is difficult, and may require plastic surgical reconstruction, such as in acral lentiginous melanoma (ALM) (339).

Adoptive cell therapy (ACT)

Adoptive cell therapy (ACT) is based on collecting autologous T cells reactive against melanoma antigens (called tumour-infiltrating lymphocytes, or TILs). TILs are expanded and re-activated in vitro prior to re-infusing in patients (323). In one study of 93 melanoma patients treated with ACT, ORR was 56% (340). Additionally, T cells can be genetically engineered to target the antigen of choice. Either a T cell receptor (TCR) reactive with a shared melanoma antigen, or a chimeric antigen receptor (CAR), is introduced by gene therapy. The main advantage is that peripheral blood T cells rather than TILs can be used, so no surgery is required. The resulting population of T cells will uniformly recognize one tumour antigen bound to one HLA molecule, which limits the applicability of this approach to HLA-compatible patients. Another limitation is identification of tumour antigens whose expression is restricted to the tumour tissue, in order to avoid off-target toxicities (341). A successful clinical trial using a melanoma-specific TCR (MART-1 or gp100) demonstrated good clinical efficacy (30% for MART-1 TCR, 19% for gp100 TCR), but also a substantial incidence of autoimmune manifestations such as uveitis and hearing loss due to the destruction of melanocytes of the eye and ear (41.7 %) (341).

Immune checkpoint blockade

To prevent excessive tissue damage at the site of inflammation, activated T cells need feedback mechanisms that slow down the immune response. Negative regulators (“inhibitory checkpoints”) such as cytotoxic T lymphocyte-associated

antigen-4 (CTLA-4), programmed cell death protein-1 (PD-1), lymphocyte activation gene-3 (LAG-3) and TIM-3 are upregulated on activated T cells and block T cell activation and/or effector differentiation after ligand binding (342). Therapies targeting these negative checkpoints are being trailed in many cancers, including melanoma, and details regarding the activity of PD-1 and CTLA-4 inhibitors in melanoma are described in Section 6. Several co-stimulatory immune molecules are also being targeted therapeutically and include the TNF receptor superfamily members OX40 (CD134) and glucocorticoid-induced TNFR family related gene (GITR), CD40 and CD122 (323).

Table 1.3 Summary of treatment options for melanoma

Treatment	Drug	Target	Mechanism of action
Chemotherapy	Dacarbazine Temozolomide Fotemustine	DNA	They cross-link DNA during all phases of the cell cycle, resulting in Methylation and/or alkylation of DNA, disruption of DNA function, cell cycle arrest, and apoptosis (343)
Targeted therapy	Vemurafenib Dabrafenib Encorafenib	*BRAF*$^{V600E/K}$	Selective inhibitors of the *BRAF*$^{V600E/K}$ which may result in an inhibition of an over-activated MAPK signalling pathway and a reduction in tumour cell proliferation (316, 319, 344)
	Imatinib, Nilotinib	c-KIT	Small molecular inhibitor of KIT tyrosine kinase. Their activity results in decreased proliferation and enhanced apoptosis in malignant cells (345)
	Trametinib, Cobimetinib	MEK	Allosteric MEK1/2 inhibitor, resulting in an inhibition of growth factor-mediated cell signalling and cellular proliferation in cancers (346)
Immunotherapy	Ipilimumab Tremelimumabx	CTLA-4	Monoclonal antibody against the CTLA-4 immune checkpoint
	Pembrolizumab Nivolumab	PD-1	Monoclonal antibodies against the PD-1 immune checkpoint
	Avelumab Atezolizumab	PD-L1	Monoclonal antibodies against PD-L1 immune checkpoint which inhibit binding to PD-1. This may restore immune function through the activation of cytotoxic T lymphocytes (347)
	Epacadostat	IDO	Inhibits IDO function, increases and restores the proliferation and activation of various immune cells, including DCs, NK cells, and T-lymphocytes, as well as IFNs production, and a reduction in tumour-associated TRegs (348)
	BMS-986016 LAG525	LAG-3	These monoclonal antibodies inhibit LAG-3 and activate antigen-specific T-lymphocytes and enhance cytotoxic T-cell-mediated tumour cell lysis, leading to a reduction in tumour growth (349)

	MBG453, TSR-022	TIM-3	A monoclonal antibody against TIM-3, Inhibits TIM-3 function so increase duration and scale of Th1 and Tc responses, abrogates T-cell inhibition, activates antigen-specific T lymphocytes and enhances cytotoxic T-cell-mediated tumour cell lysis, which results in a reduction in tumour growth (350)
	MEDI0562	OX40 (CD134)	An agonistic, humanized monoclonal antibody against receptor OX40 (CD134), with potential immunostimulatory activity, induction of proliferation of memory and effector T-lymphocytes (351)
	TRX518	GITR	A monoclonal antibody with immune stimulatory activity, induce both the activation of tumour-antigen-specific T effector cells, as well as abrogates the suppression induced by inappropriately activated T regulatory cells (352)
	Lirilumab	KIR	A fully human monoclonal antibody against killer-cell immunoglobulin-like receptors. It prevents the binding of KIR ligands to KIR on NK cells. By blocking these inhibitory receptors, NK cells become activated and attack cancer cells leading to tumour cell death (353)
	IL-2	Immune-stimulating activity	Stimulation of immune responses (354)
	T-VEC	Tumour cells	Oncolytic virus encodes the immunostimulating GM-CSF. Upon intertumoral injection, T-VEC kills tumour cells directly and boosts immune response by expressing GM-CSF (355)
	BCG vaccine	Immune-stimulating activity	Adjuvant effect (improves immune activity), immunization with BCG vaccine activates a Th1 cytokine response that includes the induction of interferon (356, 357)
	Imiquimod	TLR7	Activates local immunity via TLR7 stimulation, stimulates cytokine production, especially interferon production, and exhibits antitumor activity, particularly against cutaneous cancers (358)

MEK, mitogen-activated protein kinase kinase; CTLA-4, cytotoxic T-lymphocyte-associated protein-4; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; IDO, indoleamine 2,3-dioxygenase; LAG-3, lymphocyte activation gene 3; TIM-3, T cell immunoglobulin and mucin domain containing-3; GITR, glucocorticoid-induced TNFR family related gene; KIR, killer-cell immunoglobulin-like receptors; GM-CSF, granulocyte-macrophage colony-stimulating factor; BCG , Bacillus Calmette–Guérin; TLR, toll like receptor; Th, T helper; T-VEC, Talimogene laherparepvec

1.6 Immune checkpoint blockade in melanoma

CTLA-4 was the first immune checkpoint to be therapeutically targeted (359, 360). CTLA-4 is expressed on the surface of activated T cells and is constitutively expressed on TRegs. CTLA-4 ligands, B7-1 (CD80) and B7-2 (CD86) are expressed on antigen-presenting cells and bind not only CTLA-4 but also CD28, a co-stimulatory molecule required for T cell activation (361) (Figure 1.5). Engagement of CTLA-4 delivers an inhibitory signal to responding T cells that is capable of overriding TCR- and CD28-mediated signals (362). CTLA-4 binds both CD80 and CD86 with higher affinity than CD28; approximately 20-fold higher affinity for CD80 and approximately 8-fold higher affinity for CD86 (363). Binding of CTLA-4 results in suppression of T cell activation (364, 365) hence CTLA-4 blockade enhances T cell-mediated anti-tumour immunity.

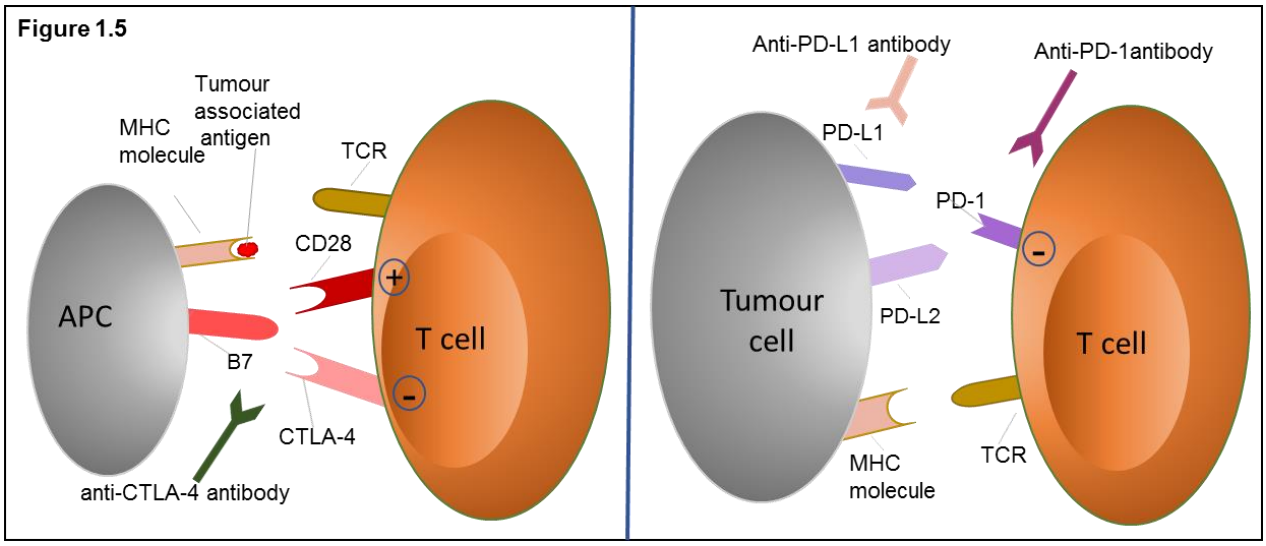

Figure 1.5 Immune inhibitory checkpoints targeted by therapeutic antibodies

(A) T cell activation is blocked when T cell inhibitory receptors PD-1 and CTLA-4 bind their respective ligands, PD-L1/L2 and B7, in the tumour microenvironment. (B) These inhibitory interactions are blocked by immunotherapeutic antibodies (anti-PD-1, anti-PD-L1, anti-CTLA-4), resulting in T cell activation and tumour cell killing. TCR, the T cell receptor; MHC, major histocompatibility complex; CD28, T cell co-stimulatory receptor; B7, a co-stimulatory ligand for CD28 that also binds CTLA-4 thereby preventing B7-CD28 interaction and T cell activation.

Anti-CTLA-4 treatment increased IFNγ production by T cells in mouse models and in bladder cancer patients treated with anti CTLA-4 (288, 366). Ipilimumab is a fully human monoclonal antibody of the IgG1 isotype that binds CTLA-4 and prevents its interaction with its ligands (367, 368). In a randomized phase III trial for patients with previously treated, unresectable stage III or stage IV melanoma, treatment with ipilimumab increased survival compared to treatment with glycoprotein 100 (gp100) peptide vaccine (45.6 % vs. 25.3 % at one year, 23.8 % vs. 16.3 % at two years) (369).

Patients with unresectable stage III or IV melanoma treated with ipilimumab with or without glycoprotein 100 (gp100) peptide vaccine, showed a 10.1 months improvement in overall survival and a 10.9% improvement in response rate, including

complete and partial responses compared with gp100 alone, leading to FDA approval for treatment of patients with late stage, unresectable melanoma (369). In Australia, ipilimumab was TGA-approved as first line therapy for patients with unresectable or metastatic melanoma in 2015. In Phase II and III clinical trials, advanced melanoma patients treated with ipilimumab showed 11.4 months OS with durability of response (370). Ipilimumab treatment-related adverse events are often mild to moderate and occur in 70-80% of patients (370, 371). Experiments in mice demonstrated that anti-CTLA4 antibodies of certain isotypes induced a selective depletion of intratumoural Tregs via andibody-dependent cell mediated cytotoxicity (ADCC) (372, 373). However, there is no evidence that this mechanism also operates in people (374).

Inhibitors of the immune checkpoint inhibitor PD-1 have also improved the survival of patients with advanced melanoma. PD-1 is expressed on the surface of activated T and B lymphocytes, NK cells and myeloid cells (375, 376). After persistent TCR stimulation, PD-1 is induced on T cells, and its ligation triggers lymphocyte apoptosis (377, 378). Persistent T cell stimulation promotes high levels of PD-1 expression that can lead to T cell exhaustion, a state where T cells gradually lose their effector functions (368, 370). PD-1 expression on CD4+ T cells promotes their conversion to suppressive TRegs (379). PD-1 also plays a role in inhibiting B cell clonal responses (379, 380), but the relevance of this observation in cancer treatment is unclear (226).

Due to its suppressive activity, PD-1 has been implicated in progression of several cancers including melanoma, non-small cell lung cancer (NSCLC), breast and renal cell carcinoma (379, 381). Hence, monoclonal antibodies that block PD-1 interaction have shown potent effects in abrogating immune suppressive functions and promoting anti-cancer immunity (382).

PD-1 is normally expressed on memory CD4+ T cells and Tfh cells. Follicular helper cells (Tfh), which are key cells to provide B cell help and promote the germinal centre (GC) reaction, express high levels of PD-1 due to continuous TCR triggering by interactions with cognate B cells. PD-1 deficient Tfh cells showed change in cytokine secretion specifically reduction in the production of IL-21. It's been shown that PD-1 signaling contributes to CD4 T cell differentiation and function of Tfh cells (383).

The cytoplasmic domain of PD-1 contains two tyrosine-containing motifs, a tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (384). Following TCR stimulation and PD-1 ligation, SRC Homology 2-Domain-Containing protein Tyrosine Phosphatase 2 (SHP-2) is recruited to the C-terminal tyrosine in the ITSM. SHP-2 dephosphorylates TCR-associated molecules such as ZAP-70, limiting its downstream signalling (384). PD-1 ligation inhibits TCR-induced proliferation, cytokine secretion and expression of pro-survival proteins such as Bcl-xL (368, 385, 386), and conversion of effector CD4 T cells into TRegs (387).

Two PD-1 inhibitors, nivolumab and pembrolizumab, have been approved for use in malignant melanoma (388). PD1 blockade demonstrated better response rates, PFS, and OS than CTLA-4 blockade (389-392). In advanced melanoma, pembrolizumab treatment has objective response rate of 33%, 12-month PFS of 35% and median overall survival of 23 months (393).

There is no overlap between the mechanisms of T cell suppression by CTLA-4 and PD-1 and they act during different stages of the T cell response. CTLA-4 mainly affects T cell priming while PD-1 affects the effector phase. So, combination of anti-PD-1 and anti-CTLA-4 was predicted to be beneficial (368). In a phase III study of previously untreated advanced melanoma, median PFS and objective response rates were significantly higher in patients treated with combination therapy compared to

each drug alone (PFS 11.5 months for combination therapy, compared with 5.6 months for nivolumab alone and 2.9 months for ipilimumab alone; the objective response rate was 57.6%, 43.7% and 19%, respectively) (389). Indeed, ipilimumab combined with nivolumab in a cohort of 76 melanoma patients with brain metastasis demonstrated objective response rate of 42% compared with 20% for nivolumab alone (323, 394). Similar results have been reported in other clinical trials (391, 395).

1.7 Resistance to immune checkpoint blockade

Approximately 40-60% of melanoma patients will not respond to or relapse while on immune checkpoint blockade (396) due to primary or acquired resistance (397). Patients with primary resistance will not respond to treatment while patients with acquired resistance will progress on therapy after an initial period of response (193). Partial responses have been observed in a large number of patients treated with anti PD-1. Response rate for these patients rarely exceed 40% (398-401) and in patients treated with anti-CTLA-4, response rate is around 15% (369, 402). Moreover, 43% of responders treated with pembrolizumab acquired resistance within 3 years (403), and approximately 25% of patients acquired resistant within 21 months after initial response to therapy (393, 404). Different tumour cell intrinsic and tumour cell extrinsic factors contribute to resistance to immunotherapy in cancer patients (Figure 1.6).

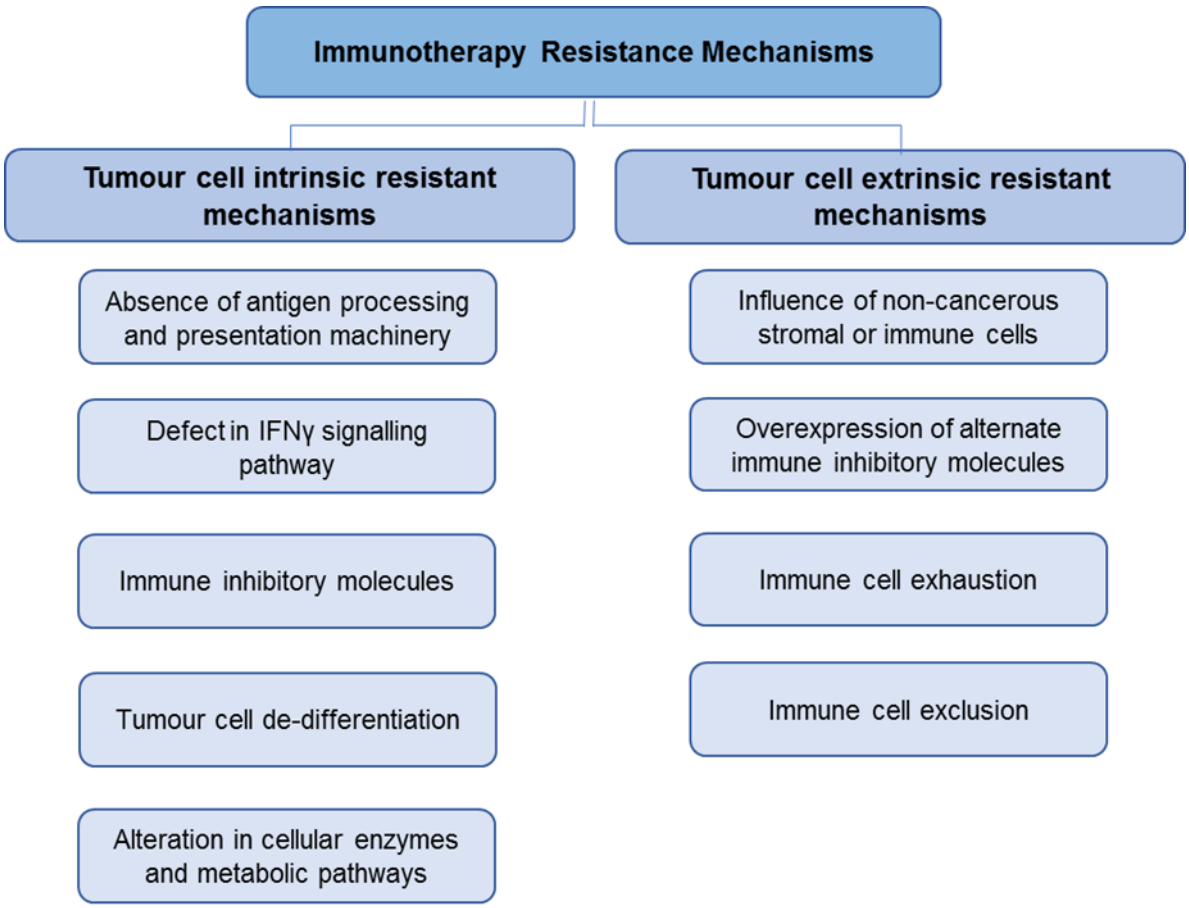

Figure 1.6 Intrinsic and extrinsic immunotherapy resistance mechanisms

Tumour **intrinsic** mechanisms include tumour specific alterations that affect the function or activity of cytotoxic T cells. These include:

1. Alterations affecting the antigen processing and presentation machinery including loss of β-2 microglobulin (B2M) or impaired expression of MHC class I molecules (193, 194).

2. Defects in IFNγ signalling pathway, including alterations affecting the IFNγ receptor (IFNGR1) and Janus kinases JAK1 and JAK2. These alterations have been identified in melanoma patients that failed to respond to PD-1 inhibition (163, 194, 289). Tumours that are resistant to IFNγ could lose their ability to upregulate antigen presenting or immune inhibitory molecules. JAK1/2 loss of function mutation in some cancer cells lead to decreased PD-L1 expression,

contributing to primary resistance to PD-1 blockade therapy (289). Loss of antigen presentation molecules protect cancer cells from T cell recognition (163).

3. The overexpression of PD-L1 and alternate immune checkpoints on tumour cells. These can dampen the efficacy of immune checkpoint inhibitors.

4. Low frequency of cancer neoantigens. Anti-tumour immune responses are more likely to occur with increasing frequency of neoantigens and these are directly proportional to the mutation burden of each cancer cell; i.e. the more single nucleotide variants the higher likelihood of neoantigen formation. Tumour types like melanoma, lung and bladder cancers with high levels of non-synonymous mutations (405) display high response rate to immune checkpoint blockade therapy (193).

5. Tumour cell mediated secretion of immune inhibitory molecules such as the stimulation of the kynurenine pathway via IDO1 enzyme overexpression (406).

6. Melanoma de-differentiation. Several clinical trials have demonstrated an association between the AXL^{high}/$MITF^{low}$ melanoma phenotype and resistance to anti-PD-1 therapy (389, 407, 408). MITF regulates melanin production after UV exposure (101), and its loss often occurs in the presence of elevated expression of the AXL receptor tyrosine kinase and the low affinity neurotrophin receptor, NGFR. AXL promotes cancer cell invasion, metastasis, epithelial to mesenchymal transition (EMT) and suppression of myeloid cell activation and function (409). AXL overexpression is associated with poor prognosis in cancer patients (409-411) and melanoma de-differentiation. Similarly, NGFR, which binds the β-Nerve Growth Factor (β-NGF) (412), is a marker of inflammation-

induced de-differentiation in melanoma cells (149). Thus, overexpression of NGFR on melanoma cells has a suppressive effect on melanoma specific CTLs via the downregulation of melanoma antigens (413).

7. Alteration in cellular enzyme and metabolic pathways including activation of the PI3K-AKT cascade via loss of PTEN expression (414), and overexpression of the Wnt-β-catenin pathway (415). These pathways can have multiple effects and may suppress T cell activation, or inhibit the recruitment of T cells into the tumour microenvironment (416).

Tumour **extrinsic** mechanisms of immune checkpoint inhibitor resistance include:

1. The influence of non-cancerous stromal or immune cells (193). Cells such as MDSCs, TRegs and M2 polarized tumour-associated macrophages (193, 417) can suppress effector T cell responses by secretion of cytokines, including IDO1, IL-10, IL-35 and TGFβ or by direct cell-to-cell contact (160)

2. Overexpression of alternate immune checkpoints such as TIM-3, LAG-3, B and T lymphocyte attenuator (BTLA), CTLA-4 and T cell Ig and ITIM domain (TIGIT) (418). In NSCLC co-expression of TIM-3, LAG-3, CTLA-4 , BTLA and PD-1 is associated with resistance to anti-PD1 therapy (419).

3. Immune cell exclusion driven by non-cancer signalling and secreted factors, such as TGFb secretion by tumour associated fibroblasts (415, 420). Non-T cell inflamed tumour microenvironment is characterised by absence of T cells, T cell markers, chemokines and regulatory factors. New methods should be developed to induce de novo inflammation in non-T cell-inflamed tumour microenvironment such as through STING agonists, or by directly argeting

pathways that mediate immune exclusion such as the Wnt/β-catenin pathway to restore immune access to the tumour microenvironment (421).

1.8 PD-1 ligands as immunotherapy target

After tumour antigen recognition, T cells secrete cytokines such as IFNγ into the tumour microenvironment. IFNγ has a number of anti-tumour effects but also induces expression of PD-L1 and PD-L2 on cancer cells, myeloid cells and lymphocytes in the TME. Induction of PD-L1/2 expression by IFNγ leads to adaptive immune resistance, or inhibition of adaptive immunity mediated through the PD-1 pathway (342, 422) that could be disrupted by PD-1 or PD-L1 blockade (196, 423).

1.8.1 PD-L1 and PD-L2 expression

PD-L1

The *CD274* gene located on chromosome 9p24 (424) encodes the PD-L1 protein, which is a 290 amino acid, transmembrane glycoprotein with broad expression pattern (381, 425). PD-L1 is expressed on tumour cells and is induced following IFNγ stimulation in different cancer types like prostate cancer, colorectal cancer, gastric cancer, lung cancer, ovarian cancer, renal cell carcinoma, multiple myeloma, leukemia and melanoma (426-431). PD-L1 can also be expressed on multiple cell types including T cells, B cells, myeloid dendritic cells, macrophages, placental trophoblasts, mesenchymal stem cells, myocardial endothelium, cortical thymic epithelial cells, and as recently shown, brown adipocytes (375, 377, 432-434).

PD-L1 expression is associated with tumour aggressiveness and adverse patient outcome (430, 435, 436). However, IFNγ-induced expression of PD-L1 is also

indicative of active cellular immunity and can be reflective of better patient prognosis, particularly in the context of anti-PD-1 treatment (437, 438).

PD-L2

The PDCD1LG2 gene, which encode the PD-L2 protein, is located on chromosome 9p24, separated from CD274 gene (PD-L1) by only 42 kilobases. PD-L1 and PD-L2 share 40% amino acid sequence homology (439). PD-L2 expression was initially reported in lymphoma (429, 431, 440, 441) but to date, less is known about the expression patterns of PD-L2 compared to PD-L1. In oesophageal adenocarcinomas and colorectal cancer, PD-L2 expression was remarkably higher (in 57% and 40% of patients, respectively) compared to PD-L1 expression (in 2% and 12% of patients, respectively) (429, 442). Moreover, PD-L2 expression was associated with early stage, smaller tumour size and a well-differentiated state (442). Constitutive expression of PD-L2 was also observed in cancer-associated fibroblasts (443). The relevance of PD-L2 expression in epithelial cancers is not known and high expression of PD-L2 in solid tumours has rarely been reported (429).

In non-cancerous cells, PD-L2 function as an immune suppressor molecule; however, PD-L2 positive B cells has the ability to augment Th1 and Th17 responses for anti-cancer protection (444), and in another study, PD-L2 positive dendritic cells increased ICOS expression on T cells to initiate protective immunity (445).

1.8.2 PD-L1 and PD-L2 interaction

PD-L1 binds to PD-1 on T cells, but it can also bind to CD80 (Figure 1.7) (446). PD-L1 expression on lymphocytes negatively regulates immune responses through binding of CD80 and production of IL-10 (377, 447, 448). PD-L1 binding to CD80 on T cells recruits CTLA-4 to the immunological synapse thus enhancing the immune

inhibitory effect, while binding of PD-L2 to PD-1 excludes CD80 and CTLA-4 from the synapse, instead recruiting CD3 and ICOS thus reducing immune inhibition (445).

In the absence of PD-1 expression, such as in PD-1 deficient T cells, PD-L2 stimulates T cell proliferation and cytokine production (449-451) suggesting an alternative receptor for PD-L2 (Figure 1.7). This alternative PD-L2 receptor has been identified as Repulsive Guidance Molecule b (RGMb), a member of the RGM family. The RGM family consists of RGMa, RGMb, and RGMc/hemojuvelin (452). Expression of RGMb has been reported in mouse hematopoietic cells and human breast, NSCLC and renal carcinoma cell lines (453). Since PD-L2 competes with PD-L1 for binding to PD-1, PD-L2 levels may impact on response to immunotherapy. In melanoma, PD-L2 expression was associated with improved overall survival (377).

PD-1 interaction with its ligands can induce T cell dysfunction through several mechanisms. These include induction of apoptosis in activated T cells, induction of T cell anergy, functional exhaustion with loss of effector function, and increase in IL-10 production (reviewed in (454)). PD-1 can promote differentiation of T regs from conventional CD4 T cells, and suppress dendritic cell function (455).

PD-L1 can also induce tumour resistance to T cell killing following interaction with PD-1. Overall, the PD-1:PD-L1 interactions provide a means for cancer cells to protect themselves from killing by immune cells (456).

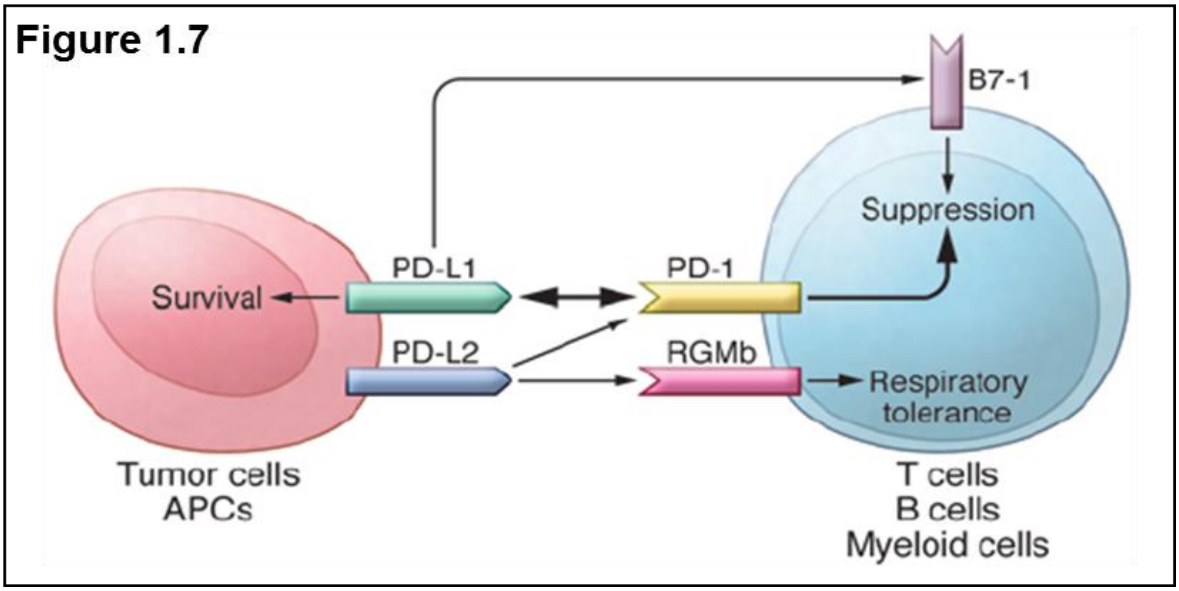

Figure 1.7 The PD-1 pathway

PD-L1 and PD-L2 are ligands for PD-1 and the interaction of PD-L1 or PD-L2 with PD-1 suppresses T cell functions. Both ligands bind a second receptor: PD-L1 binds B7-1 (CD80) on activated T cells, or on antigen-presenting cells, and PD-L2 binds RGMb. PD-L1 on tumour cells can also act as a receptor and deliver a signal that protects tumour cells from T cell-mediated lysis. CD80 also binds to CTLA-4 and CD28 with higher affinity to bind to CD28. Figure derived from (454).

1.8.3 PD-L1 and PD-L2 regulation

PD-L1 regulation

PD-L1 expression is regulated at multiple levels as outlined below.

1. Copy number alterations of chromosome 9p24 and amplification of CD274 gene and its transcriptional activator JAK2 can increase the expression of PD-L1 mRNA (375, 457) (424, 458, 459).
2. Binding of microRNAs miR-34a or MiR-152 to the PD-L1 mRNA suppress PD-L1 expression, through the degradation of PD-L1 mRNA (460).

3. Methylation of the PD-L1 promoter suppresses CD274 gene transcription and decreases its expression (457).
4. Transcription factors and regulators that modulate PD-L1 transcription include the hypoxia-inducible factors (HIF-1), PTEN, STAT3, NF-κB, Myc, AP-1, p53, bromodomain extra terminal (BET), and histone deacetylases (HDACs) are important transcription factors that regulate PD-L1 expression (375).
5. Activation of oncogenic signalling pathways like IFN/JAK/STAT, MAPK, and PI3K/AKT pathways is associated with increased expression of PD-L1 (375, 381, 461, 462).
6. Cytokines such as IFNγ, IL-4, IL-10, growth cell stem factors, bacterial LPS, and VEGF upregulate PD-L1 mRNA and protein expression, through activation of signalling pathways (381, 463-466).
7. Post-translational modifications such as ubiquitination, lysosomal-mediated degradation, glycosylation, tyrosine or serine/threonine phosphorylation, acetylation also contributes to PD-L1 regulation (375).

The majority of PD-L1 is N-glycosylated at the N35, N192, N200, and N219 residues (467). Glycosylation determines protein structure and function and modulates protein activities and protein-protein interactions by altering protein conformation (468). Glycosylation of PD-L1 leads to its phosphorylation and ubiquitination, and inhibition of glycosylation effectively decreased PD-L1 expression in cancer cells (467).

Phosphorylation of PD-L1 leads to proteasomal degradation, acetylation and ubiquitination of this molecule (469). Glycogen synthase kinase 3beta (GSK3β), a serine/threonine protein kinase, is a multifunctional switch that mediates the direct phosphorylation of a large group of substrates including PD-L1 through phosphorylation motif on this ligand (457, 467).

Membranous expression of PD-L1 can be negatively affected by ubiquitination-dependent proteasomal degradation (470). CMTM6, a positive regulator of PD-L1 is a transmembrane protein that interacts with PD-L1 on the cell surface, interfering with its ubiquitination and increasing its half-life and stability (471). CMTM6 also protects PD-L1 from lysosome-mediated degradation (472).

Deubiquitination of PD-L1 leads to its stabilization from protein degradation (457). TNFα induced activation of NF-Kb induced expression of COP9 signalosome 5 (CSN5) leading to enhanced de-ubiquitination of PD-L1 and increased expression (473). PD-L1 can translocate between the membrane and cytoplasm. The dynamic recycling and release of PD-L1 by transporter proteins determine the level of PD-L1 expression on cell membrane. Defect in this process results in continuous internalization and degradation of PD-L1 and decreased expression (457, 474).

PD-L2 regulation

Compared to PD-L1, little is known about the mechanisms regulating PD-L2 expression at the transcriptional and translational levels. It has been established that cytokines, IL-4, IFNγ and IFNβ, regulate PD-L2 expression on different cell and cancer types. For instance, IL-4 upregulated PD-L2 expression on DCs and macrophages (379, 430, 453), via downstream STAT6-dependent NF-kB activation (443, 475). PD-L2 is also regulated by IFNγ and IFNβ signalling through the IRF1 and STAT3 transcription factors, both of which bind the PD-L2 promoter (265). In melanoma, IFNγ exposure induced higher levels of PD-L1 compared to PD-L2 while IFNβ showed stronger induction of PD-L2 compared to PD-L1 (265).

PD-L2 expression is also stabilised via glycosylation (476). In colorectal cancer reduction in the expression of PD-L2 is because of a shift in molecular weight of this molecule through the glycosylation. In other words, glycosylation increases molecular weight of PD-L2 and enhance its stabilization indicating the role of glycosylation in the stabilization of PD-L2 in colorectal cancer (476).

In NSCLC, expression of PD-L2 is regulated intrinsically by activation of EGFR mutation or by the echinoderm microtubule associated protein like 4 (EML4)–ALK receptor tyrosine kinase fusion, suggesting that the RTK-MAPK signalling pathway may be involved in PD-L2 regulation. PD-L2 expression can also be induced by IFNγ through STAT1 activation in NSCLC (477).

1.9 Thesis

Despite dramatic responses to immune checkpoint blockade, some patients do not respond (termed “innate resistance”) and many develop resistance to immunotherapy (termed “acquired resistance”). A deeper understanding of immunotherapy resistance effectors, including melanoma cell-intrinsic and -extrinsic mechanisms, will help to improve immunotherapy outcomes. Our broad research question is to define melanoma cell-intrinsic mechanisms that may contribute to immunotherapy resistance.

Our hypotheses are that

1. Altered responses to immune-derived cytokines IFNγ and/or TNFα, could drive melanoma immunotherapy resistance

2. Increased expression of immune inhibitory molecules escapes from immune recognition, and alterations in antigen presentation machinery could differentially contribute to melanoma cell immunotherapy resistance.

In this thesis we investigated the effects of two proinflammatory cytokines, IFNγ and TNFα in a large panel of immunotherapy naïve melanoma cells in order to explore melanoma responses and how these responses may be altered during progression on PD-1 based therapies. In particular, we examined basal, IFNγ-induced and TNF-induced expression of immune inhibitory ligands PD-L1 and PD-L2, antigen presenting molecules HLA-ABC and HLA-DR, and nerve growth factor receptor (NGFR). We also characterized established resistance mechanisms in a panel of 16 short-term melanoma cell lines (PD-1 PROG cell lines) derived from patients who progressed on anti-PD-1, or a combination of anti-PD-1 and anti-CTLA-4 based immunotherapy. The mechanisms of resistance specifically addressed in this thesis included altered expression of melanocytic lineage differentiation antigens, altered expression of antigen presentation molecules, increased expression of immune inhibitory molecules (PD-L1, PD-L2), response to IFNγ stimulation, and lack expression of the IFNγ receptor. In addition, we addressed the regulation of PD-L1 and PD-L2 in melanoma in more detail, examining inducibility, co-regulation, kinetics of expression and protein stability.

Further research into immunotherapy resistance mechanisms will help to uncover additional actionable targets, that could be modified to prevent or circumvent resistance, leading to improved clinical outcomes for melanoma patients.

Chapter 2
Interferon signalling is frequently downregulated in melanoma

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2.1 Introduction

The identification of checkpoint signalling pathways that dampen anti-cancer immune responses and the subsequent development of checkpoint inhibitors have transformed the treatment of patients with metastatic cancer. Antibodies blocking immune checkpoints such as the cytotoxic T-lymphocyte-associated protein-4, the programmed cell death protein 1 (PD-1), and its ligand PD-L1 induce durable anti-tumour immune responses in many advanced malignancies, including melanoma, non-small-cell lung cancer, and renal cell carcinoma. PD-1 inhibition in melanoma promotes tumour regression and prolonged overall survival in 30–40% of patients with advanced disease (389, 390, 408). However, the majority of melanoma patients will not benefit from immunotherapy and 25% of responding patients will relapse within 2 years (393).

Recent studies have shown that resistance to immune checkpoint blockade involves defects in the interferon-γ (IFNγ) signalling pathway (162, 163, 194, 289, 478). Once secreted by activated T cells, IFNγ binds and activates the IFNγ receptor complex (IFNGR1/2), which is broadly expressed on many cell types, including cancer cells. Receptor binding leads to the activation of the receptor-associated Janus kinases (JAK1 and 2) which phosphorylate and activate the signal transducer and activator of transcription (STAT) proteins, STAT1 and STAT3. Nuclear translocation of STAT transcription factors promotes the transcription of hundreds of IFNγ response genes (479) including downstream transcription factors, such as IRF1, STAT1, and STAT3, genes involved in antigen presentation such as MHC class I and II molecules (289, 480), and genes that attenuate immune activity to minimize local tissue damage, such as PD-L1 and PD-L2 (194). The multifunctional effects of IFNγ are particularly important in the context of immunotherapy since enhanced antigen presentation improves immune recognition of tumours while expression of immunosuppressive molecules limits anti-tumour T cell activity.

Several genetic defects affecting the IFNγ signalling pathway are associated with melanoma resistance to immunotherapy, including checkpoint inhibition. For instance, the genetic loss of the β2M gene, the structural component of MHC class I complexes, is enriched in pre-treatment tumour samples from melanoma patients with innate and acquired resistance to checkpoint inhibitor therapy (481, 482). Genetic alterations affecting IFNGR1, IFNGR2, IRF1, and JAK2, and amplifications of the IFNγ inhibitor genes, SOCS1 and PIAS4, are also enriched in patients not responding to checkpoint inhibition (162). Furthermore, loss-of-function mutations in the upstream IFNγ-signalling regulators JAK1 and JAK2, concurrent with deletion of the wild type alleles, have been identified in two melanoma patients who failed anti-PD-1 therapy (194). The loss of IFNγ signalling limits immune cell recruitment and

immune recognition of tumour cells by suppressing the production of IFNγ-dependent chemokines and diminishing antigen presentation (163, 265, 289).

In this study, we investigated the response of a large panel of human melanoma cells to IFNγ stimulation. These cells were naïve to immune checkpoint inhibitors, and we examined whether the expression of key IFNγ downstream targets [PD-L1, PD-L2, NGFR, HLA-A, -B, -C, and HLA-DR] could serve to assess the integrity of IFNγ signalling in melanoma. We also examined the potential influence of melanoma driver oncogenes on IFNγ signalling activity and found that uveal melanoma (UVM) cells show evidence of diminished IFNγ pathway activity with minimal baseline and IFNγ induction of HLA-DR, NGFR, and PD-L2. Importantly, nearly 70% of melanoma cells included in this study showed incomplete responses to IFNγ stimulation, indicative of pre-existing resistance to immunotherapy. Furthermore, our data confirm that measuring IFNγ output with a select number of targets may be useful for detecting intrinsic defects in the IFNγ/JAK/STAT pathway, including JAK and STAT mutations which are associated with PD-1 inhibitor resistance (194, 289, 482).

2.2 Material and Methods

2.2.1 Cell lines

A total of 39 cell lines were included in this study. Oncogenic driver mutation status is shown in Table 2.1. Melanoma cell lines were provided by Prof. Nicholas Hayward and Prof. Peter Parsons at QIMR Berghofer Medical Research Institute, Australia, Prof. Bruce Ksander at Harvard Medical School, MA, Prof. Peter Hersey at the Centenary Institute, Sydney, Australia, and Prof. Xu Dong Zhang at the University of Newcastle, Newcastle, Australia. Two short-term melanoma cell lines were cultured from surgically excised, enzymatically processed melanoma lesions (SCC14-0257, SMU15-0217) in a study carried out in accordance with the recommendations of Human Research ethics committee protocols from Royal Prince Alfred Hospital (Protocol X15-0454 and HREC/11/RPAH/444). Cell authentication was confirmed using the StemElite ID system from Promega.

Table 2.1 Expression of IFNγ-target proteins at baseline and post-stimulation with IFNγ in 39 melanoma cell lines.

	Cell line	HLA-ABC		HLA-DR		NGFR		PD-L1		PD-L2	
		BSA	IFNγ	BSA	IFNγ	BSA	IFNγ	BSA	IFNγ	BSA	IFNγ
$BRAF^{V600E}$	A2058	32.9	139.1	2.5	66.7	398.7	218.6	1.0	9.8	1.9	7.7
	SKMel28	74.2	128.3	11.1	120.8	44.7	197.3	1.3	2.9	2.6	10.0
	C060M1	34.9	88.7	29.5	75.7	10.4	16.3	1.0	5.5	4.3	6.7
	SCC14-0257	15.8	64.8	12.8	102.2	347.3	722.7	0.9	2.5	1.4	5.9
	MM418	38.2	81.8	1.2	7.7	16.0	19.0	1.1	4.0	1.1	1.5
	NM16	21.7	62.5	7.0	76.5	1808.8	2833.0	0.8	3.4	7.8	17.1
	NM182	18.9	143.6	1.3	31.8	10.1	14.0	1.0	4.5	1.0	1.6
	MM200	35.1	202.0	7.2	141.0	450.7	1505.6	1.0	4.0	1.2	3.4
	NM39	43.6	122.2	27.6	123.3	90.7	46.7	1.1	3.9	4.3	17.9
	HT144	23.4	43.4	71.4	100.3	353.1	292.9	1.1	3.2	4.9	12.0
	C016M	20.9	70.3	42.7	95.9	469.6	630.2	0.9	1.3	2.2	5.5
$NRAS^{Q61R/K/L}$	MelRm	36.9	98.7	102.8	252.5	18.4	75.2	0.9	2.3	1.6	3.4
	NM47	42.1	109.0	157.9	249.0	213.6	1034.1	1.0	2.5	1.8	2.9
	NM177	56.5	99.7	76.6	92.9	3674.9	3663.2	1.0	1.9	2.0	3.4
	NM179	12.7	31.1	2.4	59.6	44.2	85.4	1.0	3.3	1.7	6.6
	ME4405	60.5	118.2	0.9	0.9	24.0	47.3	1.0	2.1	3.5	14.2
	MelAT	31.9	121.3	0.9	1.0	11.8	27.4	1.2	2.0	2.0	10.3
	D11M2	11.3	18.4	16.4	33.7	29.2	32.1	1.1	2.4	3.3	9.2
	C002M	7.2	31.4	1.0	27.0	13.7	15.6	1.2	2.3	1.4	1.5
	C013M	24.7	81.1	1.0	42.7	47.8	199.3	1.0	2.3	2.2	6.5
	D38M2	28.6	86.2	4.5	56.5	509.6	480.4	1.1	2.4	2.9	12.2
BRAF/RAS Wild Type	D22M1	28.0	25.7	1.9	1.9	5.8	5.3	1.3	1.1	1.3	1.2
	MeWo	28.9	107.6	1.4	19.1	268.9	176.2	0.9	2.2	1.3	4.2
	D24M	32.6	37.2	13.8	43.3	30.7	28.3	1.2	3.3	7.3	31.0
	C022M1	10.7	41.7	2.3	21.5	148.3	341.0	1.9	1.6	1.1	1.8
	C084M	83.6	119.0	19.5	130.4	552.5	631.5	0.9	4.6	3.2	15.7
	C086M	20.1	52.4	22.7	90.3	1.3	2.9	1.1	3.0	3.7	9.9
	D35	167.9	460.2	3.8	129.6	21.9	36.9	0.9	2.9	0.9	2.7
	C025M1	73.2	134.1	2.1	18.5	1.7	2.7	1.1	2.7	1.1	1.2
	SMU15-0217	1.5	2.1	12.8	91.4	11.0	26.0	1.2	3.4	4.4	22.3
	A04-GEH	23.9	96.6	1.5	63.3	13.8	45.9	1.0	2.9	1.2	8.2
$GNAQ^{Q209L/P}$	92.1	11.5	87.2	0.5	0.5	14.0	38.8	1.1	1.7	1.2	1.0
	MEL202	38.2	346.3	1.1	15.9	10.6	15.0	1.0	5.6	1.0	2.7
	MEL270	52.5	115.6	1.1	1.8	3.6	4.1	1.1	1.6	1.2	1.3
	MP38	73.5	329.7	1.6	10.6	10.6	15.8	1.1	2.4	2.9	25.3
	MP46	31.2	108.0	1.1	19.0	2.0	3.6	1.1	1.5	1.0	3.7
$GNA11^{Q209L}$	OMM1	26.3	44.2	0.9	3.7	2.6	3.6	0.9	1.3	1.1	2.0
	MP41	2.3	38.6	1.0	1.0	5.2	10.6	1.1	1.5	1.0	1.6
	MM28	9.4	50.8	1.1	13.1	1.3	1.7	1.0	2.1	1.0	2.1

2.2.2 Cell culture

Cell lines were cultured in Dulbecco's Modified Eagle Medium or Roswell Park Memorial Institute-1640 media supplemented with 10 or 20% heat inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 4mM L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and 10 mM HEPES (Gibco) and were maintained at 37°C in 5% CO2. For IFNγ treatment, 7×10^4 melanoma cells per well were plated in complete media in six-well plates. After an overnight incubation, the complete media was replenished, and cells treated for 72 h with 1,000 U/ml IFNγ (Peprotech, Rocky Hill, NJ, USA) or vehicle control [0.1% bovine serum albumin (Sigma-Aldrich) in phosphate-buffered saline (PBS, Gibco)]. Cells were collected, washed with PBS, and analyzed by flow cytometry.

2.2.3 Flow cytometry

Staining was performed in flow cytometry buffer (PBS supplemented with 5% FBS, 10 mM EDTA, and 0.05% sodium azide). Cells (2×10^5) were incubated for 30 min on ice with mouse anti-human antibodies against HLA-ABC (clone W6/32), HLA-DR (clone L243), CD271/NGFR (clone ME20.4), CD273/PD-L2 (clone 24F.10C12) (all from BioLegend, San Diego, CA, USA), and CD274/PD-L1 (clone MIH1; BD Biosciences, Franklin Lakes, NJ, USA) conjugated to phycoerythrin (PE), fluorescein isothiocyanate, PE-cyanine (Cy)7, allophycocyanin, and brilliant violet 421, respectively. All antibodies were titrated prior to experiment to ensure optimal concentrations were used. Fc block (BD Biosciences) was used to prevent non-specific staining due to antibody binding to Fc receptors. Fluorescence minus one control (FMO, staining with all but one antibody for each fluorochrome) were included with each experiment. Prior to acquisition, cell viability was determined by staining cells with either 5 μM DAPI (Invitrogen, Thermo Fisher Scientific), Zombie Yellow dye

(BioLegend), or Live Dead near-IR fixable dye (Invitrogen, Thermo Fisher Scientific). For the analysis of IFNGR1 and B2M expression, cells were first stained with a fixable viability dye and either PE-conjugated anti-CD119 (clone GIR-208) or PE-Cy7 conjugated anti-B2M (clone 2M2), both from BioLegend. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm kit and stained intracellularly with the same antibody that was used for cell surface stain.

Samples were acquired on BD LSRFortessa X20 flow cytometer (BD Biosciences) and the FlowJo software (TreeStar, Ashland, OR, USA) was used for data analysis. At least 10,000 live events were acquired. General gating strategy included forward and side scatter area to exclude cell debris, time parameter to exclude electronic noise, forward scatter area and height to exclude doublets and gating on viable cells (by gating on DAPI, Zombie Yellow, or Live Dead near-IR negative events). Relative marker expression levels were calculated by dividing the geometric mean fluorescence intensity (MFI) of the antibody-stained sample by the FMO control MFI (Figure 2.1). Relative MFI is used in all analyses, and a relative MFI < 1.5 was considered to reflect no antigen expression relative to the control.

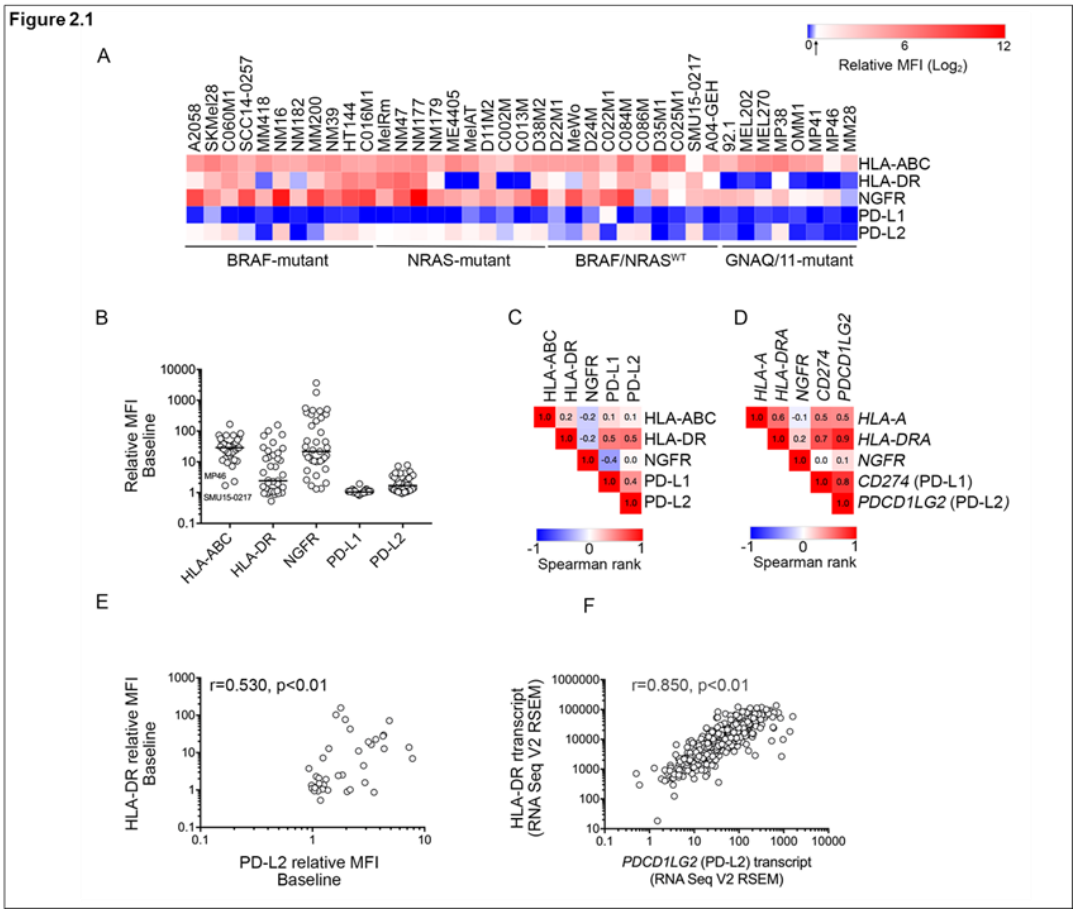

Figure 2.1 Expression of downstream interferon-γ targets in melanoma cells

(A) Heatmap showing cell surface expression [relative mean fluorescence intensity (MFI); mean of 2-5 independent experiments] of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2 in 39 melanoma cell lines with oncogenic drivers including 11 $BRAF^{V600}$-mutant, 10 NRAS-mutant, 10 BRAF/NRAS wild type (BRAF/NRASWT), and 8 GNAQ/11-mutant uveal melanoma cell lines. Relative MFI < 1.5 is indicated by the arrow on the color bar. (B) Cell surface baseline expression (relative MFI) of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2 in a panel of 39 melanoma cell lines. Each dot represents one cell line and the median expression is indicated by the horizontal line. Low cell surface expression of HLA-ABC on the MP46 and SMU15-0217 cell lines is indicated. (C) Correlation matrix showing Spearman's rank correlation analysis between cell surface expressions of markers, as indicated. Spearman's rank correlation values are shown within the similarity matrix. (D) Correlation matrix showing Spearman's rank correlation analysis between transcript levels of HLA-A, HLA-DRA, NGFR, PD-L1, and PD-L2 (The Cancer Genome Atlas (TCGA) skin cutaneous melanoma (SKCM) dataset). Spearman's rank correlation is shown within the similarity matrix. (E) Correlation between PD-L2 and HLA-DR cell surface expression and (F) mRNA transcript expression (TCGA SKCM dataset). Spearman's rank correlation coefficient and p values are shown.

2.2.4 Cell cycle and apoptosis analysis

Adherent and floating cells were combined after 72 h treatment with vehicle control or 1,000 U/ml IFNγ and cell cycle analyses were performed as previously described (483) using at least three biological replicates.

2.2.5 Gene set enrichment transcriptome analysis

Transcriptome analysis was performed on the The Cancer Genome Atlas (TCGA) human skin cutaneous melanoma (SKCM) and UVM datasets using single sample gene set enrichment analysis (ssGSEA) (484). RNA counts were normalized using the weighted trimmed mean of M-values implemented in the edgeR Bioconductor package. Normalized counts were transformed using voom, as implemented in the Limma package (485, 486). The gene sets used in ssGSEA analysis consisted of the Hallmark gene set version 6.1, a refined gene set that define specific biological processes (487).

2.2.6 Whole exome sequencing

Melanoma cell exome sequencing was performed on D22M1 and SMU15-0217 melanoma cell lines. Exonic DNA was enriched using the Illumina SureSelect technology, targeting 50 Mb encompassing protein-coding regions and sequenced on an Illumina HiSeq2000. Read pairs were aligned to the reference human genome (hg19) using BWA (488) and nucleotide variants (SNVs) and small insertion/deletions were detected by SAMTools (489). Ingenuity Variant Analysis (http://www.ingenuity.com) was used to identify mutations in genes associated with the JAK-STAT (KEGG) signalling pathway (490).

2.2.7 Statistical analysis

Statistical significance was calculated using GraphPad Prism version 7 (GraphPad software, San Diego, CA, USA). p-Values <0.05 were considered significant.

- Protocol related to “DNA extraction and Whole Exome Sequencing” are explained in section 3.2.3

2.3 Results

2.3.1 Baseline expression of IFNγ target molecules in melanoma lines with different oncogenic driver mutations

Expression of five well-defined IFNγ targets, the PD-1 ligands PD-L1 and PD-L2, NGFR, antigen-presenting HLA-A, -B, and -C (HLA-ABC), and HLA-DR molecules was examined in a panel of 39 human melanoma cell lines with defined oncogenic driver mutations (Figure 2.1A; Figure S2.1). These included 11 $BRAF^{V600}$ mutant, 10 NRAS-mutant and 10 BRAF/NRAS wild type (BRAF/NRASWT) cutaneous melanoma cell lines, and 8 GNAQ/11-mutant UVM cell lines (Table 2.1).

Analysis of cell surface marker expression (antibody-stained MFI/FMO control MFI, relative MFI) revealed a broad range of expression for all five markers (Figure 2.1; Table2.1). MHC class I molecules (HLA-ABC) were uniformly expressed on melanoma cells with the exception of the BRAF/NRASWT SMU15-0217 (relative MFI = 1.5) and the uveal MP46 cells (relative MFI = 2.3) (Figure 2.1B). HLA-DR showed a broad range of baseline expression in our panel of melanoma cells with no expression in 14 melanoma cell lines (MFI ratio < 1.5) and bimodal expression in 11/39 cell lines [i.e., only a proportion of cells (18–88%) expressed the marker]. NGFR expression was similarly variable (Figure 2.1B) with no expression at baseline in two cell lines (relative MFI < 1.5; Table 2.1). Similar to HLA-DR, NGFR was distributed in a bimodal fashion in six samples, with 42–81% cells expressing the marker. Three cell lines, the $BRAF^{V600}$ mutant C060M1 and BRAF/NRASWT D24M and SMU15-0217, had a bimodal expression of both HLA-DR and NGFR (data not shown). PD-1 ligands PD-L1 and PD-L2 were expressed at comparably low levels in our panel of melanoma cells (Table 2.1), with PD-L1 not constitutively expressed in

38/39 (relative MFI < 1.5) and PD-L2 absent in 18/39 cell lines. Seventeen melanoma lines lacked both PD-L1 and PD-L2 basal expression, including 5/10 (50%) BRAF/NRASWT, 4/11 (36%) $BRAF^{V600}$ mutant, 1/10 (10%) NRAS-mutant, and 7/8 (87.5%) uveal cell lines (Figure 2.1).

Of the targets analyzed, cell surface expression of PD-L2 was correlated with HLA-DR (Spearman's rank 0.530, $p < 0.01$) and NGFR expression (Spearman's rank 0.418, $p < 0.01$) (Figure 2.1C). The expression of HLA-DR and NGFR was also correlated (Spearman's rank 0.497, $p < 0.01$). The cell surface protein expression patterns of these markers in our melanoma panel did not precisely reflect their transcript expression patterns in the human SKCM dataset of TCGA (n = 472; Figure 2.1D), although both protein and transcript expression of PD-L2 (PDCD1LG2) and HLA-DR (HLA-DRA) were correlated (Figure 2.1E, F). It is also worth noting that PD-L1 (CD274) and PD-L2 (PDCD1LG2) transcripts were correlated (Spearman's rank = 0.793 $p < 0.01$) in the TCGA SKCM dataset, although we did not observe any correlation in their cell surface protein expression (Figure S2.2).

There was also evidence that basal marker expression in GNAQ/11-mutant UVM was distinct. In particular, HLA-DR, NGFR, and PD-L2 cell surface expression was significantly lower in the UVM cell subset compared to cutaneous melanoma (Table 2.1; Figure 2.2). To address the significance of these findings, we analyzed TCGA RNA sequencing data from 80 uveal and 472 cutaneous melanoma samples. Consistent with our cell surface expression data, the expression of HLA-DRA, NGFR, and PD-L2 transcripts was significantly lower in the 80 uveal compared to the 472 cutaneous melanoma samples from the TCGA dataset; CD274 (PD-L1) transcript expression was also different between the TCGA uveal and cutaneous datasets,

whereas HLA-A transcript expression was indistinguishable between the TCGA uveal and cutaneous tumour groups (Figure 2.2B).

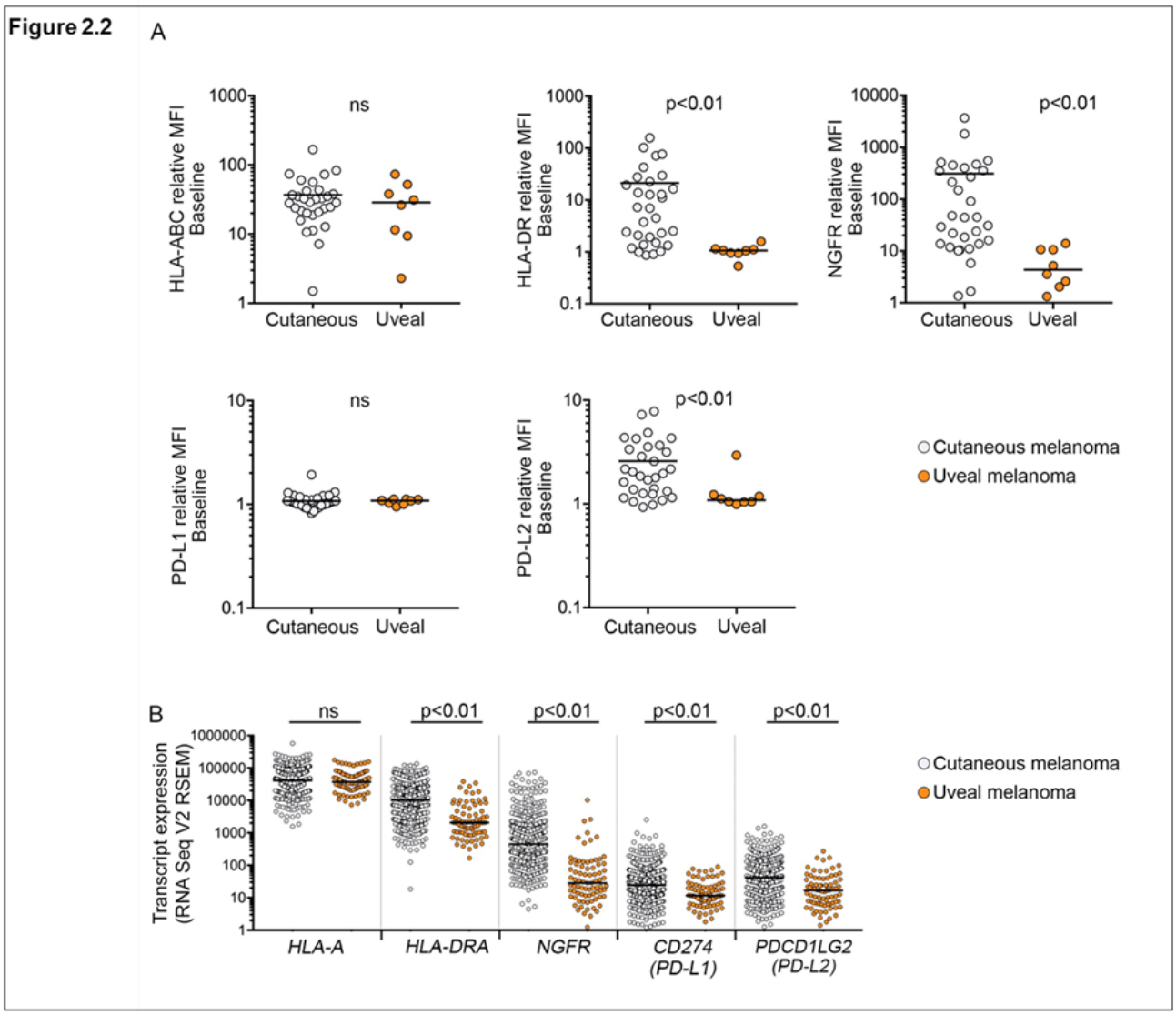

Figure 2.2 Expression of interferon-γ targets in cutaneous and uveal melanoma cells

(A) Cell surface expression [relative mean fluorescence intensity (MFI)] of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2 in cutaneous (n = 31) and UVM (n = 8) cell lines. (B) Expression of mRNA transcripts for HLA-A, HLA-DRA, NGFR, PD-L1, and PD-L2 in the 80 uveal [The Cancer Genome Atlas (TCGA) UVM dataset] and 472 cutaneous melanoma samples (TCGA skin cutaneous melanoma dataset). Each dot represents a single sample, with the median indicated by the horizontal line. Expression levels were compared using a Mann–Whitney test; ns, not significant.

2.3.2 Expression of target molecules after exposure to IFNγ

We noted that IFNγ stimulated the expression of HLA-ABC, HLA-DR, NGFR, PD-L1, and/or PD-L2 in the majority of melanoma cell lines (Figure 2.3A). The degree of IFNγ stimulation was highly variable, however, and in the case of HLA-ABC, HLA-DR, PD-L2, and NGFR, the level of stimulation was proportional to the basal expression levels (Figure 2.3B). Only IFNγ-induced PD-L1 expression was independent of its basal expression levels and all but four cell lines lacking baseline PD-L1 showed IFNγ-stimulation of PD-L1 expression (Figure 2.3B).

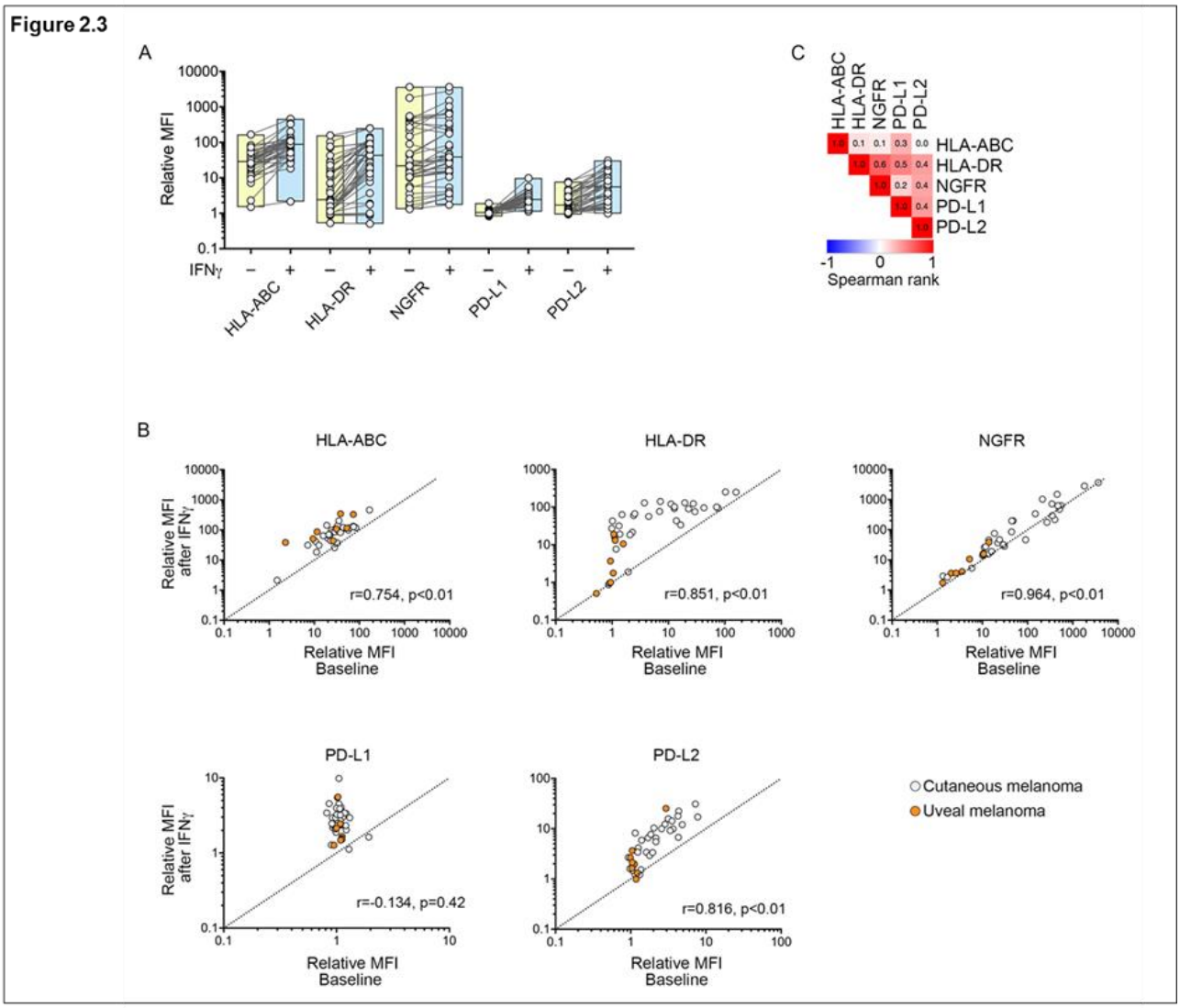

Figure 2.3 Expression of cell surface markers in response to interferon-γ treatment

(A) Change in HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2 cell surface expression [relative mean fluorescence intensity (MFI)] after exposure to IFNγ. Each dot shows one cell line before (−) and after (+) IFNγ stimulation with box plots showing the range and median. (B) Correlation of baseline and IFNγ-induced cell surface expression of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2. Each dot represents one cell line. Spearman's rank correlation coefficient and p values are shown. (C) Correlation matrix showing Spearman's rank correlation analysis between IFNγ-induced expression of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2. Spearman rank correlation values are shown within the similarity matrix.

Comparison of all five target molecules also showed positive correlation between IFNγ-induced expression of PD-L1, PD-L2, and HLA-DR. In particular, post-stimulation levels of PD-L1 and PD-L2 were correlated (Spearman's rank = 0.388, p = 0.01) (Figure 2.3C), although the degree of induction (i.e., change from pre- to post-stimulation) was not correlated (Spearman's correlation = 0.315, p = 0.05) because PD-L1 and PD-L2 showed disparate expression at baseline (Figure 2.1C). Similarly, although post-stimulation levels of NGFR were correlated with induced levels of PD-L2 (Spearman's rank = 0.358; p = 0.025) (Figure 2.3C), the degree of NGFR and PD-L2 induction (i.e., change from pre- to post-stimulation) was not correlated (Spearman's rank = −0.103; p = 0.99).

Overall, exposure of melanoma cells to IFNγ induced heterogeneous levels of all target molecules, and induction did not appear to depend on genotype in cutaneous melanomas for PD-L1, PD-L2, HLA-ABC, and NGFR (Table 1.2). In UVM lines, however, the protein expression of HLA-DR, NGFR, PD-L1, and PD-L2 post-IFNγ stimulation was significantly lower than observed in cutaneous melanomas (Figure 2.3B; Figure S2.3), and this was consistent with low baseline expression of HLA-DR, NGFR, and PD-L2 in the UVM cells (Figure 2.2 A). The transcript expression of STAT1, STAT3, and IRF1, three key transcription factors of the IFNγ signalling cascade, were also lower in the TCGA UVM dataset compared to the TCGA cutaneous melanomas (Figure 2.4).

We also explored interferon signalling pathways in the SKCM and uveal TCGA melanoma dataset using single sample gene set enrichment analysis (ssGSEA), an extension of GSEA that defines an enrichment score of a gene set for each of the sample in the dataset (484). As shown in Figure 2.4B, the enrichment scores generated for the Hallmark_interferon_alpha and Hallmark_interferon_gamma

response signatures were significantly lower in the UVM dataset, compared to cutaneous melanoma.

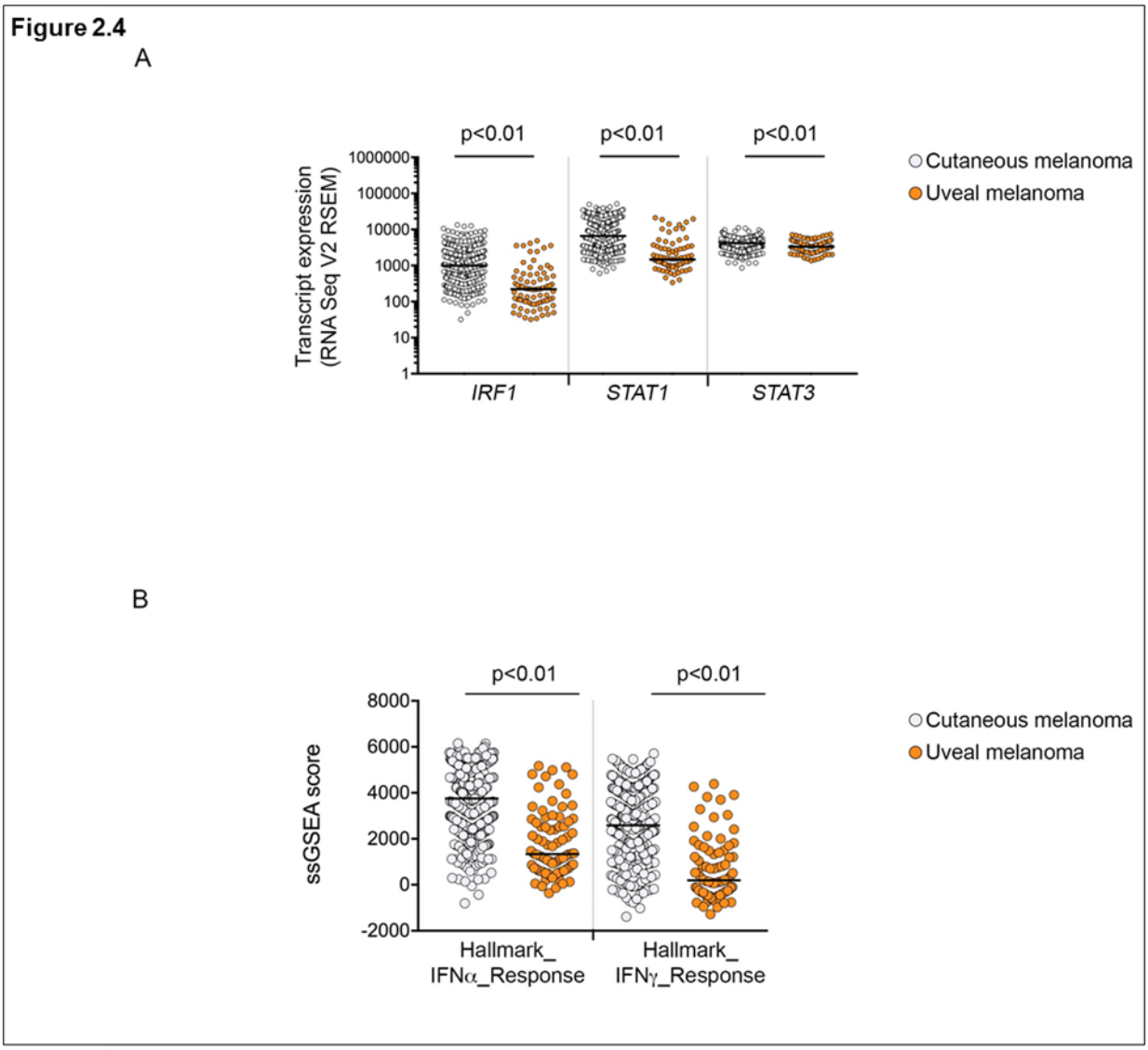

Figure 2.4 Interferon-γ signalling in cutaneous and uveal melanoma

(A) Expression of mRNA transcripts for IRF1, STAT1, and STAT3 in the 80 uveal [The Cancer Genome Atlas (TCGA) UVM dataset] and 472 cutaneous melanoma samples (TCGA skin cutaneous melanoma dataset). (B) Single sample gene set enrichment analysis (ssGSEA) scores for the Hallmark_interferon_alpha and Hallmark_interferon_gamma response signatures in the 80 uveal and 472 cutaneous melanoma samples from the TCGA datasets. Expression levels were compared using a nonparametric Mann–Whitney test, p values are indicated.

2.3.3 Downregulated response to IFNγ in a small subset of melanoma cell lines

Twenty-six of 39 cell lines (67%) demonstrated diminished response to IFNγ stimulation, usually manifested as no induction (i.e., fold induction in MFI ratio < 1.5) of one or more markers in response to IFNγ stimulation. HLA-ABC expression was absent in the BRAF/NRASWT SMU15-0217 cells even though expression of PD-L1, PD-L2, HLA-DR, and NGFR was upregulated by IFNγ (Figure 2.5A). Detailed analysis of this cell line confirmed that expression of B2M, the structural component of the MHC class I complex, was absent from the cell surface (Figure 2.5B). Among the other four markers, HLA-DR and PD-L1 expression was not induced in 7/39 cell lines, while induction of PD-L2 and NGFR was absent in 6/39 and 18/39 cell lines, respectively. One cell line, BRAF/NRASWT D22M1, showed a complete loss of response to IFNγ with no induction of any target molecules (Figure 2.6 A), suggesting an upstream defect in the IFNγ signalling pathway in this cell line. Whole exome sequencing of this cell line identified a damaging missense mutation resulting in a P44R substitution in the extracellular portion of the IFNGR1 (Figure 2.6 B). This amino acid substitution is located in the highly conserved NP linker region between the second and third beta sheets in the D1 domain (Figure 5.6 C) and is classified as deleterious by the missense substitution algorithms SIFT and Polyphen-2 (data not shown). We confirmed that IFNGR1 expression was absent on the surface of D22M1 cells although IFNGR1 expression was detected intracellularly (Figure 2.6 D), consistent with accumulation of a misfolded protein.

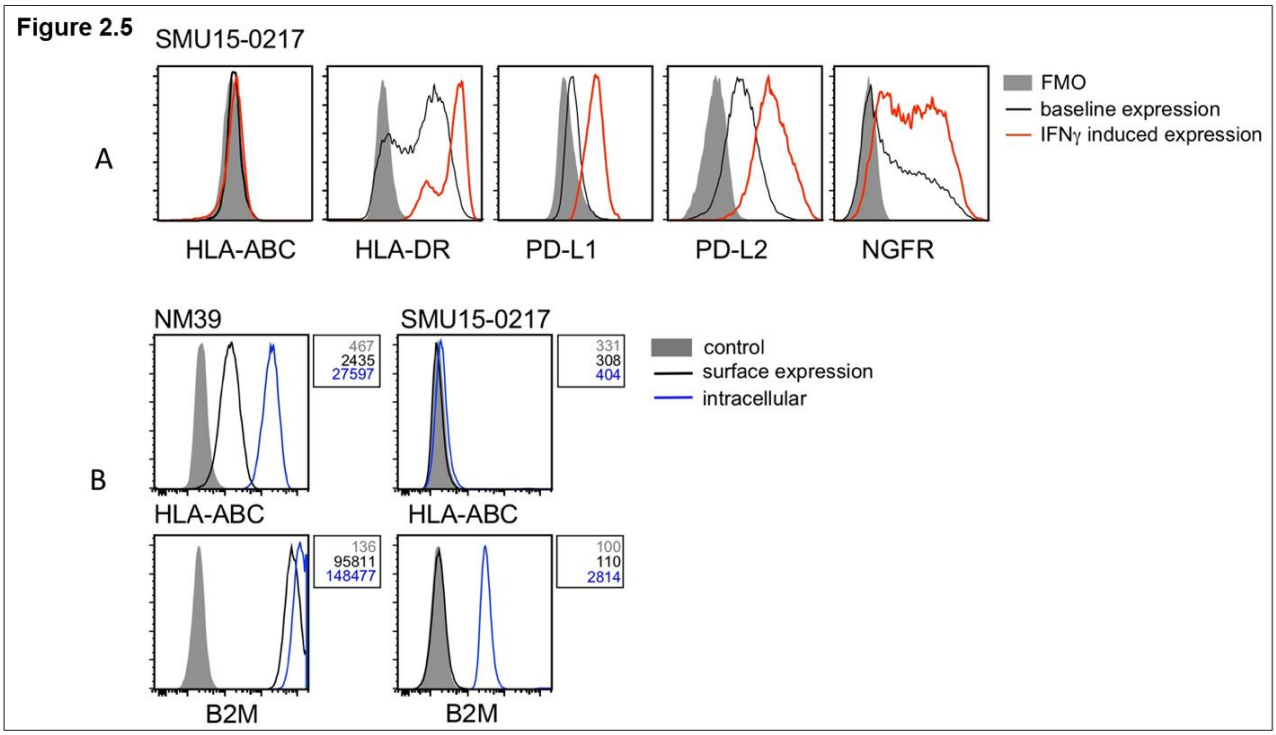

Figure 2.5 Analysis of B2M expression in the SMU15-0217 cell line

(A) Representative histograms of cell surface expression of HLA-ABC, HLA-DR, PD-L1, PD-L2, and NGFR on SMU15-0217 cells. Baseline expression is shown in black, interferon-γ (IFNγ)-induced expression in red, and FMO controls as shaded histograms. (B) Expression of HLA-ABC and B2M on the cell surface (black) and intracellular (blue) in NM39 and SMU15-0217 cells. Shaded histograms represent the mock stained control and mean fluorescence intensity values are shown next to the histograms. NM39 cells were used as a positive control.

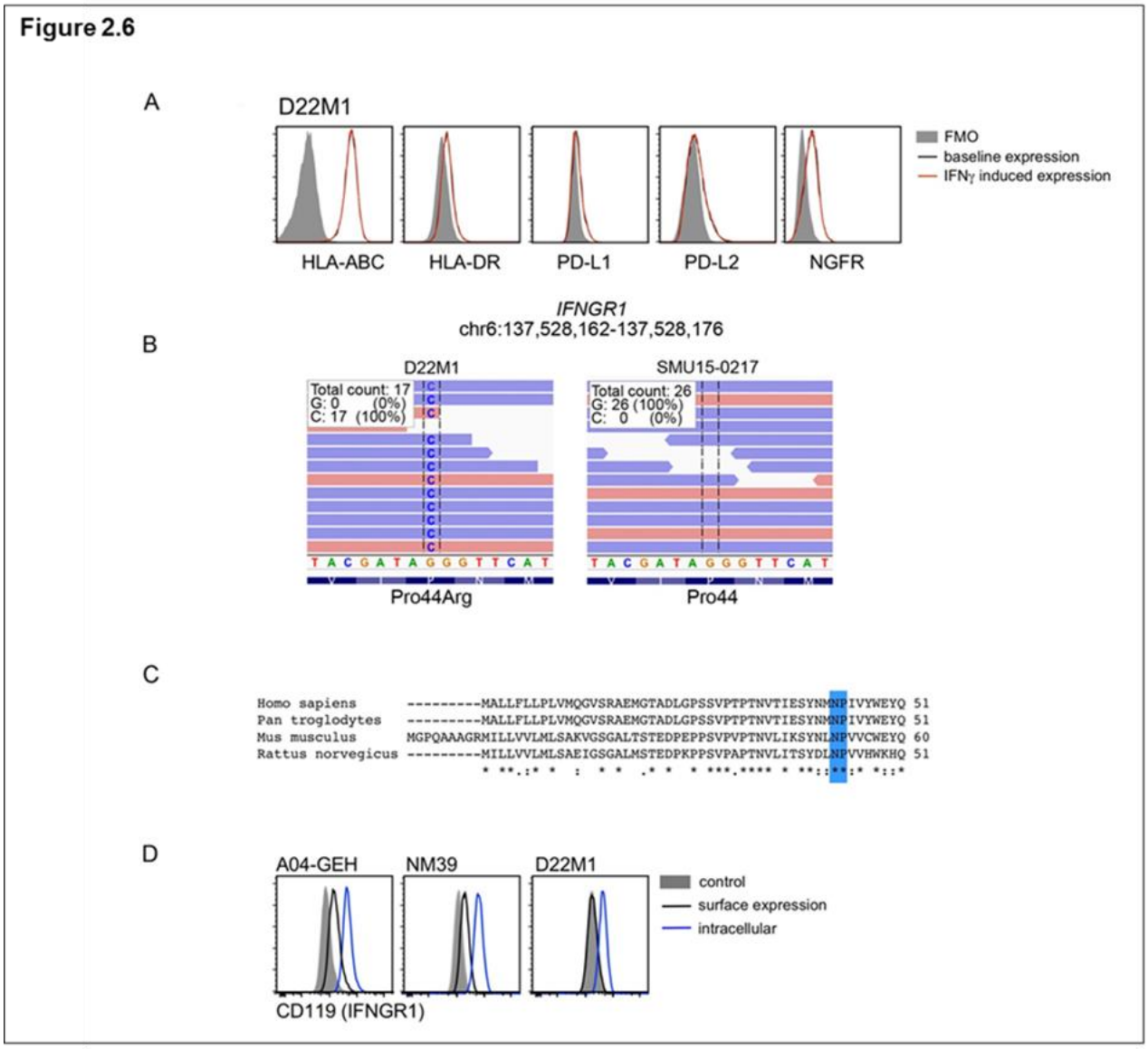

Figure 2.6 Analysis of IFNGR1 expression in the D22M1 cell line

(A) Representative histograms of cell surface expression of HLA-ABC, HLA-DR, PD-L1, PD-L2, and NGFR on D22M1 cells. Baseline expression is shown in black, interferon-γ (IFNγ)-induced expression in red, and FMO controls as shaded histograms. (B) Whole exome sequencing analysis showing Pro44Arg (P44R) substitution in the D22M1 cell line but not in the SMU15-0217 cells. (C) Alignment of IFNGR1 protein sequence of human, chimpanzee, mouse, and rat (Clustal Omega) showing the highly conserved NP linker region highlighted in blue. (D) Expression of IFNGR1 on the cell surface (black) and intracellularly (blue) in A04-GEH, NM39, and D22M1 cells, with mean fluorescence intensity values also shown. Shaded histograms represent the mock stained control.

2.3.4 Melanoma cell cycle effects in response to IFNγ treatment

We also examined the impact of IFNγ treatment on cell cycle progression in our panel of melanoma cells using flow cytometry. Of the 38 melanoma cell lines tested, three showed increasing cell death in response to IFNγ, with greater than 10% increase in sub G1 (Table 2.2). Of these, one cell line (MM200) also showed a 56% increase in the proportion of cells undergoing DNA replication (i.e. S phase cells), along with another six cell lines that showed a greater than 30% increase in S phase cells. Another six cell lines, including 5/8 UVMs, showed diminished DNA replication post-IFNγ treatment (Table 2.2). The remaining 23 melanoma cell lines, including the IFNGR1-mutant D22M1 cells, showed minimal cell cycle profile changes when exposed to IFNγ (Table 2.2). It is worth noting that 5/7 melanoma cell lines with no IFNγ-mediated PD-L1 induction also showed no cell cycle profile changes in response to IFNγ treatment (Table 2.2).

Table 2.2 IFNγ-mediated cell cycle effects in melanoma cell

	Cell line	Sub G1 phase		G1 phase		S phase		G2 phase		IFNγ effect
		BSA	IFNγ	BSA	IFNγ	BSA	IFNγ	BSA	IFNγ	
$BRAF^{V600E}$	A2058	0.6	1.3	68.2	63.3	19.2	26.7	12.7	10.1	↑ S phase
	SKMel28	1.7	3.8	72.4	73.5	19.6	13.3	8.0	13.3	↓ S phase
	C060M1	1.1	2.4	72.1	68.2	12.1	11.9	16.1	19.9	
	SCC14-0257	1.6	5.3	63.4	50.5	20.0	26.1	16.6	23.4	↑ S phase
	MM418	0.9	9.5	61.8	54.9	24.8	32.4	13.4	12.7	↑ S phase
	NM16	0.8	4.1	65.6	70.1	26.3	26.8	8.1	3.1	
	NM182	2.5	4.1	60.8	56.3	28.4	34.8	10.8	8.9	
	MM200	1.1	17.1	69.9	62.2	19.9	31.1	10.2	6.7	↑ sub G1, ↑ S phase
	NM39	0.7	2.3	85.2	86.4	9.7	9.8	5.0	3.8	
	HT144	1.6	12.9	65.4	61.7	24.3	24.4	10.4	13.9	↑ sub G1
	C016M	4.0	6.8	73.2	65.4	19.8	22.9	7.0	11.7	
$NRAS^{Q61R/K/L}$	MelRm	0.7	4.1	62.9	64.9	26.8	24.2	10.3	10.8	
	NM47	0.4	6.5	63.0	65.3	25.8	24.3	11.2	10.4	
	NM177	2.6	2.9	67.6	59.5	23.3	28.4	9.0	12.2	
	NM179	1.7	6.7	60.1	52.5	24.8	32.9	15.0	14.7	↑ S phase
	ME4405	0.3	0.7	59.7	63.6	28.2	26.1	12.1	10.2	
	MelAT	0.5	0.8	58.2	68.0	29.7	22.7	12.2	9.3	
	D11M2	7.8	8.7	41.7	39.7	33.4	28.2	24.9	32.2	
	C002M	1.4	2.5	73.3	65.5	17.7	27.0	8.9	7.5	↑ S phase
	C013M	19.0	35.5	57.1	54.2	26.4	23.8	16.6	21.9	↑ sub G1
	D38M2	0.7	1.3	65.1	59.9	18.2	21.3	16.7	18.9	
BRAF/RAS Wild Type	D22M1	1.2	1.2	52.7	50.9	37.9	39.5	9.4	9.5	
	MeWo	1.1	1.7	49.2	52.9	25.5	24.1	25.4	22.9	
	D24M	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
	C022M1	1.4	4.1	80.2	68.3	11.5	17.6	8.2	14.1	↑ S phase
	C084M	0.6	1.2	37.0	37.8	22.5	16.4	40.6	45.8	
	C086M	4.7	13.4	50.4	48.8	34.0	25.9	15.6	25.3	
	D35	0.3	1.3	71.9	72.8	20.7	23.5	7.5	3.7	
	C025M1	1.2	1.4	75.5	78.0	17.7	16.1	6.8	5.6	
	SMU15-0217	0.6	1.4	69.5	67.5	22.2	21.0	8.3	11.5	
	A04-GEH	1.0	7.7	60.0	56.9	25.7	24.7	14.3	18.4	
$GNAQ^{Q209L/P}$	92.1	0.7	8.3	60.6	87.0	31.6	10.3	7.9	2.7	↓ S phase
	MEL202	0.4	5.2	57.5	72.8	26.8	17.3	15.7	9.9	↓ S phase
	MEL270	0.8	1.4	68.7	69.9	21.8	20.8	9.5	9.3	
	MP38	0.6	2.2	72.7	88.4	12.0	4.2	15.4	7.4	↓ S phase
	MP46	1.4	1.4	53.4	52.3	35.8	36.4	10.9	11.4	
$GNA11^{Q209L}$	OMM1	1.2	4.1	60.7	84.0	28.3	12.1	11.0	3.9	↓ S phase
	MP41	1.2	1.8	28.7	29.1	10.4	10.1	57.3	61.4	
	MM28	0.7	1.2	85.6	92.4	7.1	3.4	7.3	4.3	↓ S phase

% cells in each cell cycle phase is shown. Data are the average of at least three independent experiments. S phase data indicate either 30% increase (↑) or decrease (↓) in the proportion of cells undergoing DNA replication, calculated as [(S phase IFNγ − S phase BSA)/S phase BSA]. ↑ sub G1 indicates a greater than 10% increase in sub G1 cells in response to IFNγ treatment (sub G1 IFNγ − sub G1 BSA). n.d. not determined; IFNγ, interferon-γ; BSA, bovine serum albumin.

2.4 Discussion

Analysis of the IFNγ target proteins, HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2, in a panel of 39 melanoma cell lines revealed that IFNγ stimulated cell surface expression of all five markers in only 13 melanoma cell lines tested. The degree of IFNγ-mediated induction was highly variable for all five markers but closely reflected the corresponding basal expression levels for HLA-ABC, HLA-DR, PD-L2, and NGFR. By contrast, PD-L1 expression was frequently absent at baseline (relative MFI < 1.5) but was still induced to high levels after IFNγ treatment in the majority of cell lines. Consequently, although the JAK/STAT/IRF1 pathway is critical for the IFNγ-mediated induction of HLA-ABC, HLA-DR, and the two PD-1 ligands (265, 475), the low constitutive expression of PD-L1 suggests that this pathway has low baseline activity in melanoma and that the constitutive expression of HLA-ABC, HLA-DR, PD-L2, and NGFR may be regulated via alternate pathways or downstream elements.

The IFNγ-induced expression of several markers, including PD-L1 and PD-L2, was correlated, although we did not detect significant correlation when the degree of IFNγ stimulation (i.e., change from pre- to post-stimulation) was compared. This may reflect disparate baseline expression levels due to IFNγ-independent regulatory influences but also the complexity and redundancy of the IFNγ signalling pathway. For instance, whereas the JAK–STAT1/2/3–IRF1 axis is critical for PD-L1 regulation, the JAK–STAT3–IRF1 node is important for PD-L2 stimulation (265). We also noted that cell surface expression of HLA-DR, NGFR, and PD-L2 was significantly lower in UVM compared to cutaneous melanoma, both at baseline and post-IFNγ stimulation. The transcriptomic analysis of the TCGA cutaneous and UVM datasets confirmed that UVM expressed lower levels of HLA-DRA, NGFR, CD274 (PD-L1), and PDCD1LG2 (PD-L2) transcripts, and this was associated with reduced transcript

expression of the IFNγ master transcription factors STAT1, STAT3, and IRF1 and with reduced IFNγ transcriptome signatures. It is worth noting that although transcriptome data are derived from high quality tumour samples with at least 60% tumour nuclei, they do contain variable levels of tissue-infiltrating immune and stromal cell populations that may influence the level of transcript expression (79). Nevertheless, collectively the transcriptome and flow cytometric analysis indicate diminished IFNγ activity in UVM.

Incomplete responses to IFNγ-stimulation, usually manifested as lack of induction of one or more markers were evident in 26 of 39 (67%) melanoma cell lines. Although it is still not clear whether incomplete IFNγ stimulation in melanoma cells has significant impact on patient responses to immunotherapy, it is evident that this pathway is important for response to PD-1 blockade. In particular, nuclear expression of the IFNγ transcription factor IRF1 (491) is associated with better response to anti-PD-1 therapy in melanoma (492) and loss-of-function mutations in IFNγ pathway modulators (JAK1, JAK2) are associated with resistance to anti-PD-1 treatment. Moreover, murine B16 melanoma cells deficient in JAK1 or IFNGR1 grew faster than control B16 cells in response to immune therapy (291). Metastatic UVM respond poorly to immune checkpoint inhibition (493, 494), and although there appears to be no difference in the level of infiltrating CD8+ T cells between uveal and cutaneous melanoma (495), our data suggest that UVM may have diminished capacity to respond to IFNγ, with lower expression of targets including PD-L1 (496), PD-L2, HLA-DR, and NGFR (this study). It is therefore provocative to suggest that inducibility of multiple IFNγ targets may inform or predict immunotherapy response.

It is worth noting that of the 26 melanoma cell lines displaying incomplete induction of the 5 target proteins, 8 showed cell cycle distribution changes in response to IFNγ

treatment. Importantly, 5/7 melanoma cell lines with no IFNγ-mediated PD-L1 induction showed no cell cycle profile changes after treatment with IFNγ. This may reflect the critical role of the STAT1 transcription factor in promoting PD-L1 expression and mediating IFNγ-induced cell cycle effects (265, 497). Five of eight UVM cell lines responded to IFNγ treatment with a decreased proportion of S phase cells and this was not a common response in our panel of cutaneous melanoma cells. This may be due to IFNγ concentration effects, as previous reports have shown that 50 U/ml IFNγ was sufficient to arrest UVM cells, whereas concentrations exceeding 1,000 U/ml IFNγ were required to inhibit the growth of the cutaneous A375 melanoma cells (497, 498). The unique responses of UVM cells to IFNγ stimulation require further investigation.

Interestingly, although most of our cell lines did not display baseline PD-L1 expression, PD-L1 was induced in the majority of cell lines. This is significant, as PD-L1 expression is sufficient to mediate melanoma escape from immune checkpoint inhibition (499). Loss of MHC class I expression is another established mechanism of immune escape, often involving genetic alterations in the B2M gene (194, 397, 482) and we noted that the SMU15-0217 melanoma cell line showed loss of B2M expression, concurrent with loss of HLA-ABC expression. Only one cell line (i.e., D22M1) failed to respond to IFNγ, and this was associated with a homozygous, predicted loss-of-function mutation in the IFNGR1 gene.

In conclusion, our study demonstrates that expression analysis of IFNγ targets pre- and post-IFNγ stimulation can identify incomplete IFNγ pathway activity in melanoma cells. We show that incomplete IFNγ signalling occurs in almost 70% of immunotherapy-naïve melanoma, and previous reports have confirmed that pre-existing alterations affecting IFNγ signalling have the potential to confer resistance to

immune checkpoint inhibitors (163, 194). In fact, we identified two well-recognised mechanisms of immunotherapy resistance; the loss of B2M expression, resulting in absence of cell surface HLA-ABC, and a missense mutation in the IFNGR1 gene, resulting in loss of cell surface IFNGR1. We also report that UVMs, which show poor responses to PD-1-inhibitor therapies, display an inherently weaker response to IFNγ signalling with reduced JAK–STAT pathway activity.

2.5 Supplementary Material

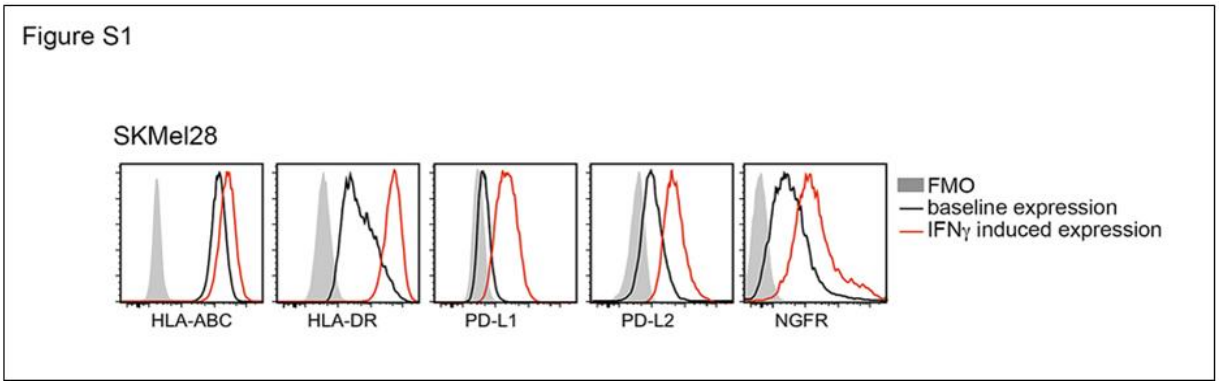

Figure S2.1. Flow cytometric analysis in melanoma cells

Representative histograms of baseline (solid black line) and IFNγ-induced expression (solid red line) of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2 in SKMel28 melanoma cells. FMO are shown as shaded histograms

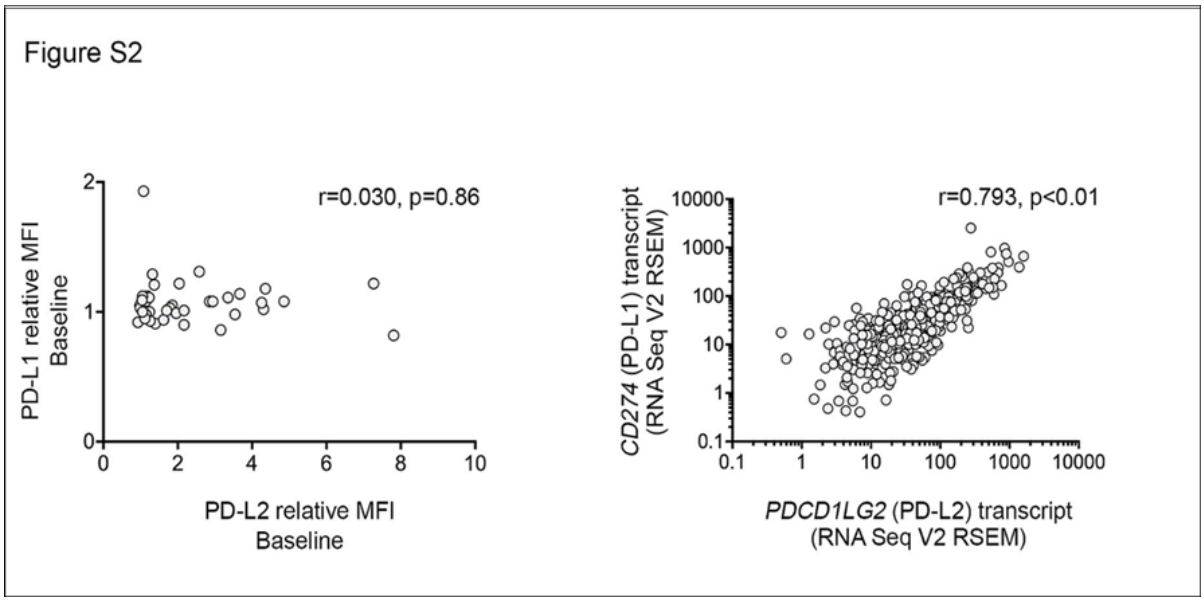

Figure S2.2. PD-L1 and PD-L2 protein and transcript expression in melanoma cells Correlation of cell surface protein [relative mean fluorescence intensity (MFI); left panel] and CD274 (PD-L1) and PDCD1LG2 (PD-L2) mRNA transcript expression derived from The Cancer Genome Atlas skin cutaneous melanoma dataset; right panel. Each dot represents one cell line. Spearman's rank correlation coefficient and p values are shown.

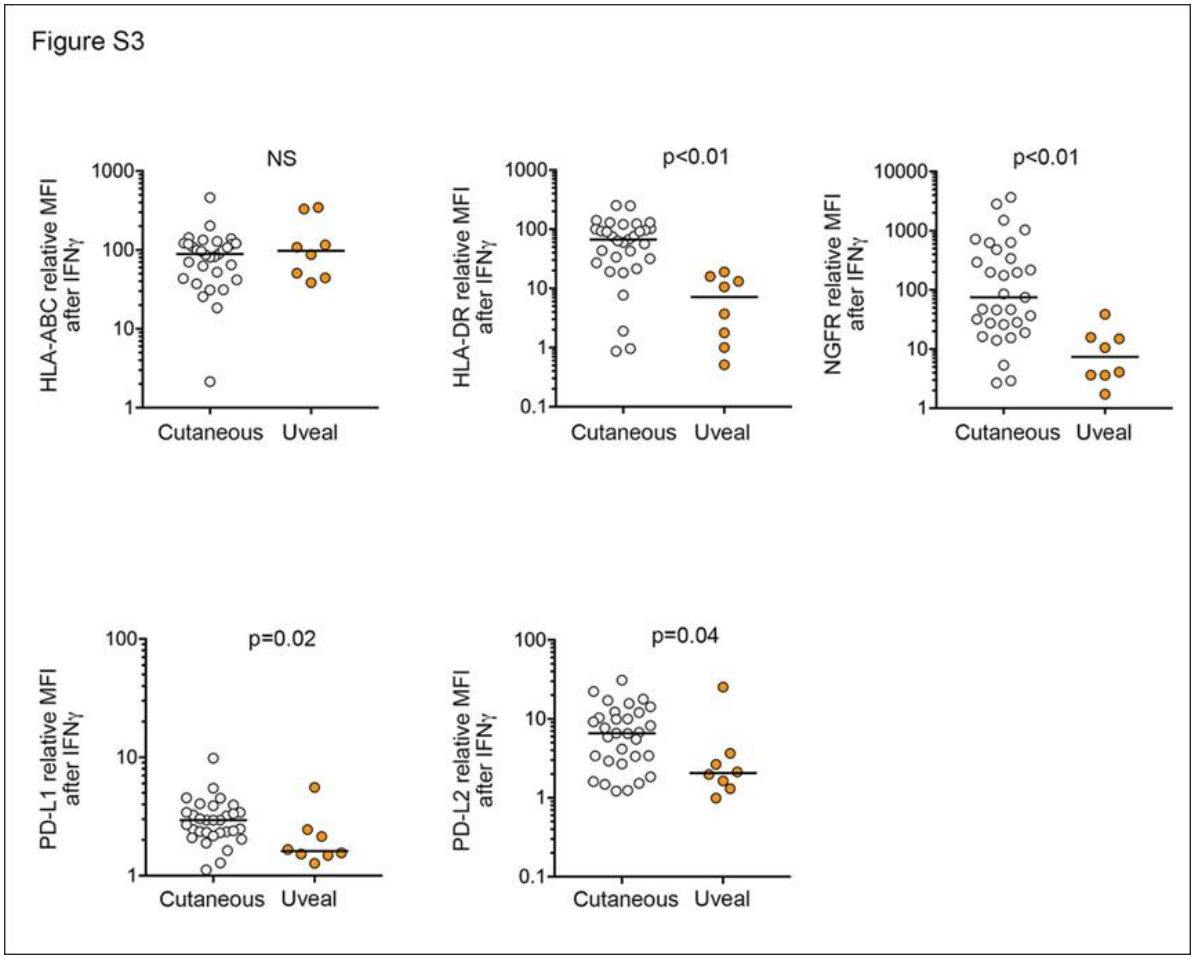

Figure S2.3. Expression of downstream IFNγ targets post-IFNγ stimulation in cutaneous and uveal melanoma cells

Cell surface expression post-IFNγ stimulation (relative MFI) of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2 in cutaneous (n = 31) and uveal melanoma (n = 8) cell lines. Bars represent medians. Mann–Whitney test, p values are indicated.

Chapter 3
TNFα effects on immune marker expression in melanoma cells

3.1 Introduction

Growing tumours develop an immunosuppressive microenvironment that may interfere with immune recognition and destruction of tumour cells. A variety of inflammatory cytokines produced by tumour-infiltrating immune cells mediate tumour response to immunotherapy via direct and indirect effects. Understanding cytokine-mediated immune modulation may provide novel strategies to overcome immunotherapy resistance.

Tumour necrosis factor alpha (TNFα) is a pro-inflammatory cytokine secreted into the tumour microenvironment by both tumour cells and stromal cells including macrophages, mast cells, fibroblasts, endothelial cells and T cells (500, 501). TNFα is one of 19 members in the large tumour necrosis factor superfamily (293). Like many other members of the TNF superfamily, it is predominantly expressed in a membrane-bound form that can be converted to soluble TNFα by the action of matrix metalloproteinases (502). TNFα acts on target cells by binding its two receptors, TNFR1 (also known as p55 or TNFRSF1A) or TNFR2 (also known as p75 or TNFRSF1B) (300, 500). TNFR1 is ubiquitously expressed (293) while expression of

TNFR2 is mainly restricted to hematopoietic cells, lymphocytes such as TRegs (502), microglia, olidodendrocytes and endothelial cells (503, 504). TNFα expression has been detected in tissue biopsies of breast, ovarian and renal carcinomas, both on cancer cells and the adjacent stroma (504), and elevated serum levels of TNFα have been reported in patients with breast and ovarian cancers (500, 505).

TNFα has a wide range of activities that can be both pro-tumourogenic (293) and anti-tumourogenic (506). TNFα pro-tumourigenic properties include:

1. Maintaining a state of chronic inflammation which is associated with cancer progression (306). TNFα supports angiogenesis by activating the mTOR pathway in endothelial cells (507) and enhancing remodelling of the tumour microenvironment by increasing vascular permeability and matrix metalloproteinase production (293, 508, 509). These factors may also contribute to tumour invasiveness and dissemination in chronic inflammation-induced cancers, such as breast and esophageal cancer (500).

2. Direct effect on tumour growth by increasing cancer cell survival and proliferation (510) via activation of the mTOR (473, 507), Hedgehog (473, 510), AKT (473, 511) and MAPK pathways (512). The TNFα/mTOR/S6K1 pathway promotes activation of downstream effectors of the Hedgehog pathway, which mediate the development of certain cancers such as esophageal adenocarcinoma (510). TNFα-induced AKT activation reduces keratinocyte susceptibility to proapoptotic stimuli and enhances cell growth in squamous cell carcinoma (511).

3. Induction of EMT in tumour cells via activation of NF-κB transcription factors (473, 513). In malignant melanoma, TNFα-induced EMT is associated with

melanoma cell de-differentiation and loss of melanocyte differentiation antigens (MDAs) (413). Loss of MDAs results in impaired CD8+ T cell immune responses, reduced tumour immunogenicity, and ultimately, tumour immune escape.

4. Reducing accumulation of cytotoxic T cells in the tumour microenvironment by preventing CD8+ T cell infiltration into tumours (304, 514). TNFα can also trigger CD8+ T cell death (306) and TNFα-induced MAPK activation prevents maturation of antigen-presenting DCs and reduces IL-12 production, inhibiting T cell activation and reducing T cell immune responses (512).

5. Promoting accumulation of immunosuppressive cell subsets, such as TRegs (508), BRegs (300, 302) and MDSCs (300, 303) in the tumour microenvironment.

6. Promoting the expression of immune inhibitory checkpoints on both tumour and immune cells. TNFα-mediated induction of immune-suppressive molecules such as IDO enables tumour cell escape (202). TNFα also stabilizes PD-L1 expression on melanoma, breast, colon and lung cancer cells via the COP9 signalosome 5 (CSN5) and NF-κB p65 activation (473).

On the other hand, TNFα has tumour suppressing activities which include:

1. Inducing haemorrhagic necrosis of tumours in vivo (500) and acting synergistically with IFNγ to induce cancer cell death (515) or senescence in cancer cells (514, 516).

2. Enhancing CD8+ T cell-mediated anti-tumour immunity by facilitating DC maturation (517) and therefore increasing naive CD8+ T cell proliferation and effector CD8+ T cell-triggered death of cancer cells (514).

3. Inducing expression of immune regulatory genes, including molecules associated with antigen presentation (HLA-DR) and chemokines (CXCL9 and CXCL10) that promote anti-tumour immune activity (518).

In this chapter, we investigated the response of a large panel of immunotherapy naive melanoma cell lines (described in Chapter 2) to TNFα. Melanoma cell surface expression of immune inhibitory checkpoints PD-L1 and PD-L2, antigen-presenting molecules HLA-ABC and HLA-DR, and NGFR were assessed following TNFα exposure, and the effects of TNFα and IFNγ treatment on immunotherapy naive melanoma cell lines were compared. Our results indicate that TNFα was inferior to IFNγ in inducing PD-L1, PD-L2, HLA-DR and HLA-ABC expression on melanoma cells, whereas TNFα preferentially upregulated NGFR expression, implicating a role for TNFα in melanoma cell de-differentiation.

3.2 Material and Methods

3.2.1 Cell lines and cell culture

The 39 melanoma cell lines included in this study are described in detail in Chapter 2.

3.2.2 Cell cycle analysis

Cells were treated with vehicle control (0.1% BSA) or 1000 U/ml (50 ng/ml) TNFα (recombinant human TNF α, Peprotech) for 72 h, then harvested, washed twice with PBS and stained with 50 µg/ml propidium iodide containing 50 µg/ml ribonuclease A for 20 min at 37°C. Cells were acquired as described in section 2.2.2 and DNA content from at least 10,000 cells was analysed using the ModFit software (Verity Software House, Topsham, ME). Flow cytometry was carried out as described in Chapter 2.

3.2.3 DNA extraction and Whole Exome Sequencing

DNA was extracted from early-passage melanoma cells using the G-spin™ Total DNA Extraction Kit as per manufacturer's protocol (Intron Biotechnology, Seongnam, South Korea) and DNA quantified using the SmartSpec Plus Spectrophotometer (Bio-Rad). Integrity of genomic DNA was further confirmed by gel electrophoresis. Exome sequencing of melanoma cell lines was performed as follows. Exonic DNA was enriched using the Illumina TrueSeq technology, targeting the 62 Mb encompassing protein-coding regions, and sequenced on an Illumina HiSeq2000. Read pairs were aligned to the reference human genome (hg19) using Burrows-Wheeler Aligner (BWA). Duplicates were removed with Picard and applied GATK indel realignment and base quality recalibration. Single-nucleotide variants and small

insertion/deletions (INDELS) were detected by SAMTools57. To generate a list of high-quality variants, low-coverage variants (single-nucleotide polymorphism quality ≤30, read depth ≤10) and variants in the top 5% of exonically variable regions, annotated as common polymorphisms, or in the 1000 Genomes Project, were removed using Ingenuity Variant Analysis (http://www.ingenuity.com; Qiagen, Venlo, Netherlands).

3.2.4 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software version 7 (GraphPad, San Diego, CA). All values are expressed as mean of at least two independent experiments and statistical methods applied are detailed in each figure legend. Differences were considered to be statistically significant when $P < 0.05$.

3.3 Results

3.3.1 Effects of TNFα on melanoma expression of immune markers

We investigated the effect of TNFα on the expression of five immune effector molecules in a panel of 39 immunotherapy naive melanoma cell lines (Figure 3.1). We have previously investigated the effect of IFNγ on the expression of these target molecules (Chapter 2).

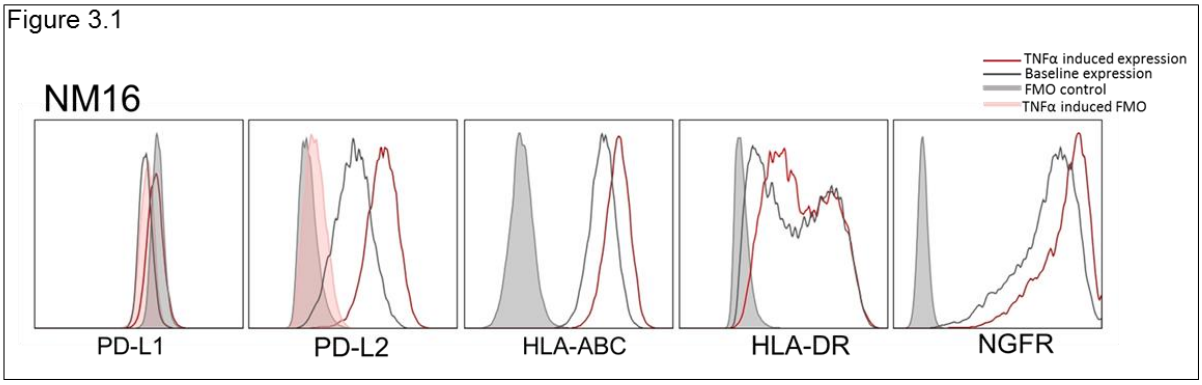

Figure 3.1 Cell surface expression of five immune markers at baseline and after TNFα exposure

A representative example of TNFα-induced expression of PD-L1, PD-L2, HLA-ABC, HLA-DR, and NGFR is shown for the NM16 melanoma cell line. Baseline expression (cells treated with 0.1% BSA) is shown by the black line, TNFα-induced expression (cells treated with 1000 U/ml for 72 h) by the red line, the relevant FMO controls are shown as gray shaded histograms, and TNFα-treated FMO controls are shown as pink shaded histograms.

Comparison of five markers at baseline and after treatment with TNFα showed that the induced expression of these markers correlated with their basal expression levels with the exception of PD-L1, which was not expressed at baseline in all but one cell line (Figure 3.2). Thus, TNFα-induced expression of PD-L1 was independent of its baseline expression. Predictably, the level of induction of all markers (i.e. expression post TNFα / baseline expression) was closely correlated (Figure 3.2).

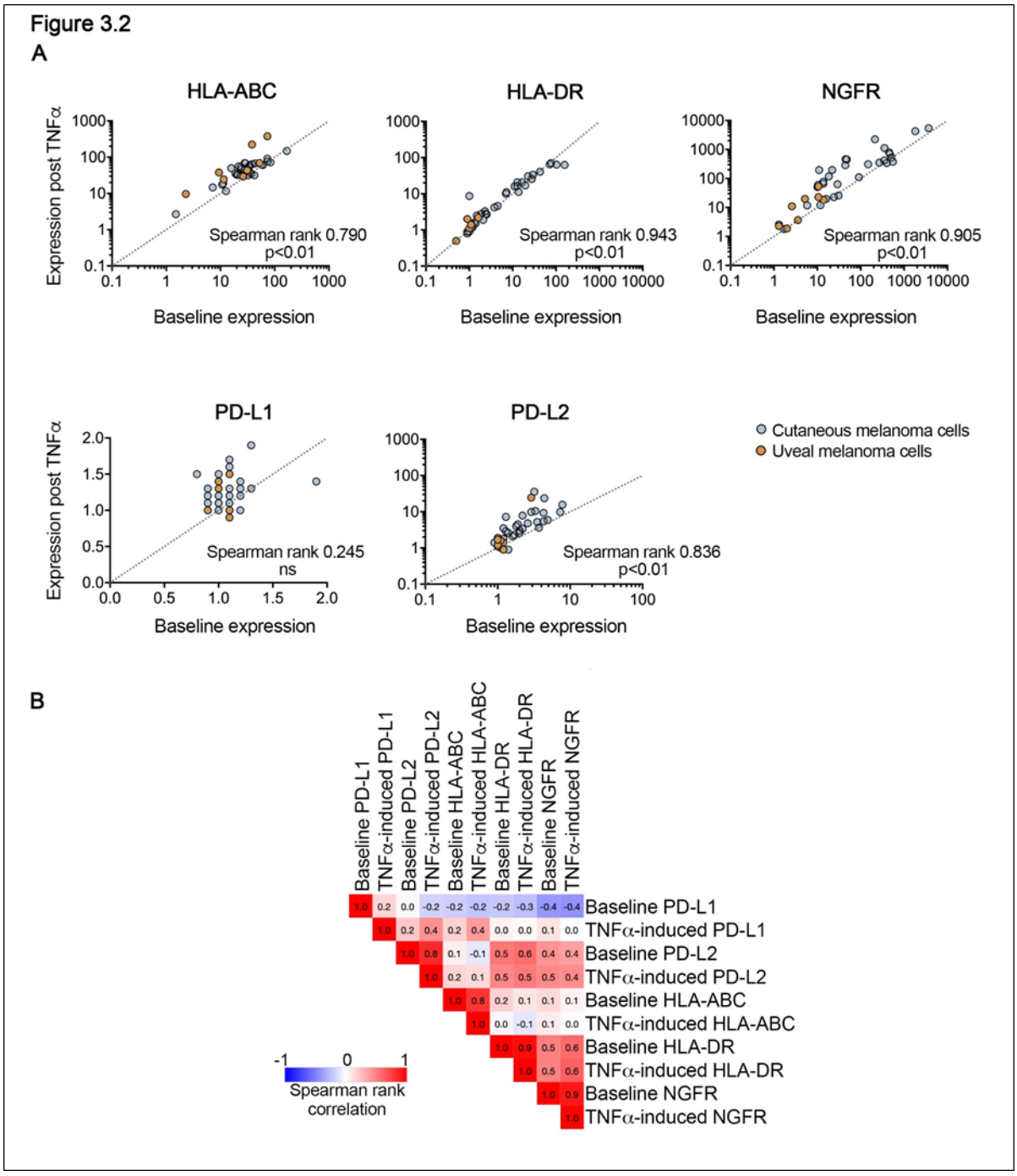

Figure 3.2 Correlation between baseline and TNFα induced expression

(A) Plot showing baseline versus TNFα-induced expression of each immune marker. Each dot represents one cell line and the average of at least two biological replicates. Orange dots indicate uveal melanoma cell lines. Spearman rank correlation coefficient and P values are shown. Dotted line corresponds to y=x. (B) Similarity matrix showing Spearman rank correlation coefficient of baseline and TNFα-induced expression of all five immune markers. Matrix generated using Morpheus tool (https://software.broadinstitute.org/morpheus).

3.3.2 Effects of TNFα on melanoma expression of immune markers

We noted that TNFα induced expression of PD-L2, HLA-ABC and NGFR in the majority of melanoma cell lines (18/39, 23/39 and 23/39, respectively; induced expression = expression post TNF / baseline expression ≥ 1.5), while PD-L1 was upregulated in only 3/39, and HLA-DR in only 5/39 cell lines (Table 3.1; Figure 3.3 F). Median level of induction (i.e. expression post TNFα / baseline expression) was below 2-fold for all markers (1.2 for PD-L1, 1.5 for PD-L2, 1.6 for HLA-ABC, 1.1 for HLA-DR) except for NGFR (median induction=2.0). Induction of NGFR expression was ≥ 2-fold in 20/23 responsive cell lines, compared with 13/18 (72%) for PD-L2 and 11/23 (48%) for HLA-ABC (Table 3.1; Figure 3.3).

Of the two inhibitory checkpoints, PD-L1 was not expressed at baseline in 38/39 cell lines analysed (expression <1.5) and remained low after TNFα exposure (expression ranging from 0.9 to 1.9, with ≥1.5 in only 7/39 cell lines). PD-L2 was expressed in 54% (21/39) of cell lines at baseline and in 74% (29/39) after TNFα exposure, with induction observed in 46% (18/39) of the cell lines. The highest induction of PD-L2 after TNFα treatment was observed for C084M (induction = 11.3), MeWo (induction = 5.7) and the uveal melanoma cell line MP38 (induction = 8.3) (Table 3.1; Figure 3.3).

Seven cell lines, two BRAF/NRAS WT cell lines C025M1 and D24M, two uveal melanoma cell lines MEL270 and OMM1 and three NRAS cell lines NM177, ME4405, MelAT showed a complete loss of response to TNFα with no induction of any of the target molecules.

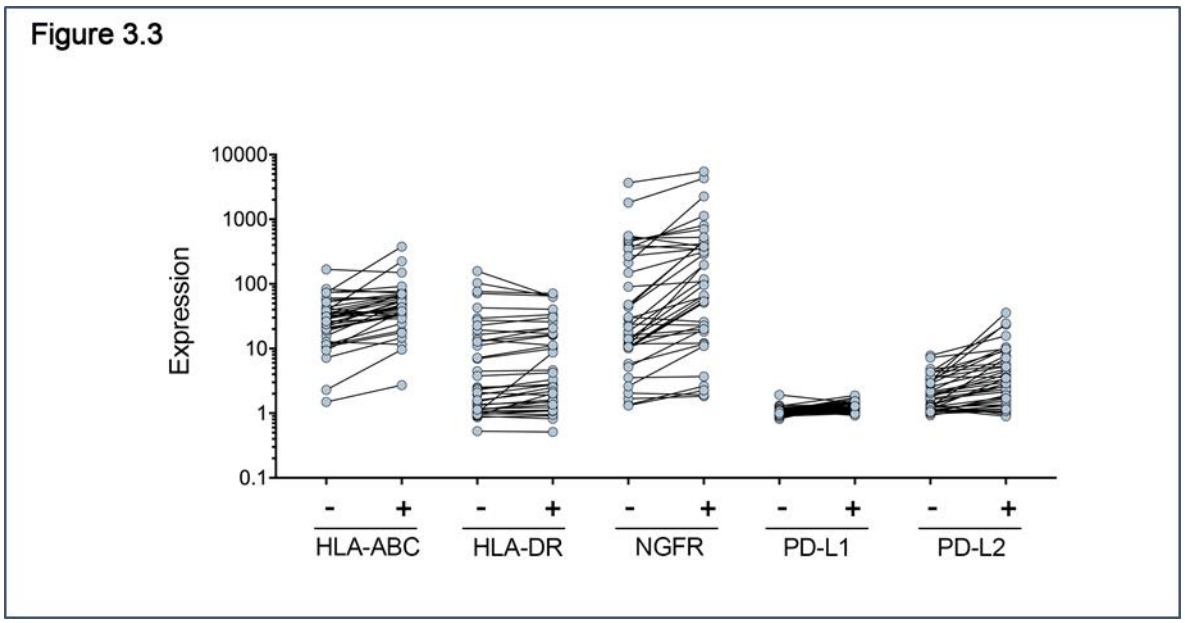

Figure 3.3 Effect of TNFα on the expression of the five immune markers

Expression of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2 at baseline and 72 h post exposure to TNFα (expression = antibody-stained MFI / MFI FMO). Each dot represents one cell line (average of at least two independent experiments) before (−) and after (+) TNFα stimulation.

Table 3.1 Expression of five immune markers at baseline and after stimulation with TNFα in melanoma cell lines

		HLA-ABC		HLA-DR		NGFR		PD-L1		PD-L2	
		BSA	TNFα	BSA	TNFα	BSA	TNFα	BSA	TNFα	BSA	TNFα
$BRAF^{V600E}$	A2058	32.9	54.9	2.5	2.7	398.7	341.7	1.0	1.5	1.9	4.5
	SKMel28	74.2	76.9	11.1	16.1	44.7	466.0	1.3	1.3	2.6	4.8
	C060M1	34.9	38.4	29.5	33.5	10.4	60.9	1.0	1.1	4.3	5.4
	SCC14-0257	15.8	49.7	12.8	16.5	347.3	419.4	0.9	1.3	1.4	2.6
	MM418	38.2	64.2	1.1	1.2	16.0	19.8	1.1	1.2	1.1	1.3
	NM16	21.7	56.0	7.0	10.0	1808.8	4306.0	0.8	1.5	7.8	15.7
	NM182	18.9	35.5	1.3	1.6	10.1	51.2	1.0	1.1	1.0	1.1
	MM200	35.1	59.6	7.1	11.1	450.7	802.6	1.0	1.1	1.2	1.8
	NM39	43.6	67.3	27.6	25.8	90.7	109.8	1.1	1.6	4.3	9.3
	HT144	23.4	32.4	71.4	65.1	353.1	1132.5	1.1	1.3	4.9	5.9
	C016M	20.9	32.6	42.7	40.7	469.6	700.0	0.9	1.0	2.2	3.4
$NRAS^{Q61R/K/L}$	MelRm	36.9	35.1	102.8	63.7	18.4	118.2	0.9	1.2	1.6	2.1
	NM47	42.1	31.7	157.9	63.0	213.6	2253.7	1.0	1.1	1.8	4.0
	NM177	56.5	62.0	76.6	71.1	3674.9	5490.6	1.0	1.0	2.0	2.6
	NM179	12.7	11.6	2.4	2.8	44.2	286.9	1.0	1.1	1.7	2.3
	ME4405	60.5	60.7	0.9	0.9	24.0	22.9	1.0	1.2	3.5	5.2
	MelAT	31.9	41.4	0.9	0.8	11.8	12.1	1.2	1.4	2.0	3.0
	D11M2	11.3	19.0	16.4	20.8	29.2	63.1	1.1	1.5	3.3	10.5
	C002M	7.2	14.7	1.0	0.9	13.7	66.2	1.2	1.0	1.4	0.9
	C013M	24.7	47.3	1.0	8.7	47.8	453.1	1.0	1.4	2.2	7.8
	D38M2	28.6	45.5	4.5	4.6	509.6	526.2	1.1	1.7	2.9	9.9
BRAF/RAS Wild Type	D22M1	28.0	69.0	1.9	2.8	5.8	11.8	1.3	1.9	1.3	2.9
	MeWo	28.9	68.5	1.4	1.5	268.9	351.7	0.9	1.3	1.3	7.2
	D24M	32.6	31.9	13.8	11.2	30.7	25.8	1.2	1.3	7.3	9.9
	C022M1	10.7	17.5	2.3	3.3	148.3	312.3	1.9	1.4	1.1	1.2
	C084M	83.6	72.0	19.5	17.0	552.5	378.8	0.9	1.2	3.2	35.8
	C086M	20.1	32.7	22.7	30.6	1.3	2.6	1.1	1.1	3.7	3.6
	D35	167.9	149.4	3.8	4.2	21.9	196.6	0.9	1.1	0.9	1.4
	C025M1	73.2	92.0	2.1	1.9	1.7	1.8	1.1	1.5	1.1	1.0
	SMU15-0217	1.5	2.7	12.8	20.8	11.0	196.6	1.2	1.2	4.4	23.6
	A04-GEH	23.9	53.1	1.5	2.5	13.8	96.1	1.0	1.2	1.2	3.5
$GNAQ^{Q209L/P}$	92.1	11.5	24.5	0.5	0.5	14.0	18.3	1.1	0.9	1.2	0.9
	MEL202	38.2	224.1	1.1	1.5	10.6	53.7	1.0	1.4	1.0	1.9
	MEL270	52.5	69.7	1.1	1.1	3.6	3.7	1.1	1.0	1.2	1.3
	MP38	73.5	378.9	1.6	2.2	10.6	22.7	1.1	1.5	2.9	24.5
	MP46	2.3	9.7	0.9	1.0	5.1	19.9	1.0	1.0	1.0	1.1
$GNA11^{Q209L}$	OMM1	31.2	43.5	1.0	1.1	2.0	1.9	1.1	1.5	1.0	1.0
	MP41	26.3	29.2	1.0	2.0	2.6	11.0	0.9	1.0	1.1	1.5
	MM28	9.4	37.8	1.1	1.4	1.3	2.3	1.0	1.3	1.0	1.7

Expression levels at baseline (BSA) and after TNFα were calculated by dividing the geometric mean fluorescence intensity (MFI) of the antibody-stained sample by the MFI of FMO control. Data shown are the average of at least two independent experiments.

3.3.3 Comparison of TNFα and IFNγ mediated induction of immune markers

We next compared the melanoma cell responses to TNFα and IFNγ treatment (Chapter 2). Expression of PD-L1, PD-L2, HLA-DR and HLA-ABC was significantly higher in response to IFNγ compared to TNFα, while there was no significant difference between the expression of NGFR post IFNγ or TNFα treatment (Figure 3.4). Of note, PD-L1 was absent in 38/39 cell lines at baseline and induced by TNFα in 3/39 (8%) cell lines. In contrast, IFNγ induced PD-L1 in 32/39 cell lines (82%). HLA-DR did not show significant change after exposure to TNFα compared to baseline, whereas this marker was induced (i.e. expression post TNFα / baseline expression ≥ 1.5) by IFNγ in 32/39 (82%) melanoma cells (Figure 3.4 and Figure 3.5). Overall, IFNγ induced higher levels of PD-L1, PD-L2, HLA-ABC and HLA-DR, while TNFα induced higher levels of NGFR (Figure 3.5).

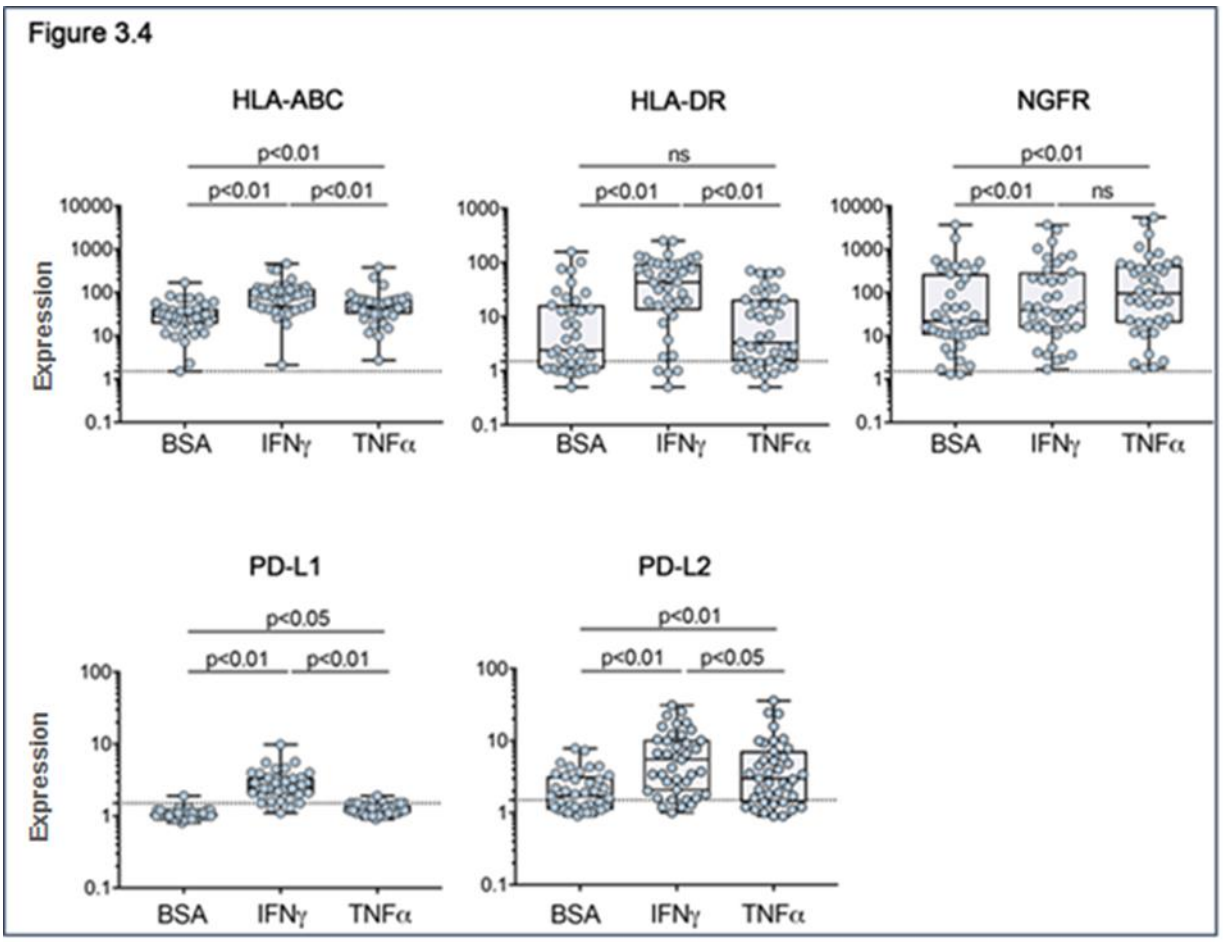

Figure 3.4 Expression of markers at baseline and in response to TNFα and IFNγ

Box plots showing the cell surface expression of markers at baseline, after IFNγ or TNFα treatment. Each dot represents one cell line (average of at least two independent experiments), box plots indicate the median and the interquartile range and the whiskers indicate the range. Expression levels (ratio, mfi stained/ mfi FMO control) were compared using a Friedman's test with Dunn's post-test. P values are indicated; ns, not significant.

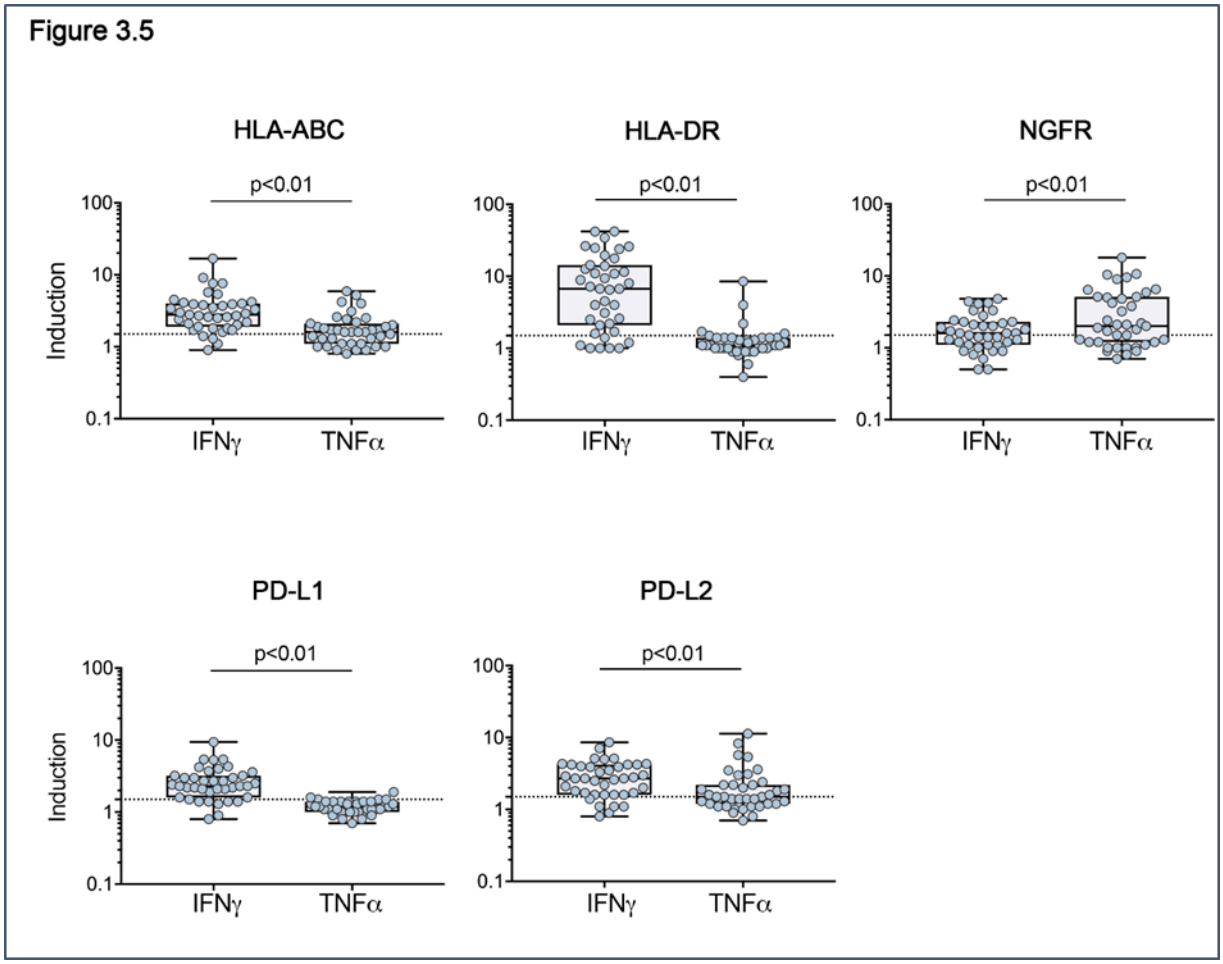

Figure 3.5 Induction of cell surface immune markers after IFNγ and TNFα treatment

Box plot showing the cell surface induction of markers after IFNγ or TNFα treatment (expression after cytokine treatment / expression at baseline control). Each dot represents one cell line (average of at least two independent experiments), box plots indicate the median and the interquartile range and the whiskers indicate the range Induction levels were compared using Wilcoxon matched-pairs signed rank test. P values are indicated; ns, not significant.

Our analysis revealed that some markers were not induced by either cytokine in a subset of cell lines. For instance, PD-L1 was not induced by TNFα or IFNγ in seven cell lines (four uveal melanomas, two BRAF/NRAS WT and one BRAF-mutant), PD-L2 was not induced in five cell lines (two uveal melanomas, one BRAF/NRAS WT, one BRAF-mutant and one NRAS-mutant), HLA-ABC was not induced in two BRAF/NRAS WT cell lines, HLA-DR was not induced in seven cell lines (three NRAS-mutant, one BRAF-mutant, one BRAF/NRAS WT, and two uveal melanomas), and NGFR not induced in ten cell lines (four BRAF-mutant, two NRAS-mutant, three BRAF/NRAS WT and one uveal melanoma) by either IFNγ or TNFα (Table 3.2).

Table 3.2 Cell lines with no response to either IFNγ or TNFα treatment

Cell lines with no marker induction after IFNγ and TNFα				
PD-L1	**PD-L2**	**HLA-ABC**	**HLA-DR**	**NGFR**
C016M*	**92.1***	**C084M**	**92.1***	A2058
C022M1	C002M*	**D24M**	**D22M1**	**C016M**
D22M1*	C025M1*		HT144	**C084M**
MEL270*	**MEL270***		ME4405*	**D24M**
MP41*	**MM418***		MelAT*	D38M2
MP46*			**MP46***	**MEL270**
OMM1*			**NM177**	MeWo
				MM418
				NM177
				NM39

Asterisks indicate lack of expression at baseline. Highlighted in bold are nine cell lines that lacked expression of two or more markers after IFNγ and TNFα treatment.

3.3.4 Changes in cell cycle profile after TNFα treatment

TNFα has been shown to decrease cell viability and induce cell death, and we explored the effect of TNFα on cell cycle distribution in our panel of cell lines. A threshold of ≥ 10% increase in sub G1 was chosen as an indicator of cell death, and ≥ 30% change in the S-phase was chosen as an indicator of change in the DNA replication as previously described in Chapter 2.

Only 1/39 cell lines analyzed, C022M, showed substantial cell death (37.1% sub G1 increase) in response to TNFα treatment (Figure 3.6). This cell line did not show sub G1 accumulation in response to IFNγ, although IFNγ induced >10% sub G1 increase in three other cutaneous melanoma cell lines (HT144, MM200 and C013M) (Figure 3.6). The effect of TNFα on S-phase was more pronounced, with nine cell lines including four cutaneous melanoma cell lines (C022M1, SMU15-0217, D22M1, MM200) and five uveal melanoma cell lines (MP28, Mel202, MP38, Mel270 and MP41) showing ≥ 30% decrease in S-phase relative to controls. In comparison, only one cutaneous melanoma cell line (SKMel28) and five uveal melanoma cell lines (Mel202, MP28, MP41, 92.1, MP38) responded to IFNγ with S-phase changes.

Finally, one cell line (C084M) showed as increase in DNA replication (≥ 30% increase in S phase) in response to TNFα, compared with five cutaneous melanoma cells lines (NM179, A2058, MM200, C002M, C022M1) after IFNγ treatment (Figure 3.6). Of note, 4/8 uveal melanoma cell lines (MEL202, MP28, MP41 and MP28) showed a decrease in DNA replication after both IFNγ and TNFα treatment.

Figure 3.6

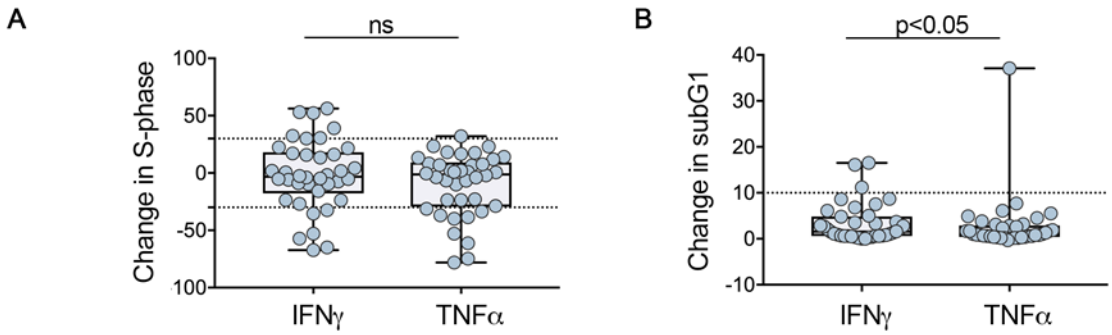

Figure 3.6 Change in cell cycle after treatment with IFNγ or TNFα

Box plots showing changes in S-phase (A) or sub G1 (B) in response to IFNγ or TNFα treatment. Each dot represents one cell line (average of at least two independent experiments), box plots indicate the median and the interquartile range and the whiskers indicate the range. The thresholds (30% for S-phase, 10% for sub G1) are indicated by the dotted lines. Changes were calculated as (S-phase after treatment - S-phase at baseline) / (S-phase at baseline) for S phase or (sub G1 after treatment – sub G1 at baseline) for sub G1. IFNγ and TNFα results were compared using Wilcoxon matched-pairs signed rank test.

3.3.5 Response of uveal and cutaneous melanoma cell lines to TNFα treatment

Response of cutaneous and uveal melanoma cells to TNFα was also compared. Induction of HLA-ABC was significantly higher in uveal cell lines compared to cutaneous melanoma cell lines (Figure 3.7). In contrast, induction of HLA-DR, NGFR, PD-L1 and PD-L2 were comparable in the uveal and cutaneous melanoma cells (Figure 3.7). There was no difference in the expression of immune markers in cutaneous melanoma cells of different genotypes after TNFα treatment (Table 3.1).

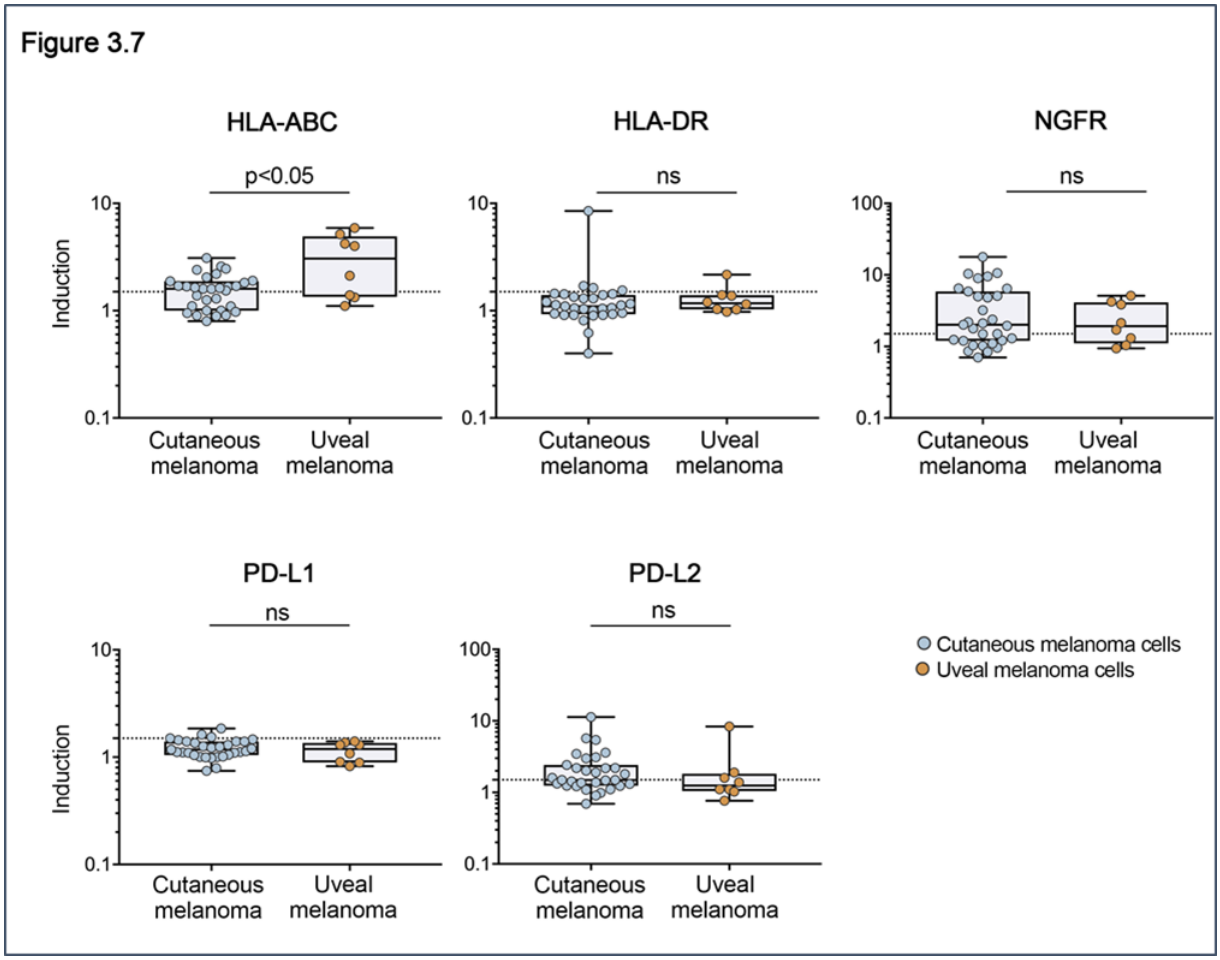

Figure 3.7 Response of cutaneous and uveal melanoma cell lines to TNFα treatment

Induction of HLA-ABC, HLA-DR, NGFR, PD-L1 and PD-L2 (expression after IFN treatment / expression at baseline) in cutaneous (n = 31) compared to uveal melanoma (n = 8) cell lines. Each dot represents one cell line (average of at least two independent experiments), box plots indicate the median and the interquartile range and the whiskers indicate the range. Induction levels were compared using Mann-Whitney test.

3.4 Discussion

Upregulation of immune inhibitory checkpoints such as PD-L1 and PD-L2 has been shown to contribute to tumour immune escape (300, 473). Expression of PD-1 ligands is regulated in part by inflammatory mediators such as IFNγ and TNFα. Several previous studies have found that TNFα induced expression of PD-L1 but not PD-L2 (519) in a range of cancers, including melanoma (473), colorectal and prostate cancer (473, 519). TNFα also regulates PD-L1 stabilization in cancer cells (457, 473). A recent study has shown that TNFα is important in mediating T cell killing of tumour cells that show loss of antigen presentation capacity, through the deletion of genes within the TNFα signalling, IFNγ signalling, and antigen presentation pathways (520).

In our study, we found that TNFα induced PD-L1 expression in only 3/39 (8%) of melanoma cell lines analysed (induction ≥ 1.5) in contrast to a previous report that showed TNFα induced PD-L1 expression in the LNCaP prostate cancer and HCT116 colon cancer cell lines (519). The discrepancies between our results and those reported by Wang et al. (519) could be due to differences in the cancer cell types tested (prostate cancer and colon cancer cell lines in the Wang et al. study (519), compared to melanoma in our study), the concentration of TNFα used (10 ng/mL in the Wang et al. study compared to 1000 U/ml in our study) and/or time of treatment (8 h, 16 h and 24 h in the Wang et al. study compared to 72 h in our study).

Intratumoural TNFα production during immunotherapy has been associated with loss of tumour immunogenicity and MHC class I antigen presentation due to increased activation of the epigenetic modifier, histone methyltransferase enhancer of zeste homolog 2 (EZH2) in melanoma cells (521). We observed decreased (induction < 1) HLA-ABC expression in 6/39 cell lines after TNFα treatment, while HLA-ABC was

upregulated in 23/39 cell lines (MFI ≥ 1.5). It is possible that the suppressive effects of TNFα on HLA-ABC expression in immunotherapy naive melanoma cells are less pronounced, and/or that the TNFα effect involved additional cell types in vivo and thus was not reproduced in our single cell type in vitro assays. We did, however, notice a reduction in HLA-DR expression in 10/39 cell lines and upregulation of HLA-DR in another 5/39 cell lines in response to TNFα. MHC class II is expressed only in professional antigen-presenting cells (B cells, DCs and macrophages) and thymic epithelial cells (202, 306). Most cancers do not express MHC class II molecules, melanoma being a rare exception (522). While primary melanomas often express MHC class II, only 50% of metastatic melanomas and cell lines express these MHC molecules (201, 523). MHC class II expression by melanoma cells is regarded by some as a potential mechanism preventing CD8+ T cell-mediated immunity by recruiting CD4+ T cells that subsequently downregulate the immune response (202). On the other hand, MHC class II positivity on tumour cells is associated with therapeutic response, progression-free and overall survival, as well as CD4+ and CD8+ tumour infiltrate (201). In melanoma, MHC class II expression is also associated with better response to anti-PD-1/PD-L1 therapy and can act as a predictive biomarker for therapeutic efficacy (201).

Overall, TNFα was less effective at stimulating expression of immune effectors in melanoma cells compared to IFNγ. IFNγ treatment led to higher upregulation of PD-L1, PD-L2, HLA- ABC and HLA-DR compared to TNFα, even though induction of NGFR by TNFα was higher. These results are consistent with earlier studies where IFNγ was superior to TNFα in upregulating PD-L1 and HLA-DR (518). Moreover, TNFα and IFNγ acted synergistically in the study (518). Selective upregulation of NGFR by TNFα may reflect its effect on melanoma cell de-differentiation. The effect of TNFα on NGFR is an interesting finding in this study. Expression of NGFR is

considered a marker of melanoma cell de-differentiation (422), and TNFα-induced de-differentiation in melanoma cells could lead to decreased expression of melanosomal antigens (149),), resulting in poor immune recognition. Decreased melanocytic antigen expression and the phenotypic plasticity of melanoma cells may contribute to tumour relapse after initial successful T cell immunotherapy (149, 514). Previous studies have shown upregulation of NGFR on melanoma cells by both IFNγ and TNFα (413), where TNFα-induced upregulation of NGFR also correlated with loss of melanoma antigens MLANA and gp100 in melanoma cells (413). A recent genome wide loss of function screen also confirmed that downregulation of MLANA conferred resistance to T cell mediated lysis (397) . Furthermore, melanoma dedifferentiation has been linked with resistance to BRAF inhibitors and immune therapies. (524) Although we did not address the effect of TNFα on melanocytic antigens, 59% of cell lines in our study upregulated NGFR after exposure to TNFα, serving as a surrogate de-differentiation marker.

In conclusion, our findings suggest that diminished response to TNFα is prevalent in melanoma cell lines. However, the majority of melanoma cells exposed to TNFα did induce NGFR expression, which may potentially contribute to immunotherapy resistance. It's been shown that acquired resistance to cancer immunotherapy can be mediated by inflammation-induced cancer dedifferentiation (525). TCGA data demonstrated that the expression of TNF was significantly correlated with AXL and inversely correlated with MITF and MLANA in the TCGA SKCM dataset, supporting a role for this cytokine in inducing melanoma de-differentiation, Although, we observed a weak correlation between TNF and NGFR, this did not reach significance (Spearman's rank = 0.089, p=0.054).

Future studies will delineate the role of TNFα in melanoma de-differentiation, including investigating the molecular signalling pathways induced by TNFα, and the differential effects of TNFα on the temporal and kinetic expression pattern of important immune markers. Outcomes of these investigations will be critical in the future development of effective cytokine-based cancer treatments. This is important as targeting TNFα could have beneficial effects by boosting anti-tumour responses via mechanisms including the inhibition of melanoma de-differentiation, preventing activity of immune suppressor cells, including MDSCs or immune inhibitory molecules like IDO, enhancing CD8+ T cell survival and infiltration into the tumour microenvironment.

These studies highlight the distinctive roles of two important immune-regulated cytokines. Whereas IFNg stimulates antigen presentation machinery, and negative feedback loops (i.e PD-L1 and PD-L2), it did not promote significant levels of NGFR in melanoma. In contrast, TNFa preferentially induced NGFR only, and thus appears to contribute to melanoma dedifferentiation as indicated by Landsberg et al (149). There is now significant data to show that this dedifferentiated melanoma phenotype is highly resistant to molecular targeted therapies (i.e BRAF and MEK inhibitors) and immune checkpoint inhibitors (524). It is therefore of critical interest to examine the precise role of TNFα in melanoma de-differentiation, including investigating the molecular signalling pathways modulated by TNFα and the TNFa downstream effectors critical to the promotion of melanoma dedifferentiation. Outcomes of these investigations may lead to the development of effective cytokine-based cancer treatments, that target the dedifferentiation-promoting effects of TNFα and improve anti-tumour immune responses.

Chapter 4
Immunotherapy resistance mechanisms in melanoma cells

4.1 Introduction

Immune checkpoint inhibitors such as anti-PD-1 and anti-CTLA-4 antibodies disrupt critical immune regulatory mechanisms to promote anti-tumour immune activity (323). These inhibitors have revolutionized cancer therapy and are widely used for the treatment of multiple malignancies including metastatic melanoma, non-small cell lung cancer, renal cell carcinoma, bladder cancer, head and neck cancer and gastric cancer (reviewed in (359). Immunotherapies are also being trialed in many other cancer types, including renal medullary cancer (ClinicalTrials.gov NCT03274258), gliomas (NCT03557359) and breast cancer (NCT02499367, NCT02892734).

In melanoma, immune checkpoint inhibitors produce long lasting responses and improve overall survival (368, 393, 397, 526). For instance, three years after the initial dose of anti-CTLA-4 (ipilimumab) the survival rate of melanoma patients is 20-25% and survival extends up to 10 years in a subset of patients (193, 370). Anti-PD-1 antibodies (nivolumab or pembrolizumab) produce objective responses in 30–40% of patients, with the many durable responses (389). The combination of immunotherapies (anti-CTLA-4 plus anti-PD-1 inhibitors) has further improved response rates and survival; 55% of melanoma patients will respond to combination anti-PD-1 and anti-CTLA-4 (389, 395) and the two-year survival rates for combination immunotherapies, nivolumab alone and ipilimumab alone were 64%, 58% and 54%,

respectively (396, 527). The increase in response rate with combination immunotherapies has come with significant adverse events however, and approximately 50% of patients treated with combination immunotherapies will experience grade 3/4 adverse events compared to 12% and 24% of patients treated with pembrolizumab or ipilimumab alone, respectively (193, 389, 395, 528).

Primary and acquired resistance remains a significant limitation of immune checkpoint inhibitors; 40-65% of melanoma patients will not respond to initial anti-PD-1 treatment and primary resistance to anti-CTLA-4 therapy is seen in more than 70% of melanoma patients (reviewed in (359). Of the patients who initially respond to PD-1 blockade, 25-45% of patients will eventually develop resistance (359, 393, 403, 404). Heterogeneity of response is also an important limitation with only 10%–12% of melanoma patients treated with pembrolizumab showing a complete response (359, 389, 390, 392).

Failure to respond to immune checkpoint blockade therapy may occur via tumour-intrinsic or tumour-extrinsic mechanisms (Table 4.1). Tumour-intrinsic mechanisms include genetic and epigenetic alterations which affect neoantigen formation, processing and/or presentation, and any change in cellular signalling pathways that disrupt the action of cytotoxic T cells (193, 398). Tumour-extrinsic mechanisms include non-cancerous stromal or immune cells, or any other systemic changes (161, 193, 398) that their function in association with cancer cells, promote tumour growth and resistance to immune checkpoint inhibitors (193). These mechanisms are described in detail in chapter 1 section 1.7. In this chapter, we sought to characterize several key resistance mechanisms in a panel of 16 short-term melanoma cell lines (PD-1 PROG cell lines) derived from patients who progressed on anti-PD-1-based immunotherapy. We investigated expression of IFNγ receptor IFNGR1, IFNγ

effectors including PD-1 ligands, PD-L1 and PD-L2, antigen presentation molecules, HLA-ABC, HLA-DR and the HLA-ABC structural element B2M. We also analysed expression of transcription factors, melanoma pigment antigens and markers of de-differentiation, including SOX10, MLANA, AXL, MITF and NGFR.

Table 4.1 Mechanisms of immunotherapy resistance

Tumour Cell intrinsic Resistant Mechanisms		Tumour cell extrinsic resistant mechanisms	
Mechanism	**Examples**	**Mechanism**	**Examples**
Absence of antigenic peptides	Low mutational burden Lack of cancer-testis antigens	Presence of immunosuppressive cells	Tumour associated macrophages Regulatory T cells Myeloid derived suppressor cells
Alterations in cellular enzymes and metabolic pathways	Induction of IDO1 Loss of PTEN expression Deregulated expression of the Wnt–β-catenin pathway	Immune suppressive cytokines and metabolites in the tumour microenvironment	Colony stimulating factor (CSF-1) Tryptophan metabolites Transforming growth factor ß (TGFβ)
Inactivation of IFNγ signalling	JAK1/2 loss of function mutations Loss of STAT1, IFNGR expression	Absence of T cells	Lack of T cells within the microenvironment
Disruption of antigen processing and presentation	Deletion in TAP transporters Deletion in B2M Suppression of HLA class I expression	Over expression of immune inhibitory molecules	VISTA, LAG-3, TIM-3, TIGIT, CTLA-4, PD-1
T cell exclusion	MAPK oncogenic signalling Stabilized β-catenin Mesenchymal phenotype Secretion of immune inhibitory molecules		

Adapted from Sharma et al (160)

4.2 Material and methods

4.2.1 Cell lines

A total of 16 melanoma cell lines (PD-1 PROG cells) were derived from melanoma patients treated with either anti-PD-1 (n=12/16; 75%) or combination anti-PD-1 and anti-CTLA-4 (n=4/16; 25%). Cells were derived from surgically excised, enzymatically processed melanoma lesions in a study carried out in accordance with the Human Research Ethics Committee (HREC) protocols from the Royal Prince Alfred Hospital (Protocol X15-0454 and HREC/11/RPAH/444). Cell line details are shown in Table 4.2.

Cell culture methods are described in detail in Chapter 1, Section 2.2.2

4.2.2 Coculture

Melanoma cells and matched T infiltrating lymphocytes (TILs) derived from the same tissue biopsy were used in the coculture assay. TILs were cultured in TIL media (RPMI1640 media supplemented with 10% heat inactivated human serum from male AB plasma (Sigma), 25mM HEPES, 100U/ml penicillin, 100ug/ml streptomycin, 10ug/ml gentamycin, 4mM L-glutamine and 1000U/mL IL-2 (Peprotech)) and expanded with addition of DynaBeads Human T activator CD3/CD28 (ThermoFisher, 25ul/1mL media). For the coculture assay, 1x104 MLM were cultured with 1x104 TILs (1:1 ratio) in 96 well plate in a total volume of 100ul TIL media, and each experimental setup was performed in triplicate. After two days culture, supernatant was collected, spun down to remove cell debris, and stored at -20°C for IFNγ

analysis using the Human IFNγ DuoSet ELISA (R&D Systems). ELISA was performed according to manufacturer's protocol. For PD-L1 and PD-L2 overexpression experiments, MLM cells were transduced with PD-L1 and PD-L2 constructs before co-culture setup.

4.2.3 Antibodies and reagents

Staining for flowcytometry was performed using anti-human antibodies against HLA-ABC (1:1000; clone W6/32; BioLegend, San Diego, CA) and IFNGR (1:10; clone GIR-208; BD Biosciences; Franklin Lakes, NJ) both conjugated to phycoerythrin (PE), HLA-DR (1:100; clone L243; BioLegend, San Diego, CA) conjugated to fluorescein isothiocyanate (FITC), CD271/NGFR (1:40; clone ME20.4; Biolegend; San Diego, CA) and B2M (1:200; clone 2M2; BioLegend; San Diego, CA) conjugated to PE-cyanine (Cy)7, CD273/PD-L2 (1:40; clone 24F.10C12; BioLegend; San Diego, CA) conjugated to allophycocyanin (APC) and CD274/PD-L1 (1:50; clone M1H1; BD Biosciences; Franklin Lakes, NJ) conjugated to brilliant violet.

Recombinant Human IFNγ (300-02) was purchased from Peprotech. Live dead stain 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Invitrogen, Thermo Fisher Scientific.

Western blotting was performed using Goat anti-Human AXL (1:200, R&D Systems), Rabbit anti-human SOX10 mAb (1:1000, cell signalling technology; MA, USA) and Rabbit anti-human MLANA/MART-1(1:1000, cell signalling technology; MA, USA); MITF (1:1000; Calbiochem); Goat anti-Mouse and goat anti-rabbit IgG (H+L) secondary antibody conjugated to Alexa Fluor 488 both obtained from Thermo Fisher Scientific; ß-actin (1:6000; Sigma Aldrich); IRDye 800CM and IRDye 680RD (1:20,000; LI-COR Biosciences, Lincoln, NE, USA),

Flow cytometry method details are described in Chapter 2, Section 2.2.3

4.2.4 Western blotting

Total cellular proteins were extracted at 4°C using RIPA lysis buffer (20nM HEPES, PH 7.4, 1% TritonX-100, 2mM EDTA) containing protease inhibitors (10 µg/ml NaVO3, 100mM NaF, 1mM Na2MoO4 and 10mM Na2P2O7.

Proteins were resolved on 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred to Immobilon-FL membranes (Millipore, Bedford, MA). The blots were blocked with 5% non-fat dry milk or 5% BSA in TBS buffer (Tris-buffered saline; 20mM Tris-CL, pH 7.4; 150mM NaCl) for 1h at room temperature.

ß-actin or REVERT total protein (LI-COR Biosciences, Lincoln, NE) staining was performed to ensure a comparable protein loading. After primary antibody incubation, blots were washed three times in TTBS (TBS with 0.1% (v/v) Tween-20) and proteins were detected using incubation for 1h in secondary antibodies and after extensive washing, antibody detection was accomplished with Supersignal West Pico Chemiluminescent substrate (Pierce). Signal was detected using the luminescent image analyser (ImageQuant LAS 4000, GE Healthcare) or the the Odyssey® CLx imaging system (LI-COR).

4.2.5 Cloning and lentivirus transductions

The PDL1 pCMV3-C-FLAG and PDL2 pCMV3 c-MYC constructs were obtained from Sino Biological (Wayne, PA). The FLAG-tagged PD-L1 and MYC-tagged PD-L2 were each cloned into pENTR1A and recombined into plenti6.3/TO/V5_DEST lentiviral vector (Thermo Fisher). Lentiviruses were produced in HEK293T cells as described previously (529). Cells were infected using a multiplicity of infection of 5-10 to provide

an efficiency of infection above 90%. Cells transduced with the PD-L1 or PD-L2 pLenti6.3/TO/V5_DEST constructs and cells were selected with 500 µg/ml geneticin (G418 Sulphate) (Life Technologies) to ensure maintenance of transgene expression. Lentiviral transduction and PD-ligand cloning was kindly performed by Ms. Ashleigh Stewart.

4.2.6 Generation of cell lines from tumor samples

Fresh tissue biopsies were obtained from melanoma patients as part of a study in accordance with the recommendations of Human Research ethics committee protocols from Royal Prince Alfred Hospital (Protocol X15-0454 and HREC/11/RPAH/444). Tumour biopsies were manually minced and cut into small pieces of 2-4 mm in an enzyme mix containing 4.7 ml RPMI 1640 with 200 µl Enzyme H, 100 µl Enzyme R and 25 µl Enzyme A (Tumor Dissociation Kit, Miltenyi Biotec (Order no. 130-095-929)) The tumour pieces were then transferred to the gentleMACS C Tube and dissociated into single-cell suspensions using the gentleMACS Dissociator (Miltenyi Biotec).

After termination of the program, C Tubes were detached from the gentleMACS Dissociator and centrifuged shortly to collect the sample material at the bottom of the tube. The cell suspension was pass through a MACS SmartStrainer (70 µm) and washed with 20 ml of RPMI 1640 media. In the next step, cell suspension was centrifuged at 1200 rpm for 5 min and supernatant was aspirated completely. Then cells were resuspended and counted and single-cell suspensions were viably frozen as tumor dissociates (TD, 1x106 cells/vial) in 10% DMSO in human serum from male AB plasma (Sigma) and plated into 24 well plates (1x106 cells/well) to isolate short term melanoma and tumour infiltrating lymphocyte (TILs) cultures.

4.2.7 Statistical analysis

Statistical significance was calculated using GraphPad Prism version 7 (GraphPad software, San Diego, CA). All values are expressed as a mean of at least three independent experiments. Mean values were compared using the Student's t test or ANOVA test for parametric data, Kruskal Wallis and Mann Whitney for non-parametric data. Non-parametric Spearman test was used for correlation analysis. P-value < 0.05 was considered significant.

Table 4.2 Characteristics of PD-1 PROG cell lines

Cell line	Site of Biopsy	Therapy	Resistance type, RECIST response
SCC11-0270	Brain	Nivolumab	Innate, PD
SCC13-0156	Retroperitoneal	Nivolumab	Innate, PD
SCC15-0111	Brain	Pembrolizumab	Acquired, PR
SCC15-0534	Neck	Pembrolizumab	Acquired, PR
SCC16-0016	Pancreas	Pembrolizumab	Acquired, PR
SMU-059	Flank	Pembrolizumab	Innate, PD
SMU-092	Abdomen	Ipilimumab + Nivolumab	Acquired, PR
SMU11-0376 M2	Brain	Nivolumab	Innate, PD
SMU11-0376 M4	Brain	Nivolumab	Innate, PD
SMU13-0183 M3	Brain	Nivolumab	Innate, PD
SMU13-0183 M7	Brain	Nivolumab	Innate, PD
SMU15-0404	Arm	Pembrolizumab	Innate, PD
SMU16-0150	Scalp	Ipilimumab + Nivolumab	Acquired, PD
WMD-084#1 and #2	Other	Pembrolizumab	Innate, PD
WMD15-083#1	Small bowel	Ipilimumab + Pembrolizumab	Acquired, PR
WMD15-083#2	Large colon	Ipilimumab + Pembrolizumab	Acquired, PR

RECIST, Response Evaluation Criteria in Solid Tumours; PD, progressive disease; PR, partial response; innate, PROG tumours showed no shrinkage during therapy; acquired, PROG tumours were newly identified metastases arising during treatment or pre-existing tumours

that initially responded but subsequently progressed on PD-1 inhibitor therapy; WMD-084#1 and #2 are two cell subclones derived from a single tumour biopsy. Acquired resistance indicates patients who initially responded to immunotherapy but after a period of time on treatment, showed disease progression. Innate resistance indicates patients who initially did not respond to immunotherapy.

4.3 Results

In order to characterize immunotherapy resistance mechanisms, we studied a panel of 16 PD-1 PROG melanoma cell lines generated in our laboratory. Cell lines were derived from tumours of patients who have failed treatment with nivolumab (n=6), pembrolizumab (n=6), combination of ipilimumab and nivolumab (n=2) or combination of ipilimumab and pembrolizumab (n=2; Table 4.2).

Each of these 16 melanoma cells were classified as (i) innate progressing tumours (n=9), that showed no shrinkage during therapy and (ii) acquired PROG tumours (n=7), that were newly identified metastases arising during treatment or pre-existing tumours that initially responded but subsequently progressed on PD-1 inhibitor therapy (Table 4.2).

4.3.1 Baseline and IFNγ-induced expression of immune effector molecules in PD-1 PROG cell lines

We initially examined the expression of immune effector molecules in the panel of immunotherapy resistant PD-1 PROG melanoma cell lines (Table 4.2). Expression of the PD-1 ligands PD-L1 and PD-L2, and antigen-presentation molecules HLA-A, -B, and -C (HLA-ABC) and HLA-DR was analysed at baseline and after stimulation with IFNγ and expressed as the ratio of mean fluorescence intensity (MFI) of antibody-stained sample/MFI FMO control. We established an expression threshold of ≥1.5 to indicate marker expression; i.e. ratio below 1.5 was considered to reflect the absence of marker expression. At baseline, PD-L1 expression was absent in 12/16 cell lines (ratio < 1.5) and was low in the remaining four cell lines (range, 1.7-3.0). Of the 16 PD-1 PROG cell lines, nine showed baseline PD-L2 expression (range, 4.8-41.1). HLA-DR was expressed at variable levels in 10/16 cell lines (range, 1.5-47.2)

whereas HLA-ABC was expressed in all PD-1 PROG cell lines with the exception of SCC15-0156 and SMU-092, which were HLA-ABC negative (Table 4.3; Figure 4.1).

Collectively we noted that 10/16 (63%) PD-1 PROG cell lines expressed PD-L2 and/or PD-L1 at baseline (SCC15-0534, SMU13-0183 M3, SMU13-0183 M7, SMU15-0404, SMU16-0150, WMD15-083#1, WMD15-083#2, SMU-059, SCC16-0016, WMD-084#1). Only two PD-1 PROG cells lacked baseline expression of HLA-ABC (SCC13-0156, SMU-092) and these cells were also negative for baseline PD-L1, PD-L2 and HLA-DR (Figure 4.1; Table 4.3).

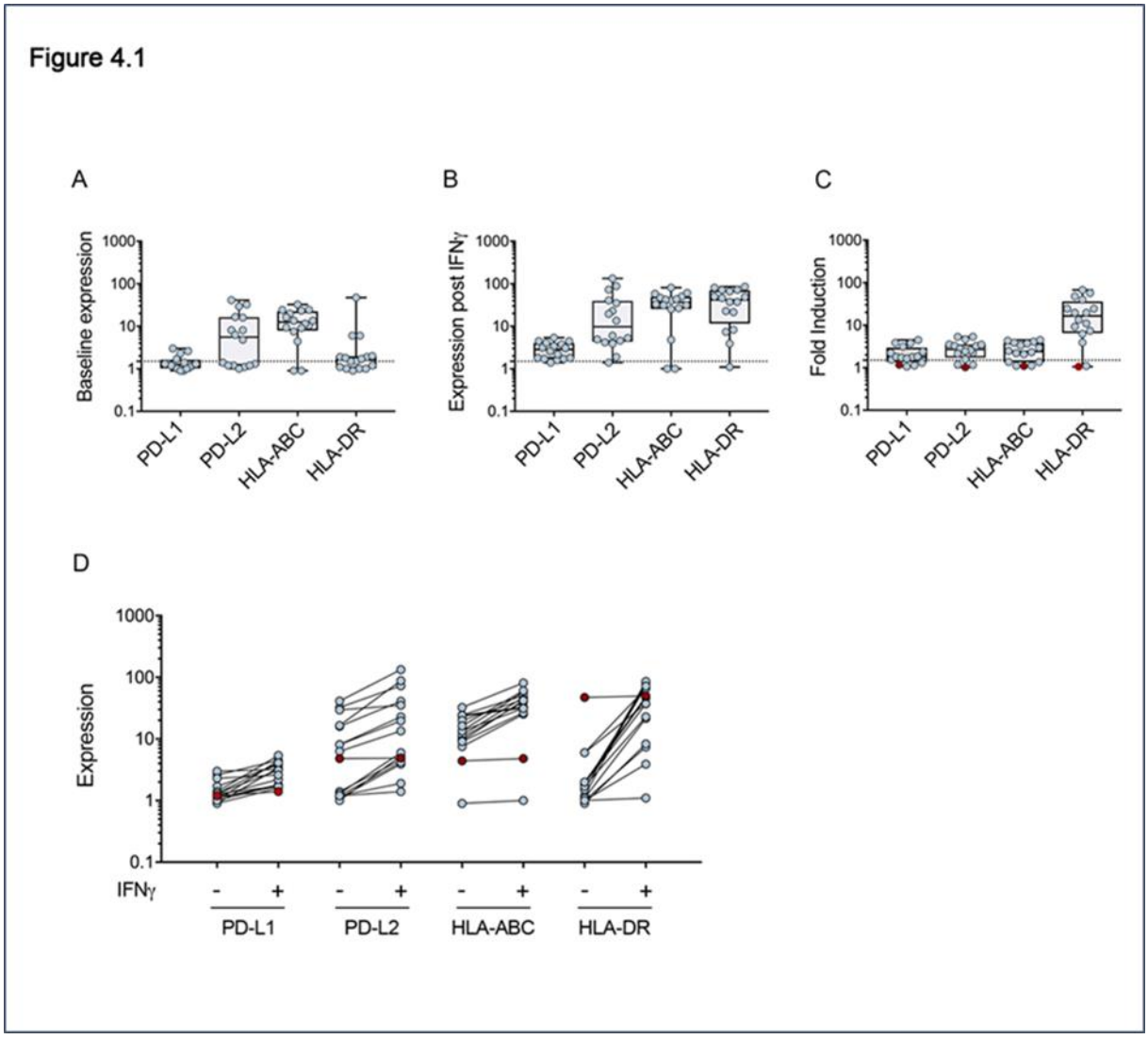

Figure 4.1 Heterogeneous baseline and IFNγ-induced expression of immune markers in PD-1 PROG cell lines

Box plots showing the (A) baseline cell surface expression, (B) expression (MFI stained sample /MFI FMO control sample) 72 h post IFNγ treatment and (C) fold induction (expression after IFN treatment / expression at baseline) of PD-L1, PD-L2, HLA-ABC and HLA-DR in a panel of 16 PD-1 PROG melanoma cell lines. Each dot represents the average expression of at least three biological replicates. Box plots show the median and interquartile ranges. (D) Change in expression of cell surface markers in response to IFNγ treatment. Dots highlighted in red indicate the SCC16-0016 cell line that showed no IFNγ-mediated induction of any target molecules.

4.3.2 Heterogeneous IFNγ-responses of PD-1 PROG cell lines

Expression of PD-L1, PD-L2, HLA-ABC and HLA-DR in response to IFNγ was examined next (Table 4.3; Figure 4.1). HLA-DR showed the greatest level of IFNγ induction with the median fold induction of 18.1 for this marker (median fold change IFNγ/baseline expression). The median fold induction of PD-L1 and PD-L2 in the 16 PD-1 PROG cell lines was similar at 1.8 and 2.7, respectively. HLA-ABC was not expressed after IFNγ stimulation in the two HLA-ABC negative cell lines, and the median fold induction in the remaining 14 PD-1 PROG cell lines was 2.7. The level of induced protein expression correlated with baseline expression for PD-L2, HLA-ABC and HLA-DR, but not PD-L1 (which was not expressed at baseline in 12/16 PD-1 PROG cell lines) (Figure 4.2).

Figure 4.2

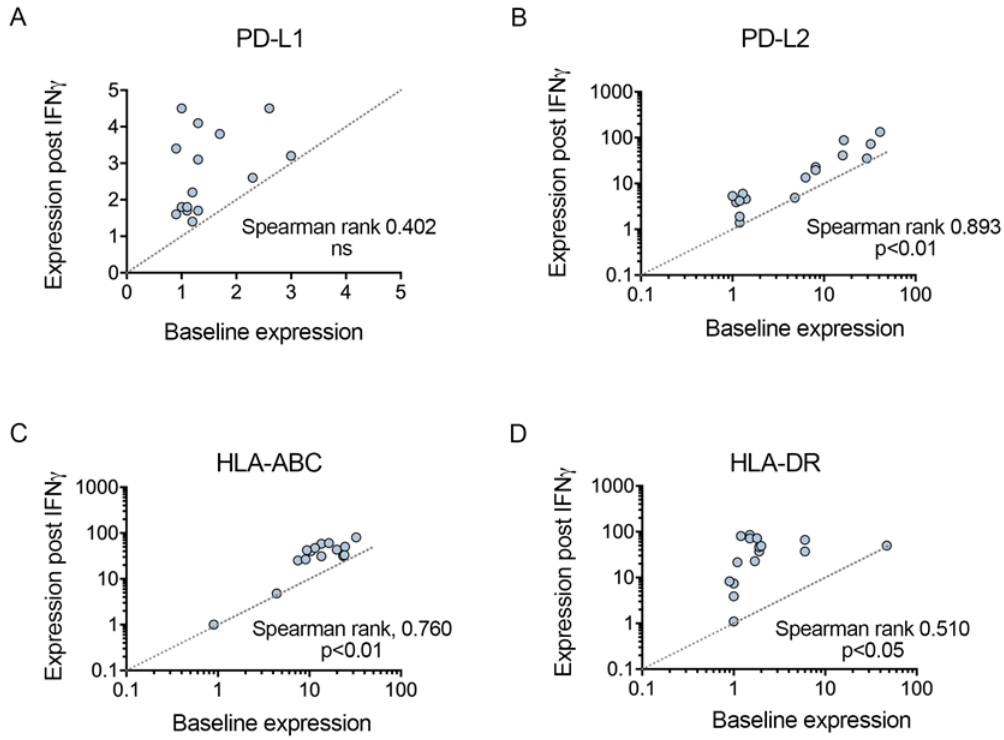

Figure 4.2 Correlation between immune marker expression at baseline and IFNγ treatment

Each dot represents the average expression of at least three biological replicates for each cell line. Spearman's rank correlation coefficient and P values are shown. ns, not significant.

For all immune markers, there was a subset of cells that did not show IFNγ-mediated induction; PD-L1 was not induced in two cell lines, PD-L2 (three cell lines), HLA-ABC (five cell lines) and HLA-DR (two cell lines). The lack of IFNγ-mediated induction did not reflect baseline protein expression, as we noted lack of IFNγ-induced expression in cell lines with and without baseline protein expression (Figure 4.2). SCC16-0016 was the only PD-1 PROG cell line that did not show IFNγ-mediated induction of any of the four markers (Figure 4.1).

4.3.3 Expression of antigen presentation molecules, interferon gamma receptor and melanoma differentiation markers in PD-1 PROG cell lines

We also examined baseline expression of three additional markers, IFNGR1, B2M and NGFR, in 15/16 PD-1 PROG cell lines (Figure 4.3). IFNGR1 was expressed in all PD-1 PROG cell lines analyzed, including SCC16-0016, which showed a lack of response to IFNγ, with no induction of any of the four target molecules (Table 4.3; Figure 4.3). B2M was expressed in 13/15 cell lines; B2M was not expressed in the two cell lines that lost HLA-ABC expression (SCC13-0156, SMU-092), indicating that loss of B2M is likely to be responsible for HLA-ABC loss in these cells (Table 4.3). As expected, there was a strong correlation between baseline HLA-ABC and B2M expression levels (Spearman $r=0.7048$, $P<0.01$). NGFR expression was expressed in all cell lines and was highly variable (range, 1.5-2035) (Table 4.3).

Figure 4.3

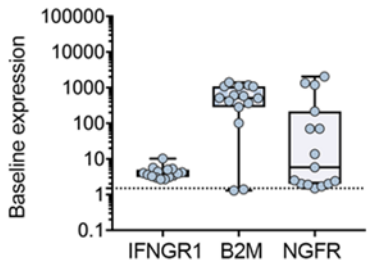

Figure 4.3 Expression of IFNGR1, B2M and NGFR at baseline level

Box plots showing the baseline cell surface expression of IFNGR1, B2M and NGFR in 16 PD-1 PROG melanoma cell lines. Each dot represents the average expression of at least three biological replicates for each cell line. Box plots show the median and interquartile ranges.

Figure 4.4

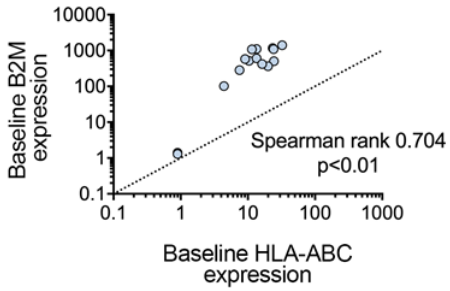

Figure 4.4 Correlation between B2M and HLA-ABC at baseline level

Each dot represents the average expression of at least three biological replicates for each cell line. Spearman's rank correlation coefficient and P values are shown.

Table 4.3 Expression of immune markers at baseline and/or after IFNγ stimulation in PD-1 PROG cell lines

Cell line	Mutation status	PD-L1		PD-L2		HLA-ABC		HLA-DR		NGFR	IFNGR1	B2M
		BSA	IFNγ	BSA	IFNγ	BSA	IFNγ	BSA	IFNγ	BSA	BSA	BSA
SCC11-0270	$BRAF^{V600E}$	0.9	1.6	1.2	1.4	7.5	25.2	1.0	7.4	1.5	3.5	282.5
SCC13-0156	$BRAF^{V600E}$	1.1	1.7	1.1	3.9	0.9	1.0	1.0	1.1	1.7	10.2	1.4
SCC15-0111	n/a	1.0	1.8	1.4	4.6	10.4	40.2	1.9	37.2	70.1	5.6	512.9
SCC15-0534	$NRAS^{Q61K}$	2.6	4.5	32.4	72.8	23.4	31.6	0.9	8.3	217.5	4	1167.2
SCC16-0016	$NRASQ^{61E/K}$	1.2	1.4	4.8	4.9	4.4	4.8	47.2	49.9	1335.0	4.4	101.7
SMU11-0376 M2	$BRAF^{V600E}$	1.1	1.8	1.0	5.3	13.5	58.2	1.2	80.7	2.0	3.0	1096.7
SMU11-0376 M4	$BRAF^{V600E}$	0.9	3.4	1.3	6.0	11.5	47.0	1.5	85.3	2.0	3.6	1075.5
SMU13-0183 M3	$BRAF^{V600E}$	1.3	1.7	16.0	41.0	24.5	50.5	1.5	71.4	2.4	3.1	504.5
SMU13-0183 M7	$BRAF^{V600E}$	1.7	3.8	16.5	88.2	24.2	33.0	6.0	66.1	1.9	4.2	1085.6
SMU15-0404	$BRAF^{G469R/S}$	3.0	3.2	41.1	133.2	32.5	80.9	1.8	72.1	70.1	3.3	1400.5
SMU16-0150	$BRAF^{V600K}$	1.0	4.5	8.1	22.8	19.9	43.6	1.9	46.4	5.8	4.0	366.3
SMU-059	$NRAS^{Q61R}$	1.4	5.4	8.1	19.6	9.4	42.2	1.1	21.5	n.d.	n.d.	n.d.
SMU-092	n/a	1.2	2.2	1.2	1.9	0.9	1.0	1.0	3.9	13.7	2.7	1.3
WMD15-083#1	$NRAS^{Q61K}$	1.3	4.1	6.3	13.5	9.1	26.7	6.0	37.3	2035.0	5.3	577.9
WMD15-083#2	$NRAS^{Q61K}$	1.3	3.1	29.5	35.1	13.5	31.3	1.7	22.8	1199.8	5.0	605.4
WMD-084	$NRAS^{Q61K}$	2.3	2.6	1.2	4.2	16.3	60.8	2.0	48.9	2.5	2.7	417.0

Baseline (BSA) and IFNγ-induced expression of each marker is shown. n.d., not determined. n/a, not available

4.3.4 Comparison of immune effector molecule expression in immunotherapy naïve and PD-1 PROG cells

In order to examine whether PD-1 PROG melanoma cell lines displayed altered patterns of immune effector protein expression and response to IFNγ, we compared the PD-1 PROGs to a panel of 31 immunotherapy naïve, cutaneous melanoma cell lines (previously described in Chapter 2). We noted several consistent differences between these cell subgroups:

1. The baseline expression of HLA-ABC was significantly lower in PD-1 PROG cells lines (median expression 12.5; range 0.9-32.5) compared to immunotherapy naïve cell lines (median expression 28.9; range 1.5-167.9, P=0.002) (Figure 4.5). HLA-ABC cell surface expression was absent in one immunotherapy naïve melanoma cell line (SMU15-0217) and two PD-1 PROG cell lines (SCC13-0156 and SMU-092).

2. Similarly, the baseline expression of HLA-DR was significantly lower in PD-1 PROG cells lines (median expression 1.6; range 0.9-47.2) compared to immunotherapy naïve cell lines (median expression 6.9; range 0.87-157.9, P=0.0086) (Figure 4.5). A larger number of PD-1 PROG cells displayed no baseline expression of HLA-DR (7/16; 44%), compared to immunotherapy naïve melanoma cells (7/31; 22.5%).

3. In contrast, the baseline expression of PD-L1 was significantly higher in PD-1 PROG cell lines (median expression 1.25; range 0.9-3) compared to immunotherapy naïve cell lines (median expression 1.05, range 0.82-1.93) (Figure 4.5). Baseline PD-L1 expression was absent in 30/31 (97%) of immunotherapy naïve melanoma cells and only in 12/16 (75%) of the PD-1 PROG cells.

4. Baseline PD-L2 expression was highly variable, with 7/16 PD-1 PROG cells and 18/39 immunotherapy naïve cells lacking this marker. There was a large subset of PD-1

PROG cells (7/16; 44%), however, that expressed very high levels of PD-L2 (expression levels >8), well above levels observed in the immunotherapy naïve melanoma cell lines (Figure 4.5).

The response of immunotherapy naïve and PD-1 PROG melanoma cell lines to IFNγ was also compared. No differences were detected in the response of PD-L1 to exogenous IFNγ, with both groups showing low induction of this immune checkpoint (PD-L1 induction range 1.1-4.7 for PD-1 PROG cells and 0.9-9.3 for immunotherapy naïve cells). PD-L2 induction in PD-1 PROG and immunotherapy naïve cells was not significantly different either (PD-L2 induction range 1.0-5.4 for PD-1 PROG cells or 0.9-14.2 for immunotherapy naïve cells) (Figure 4.5).

HLA-ABC induction by IFNγ was not significantly different between PD-1 PROG and naïve cell lines (median induction levels 2.45 and 2.68, respectively), however post-IFNγ expression levels were significantly lower in PD-1 PROG (median expression 36.6, range 1-80.9) compared to immunotherapy-naive cell lines (median expression 88.66, range 2.14-460.2, P=0.0002), which is in agreement with the lower HLA-ABC baseline levels in PD-1 PROG cells (Figure 4.5).

HLA-DR induction by IFNγ, or IFNγ-induced expression of this marker, were not significantly different between the two groups of melanoma cell lines (Figure 4.5).

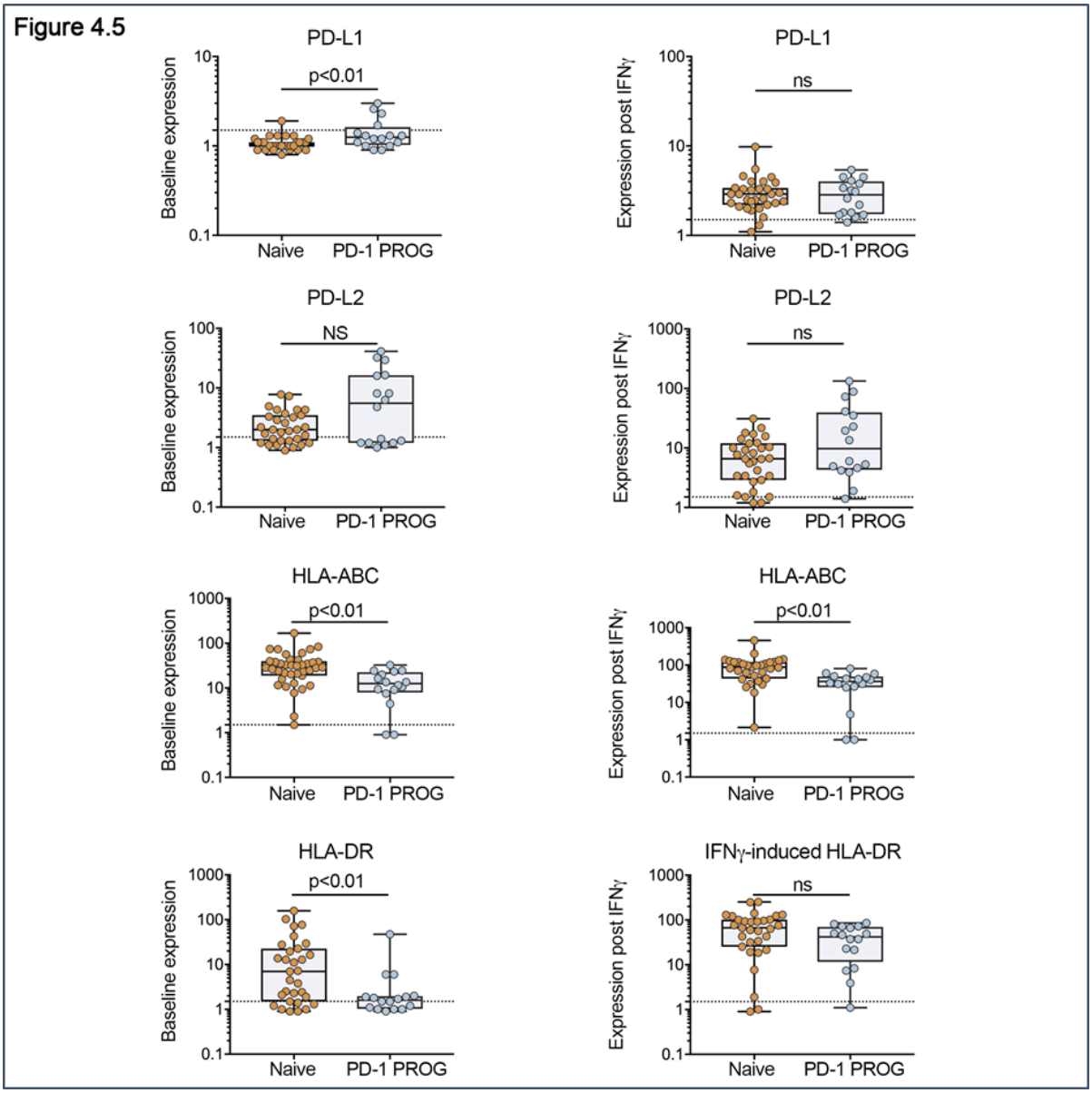

Figure 4.5 Expression of immune markers in immunotherapy naïve and PD-1 PROG cells

Expression of PD-L1, PD-L2, HLA-ABC and HLA-DR at baseline and after exposure to IFNγ (MFI stained sample /MFI FMO control sample) in 16 PD-1 PROG melanoma cell lines and 31 immunotherapy-naïve cutaneous melanoma cells. Each dot represents the average expression of at least three biological replicates for each cell line. Box plots show the median and interquartile ranges. Expression of markers in the naïve and PD-1 PROG cells was compared using Mann Whitney test.

4.3.5 Comparison between resistant and sensitive subclones of WMD-084

Two melanoma subclones (WMD-084#1 and WMD-084#2) were independently derived from a single patient biopsy. These PD-1 PROG subclones were morphologically distinct (Figure 4.6) and were compared using a co-culture assay. In this assay, the melanoma WMD-084#1 or WMD-084#2 subclones were co-cultured with the same patient-matched tumour infiltrating immune cells, and immune cell activation was measured by IFNγ release after two days of co-culture.

Figure 4.6

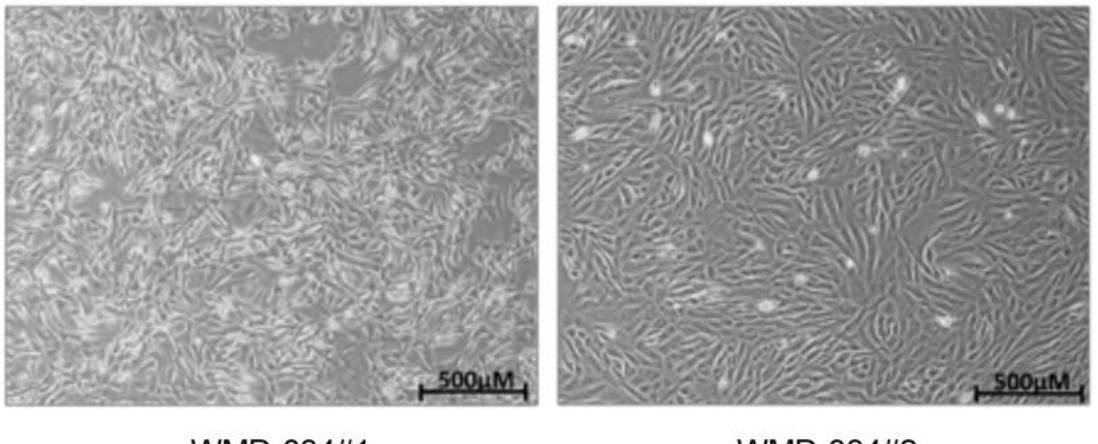

Figure 4.6 Morphology of the two WMD-084 subclones independently derived from a single patient biopsy

Two melanoma subclones (WMD-084#1 and WMD-084#2) showed distinct morphology

As shown in Figure 4.7A, co-culture with the WMD-084#1 subclone induced autologous T cells activation whilst coculture with the WMD-084#2 subclone did not (Figure 4.7A). The WMD-084 subclones were also distinct in their response to IFNγ. In particular, the WMD-084#2 subclone expressed and induced higher levels of PD-L2 whereas HLA-DR expression was significantly lower, both at baseline and after IFNγ treatment, compared to WMD-084#1 (Figure 4.7B).

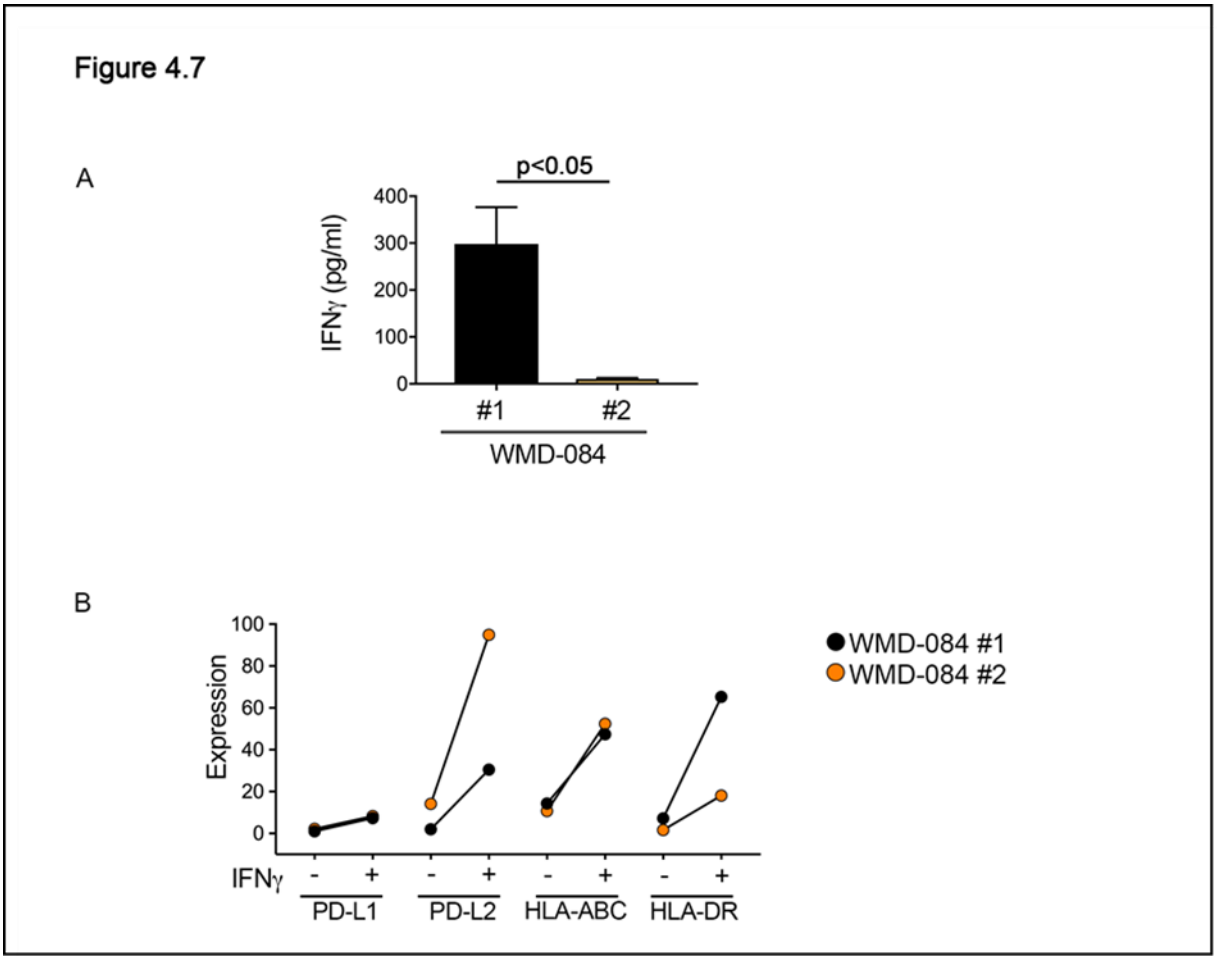

Figure 4.7 Intra-tumoural heterogeneity in immune cell response and immune effector expression in melanoma PD-1 PROG cell lines

(A) Two melanoma subclones (WMD-084#1 and WMD-084#2), that were independently derived from a single patient biopsy were mixed with the same autologous immune cells, and secreted IFNγ was measured 72 h post-seeding as a measure of immune cell activation. Unpaired t test used for comparison of immune cell response to WMD-084 subclones. Results show mean ± SEM and are representative of three independent experiments. (B) Expression (MFI stained sample /MFI FMO control sample) of cell surface markers, PD-L1, PD-L2, HLA-ABC and HLA-DR, at baseline (-) and after 72 h exposure to IFNγ (+).

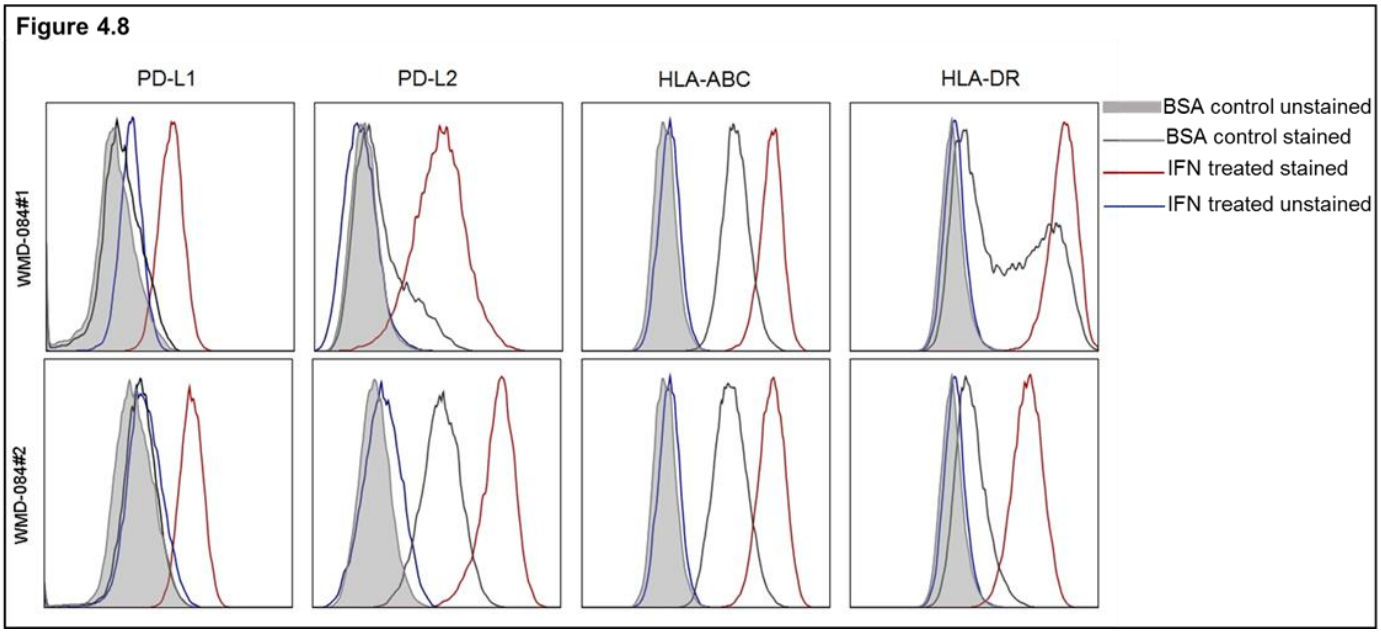

Figure 4.8 FACS data represented as histograms for WMD-084#1 and WMD-084#2
Expression of cell surface markers, PD-L1, PD-L2, HLA-ABC and HLA-DR for WMD-084#1 and WMD-084#2 represented as histogram.

To explore the potential impact of PD-L2 overexpression in conferring immune cell resistance in the WMD-084#2 subclone, the PD-L1 and PD-L2 molecules were each overexpressed in the WMD-084#1 subclone using lentiviral transduction. When co-cultured with autologous immune cells, overexpression of PD-L1 resulted in decreased levels of secreted IFNγ compared to the vector-transduced WMD-084 #1 cells. In contrast, PD-L2 overexpression did not significantly diminish the production of IFNγ (Figure 4.8).

Figure 4.9

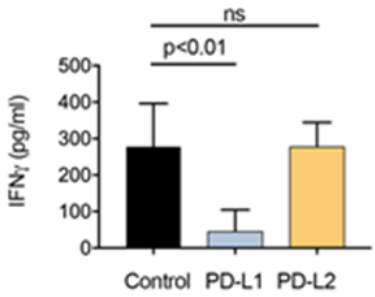

Figure 4.9 Immune activity of overexpressed PD-L1/PD-L2

IFNγ production after co-culture of PD-L1 and PD-L2 overexpressing melanoma cells with the patient-matched tumour-infiltrating lymphocytes expanded from the same tumour biopsy. IFNγ was measured by ELISA. Results are from four biological replicates. Paired t test was used for the analysis. ns, not significant

4.3.6 Expression of melanoma pigment antigens and de-differentiation markers in PD-1 PROG cells

To identify other molecules that may contribute to immunotherapy resistance in the PD-1 PROG cells, we investigated the expression of the receptor tyrosine kinase AXL, transcription factors SOX10 and MITF, and melanoma differentiation antigen, MLANA, at baseline and after IFNγ treatment. These molecules were selected as they are important markers of melanocyte differentiation (530), and melanoma de-differentiation is considered to play a role in resistance to anti-PD-1 based therapies (101, 397, 524, 531).

As expected, melanoma cells displaying markers of pigmentation and differentiation (i.e. MLANA, SOX10 and MITF) commonly showed low or absent AXL expression; seven cell lines showed expression of MLANA and MITF, and all seven displayed low/undetectable AXL (SCC11-0270, SCC13-0156, SCC15-0111, SMU11-0376 M2, SMU11-0376 M4, SMU-092, WMD-084#1). Seven melanoma cells showed AXL accumulation without MITF

and/or MLANA expression (SCC15-0534, SMU13-0183 M7, SMU-059, SMU16-0150, SCC16-0016, SMU15-0404, and SMU13-0183 M3). Four cell lines lacked any of the three pigmentation markers and showed expression of AXL (SCC15-0534, SMU13-0183 M7, SMU15-0404, SMU13-0183 M3) (Figure 4.9). PD-1 PROG cell lines with the AXL high, MLANA/MITF low phenotype also showed high baseline PD-L2 expression by flow cytometry (Table 4.3).

IFNγ treatment had little effect on expression levels of AXL, MITF and MLANA but SOX10 appeared to be reduced after IFNγ treatment in SMU-0376 M2, WMD-084, SMU16-0150, SCC11-0270 and SMU11-0376 M4 cell lines (Figure 4.9).

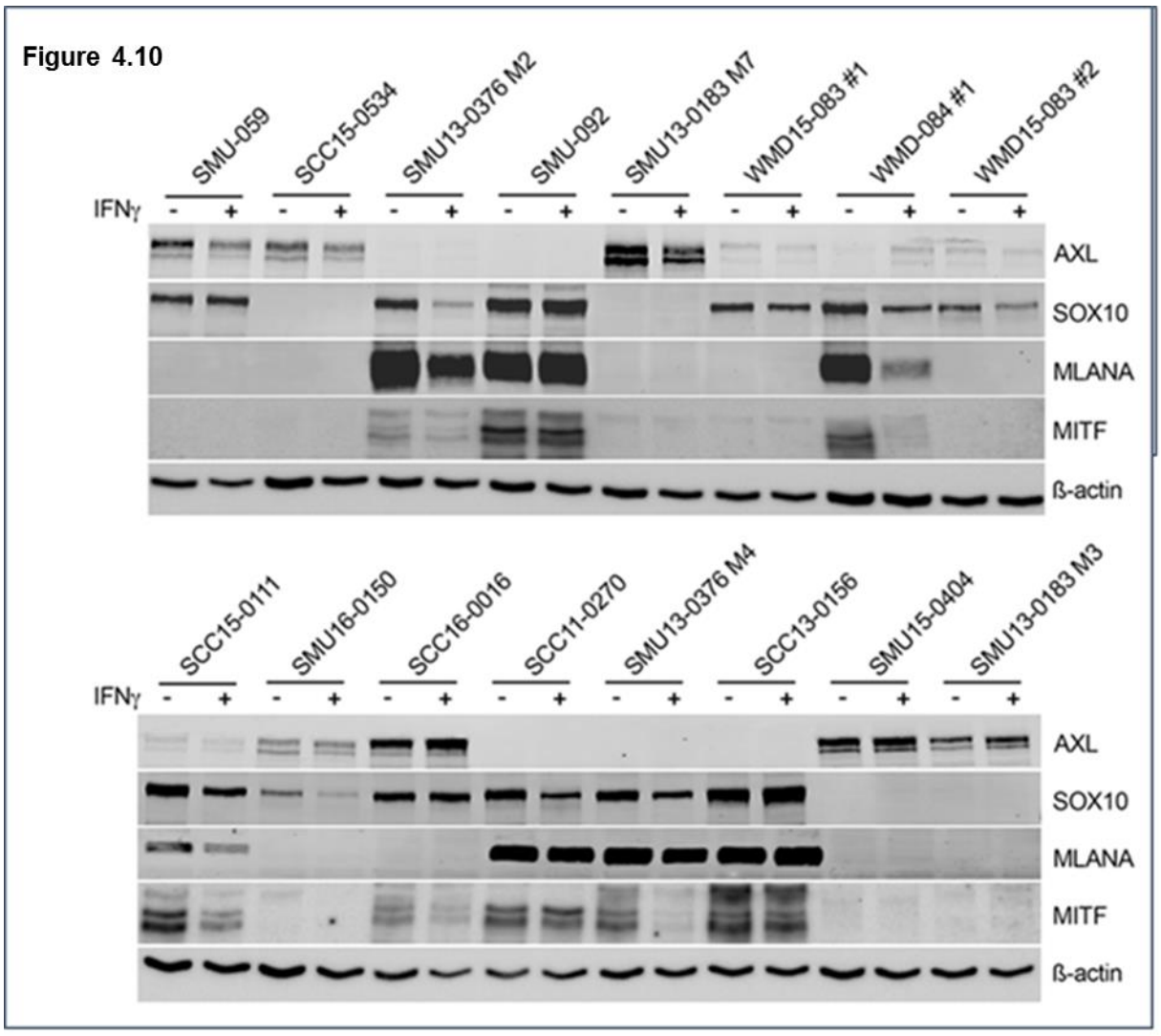

Figure 4.10 Western blotting analysis of melanoma differentiation and pigmentation markers

(-), no IFNγ treatment; (+), treated with 1000U/ml IFNγ for 72 hours

4.4 Discussion

Immunotherapies with monoclonal antibodies blocking CTLA-4 and PD-1 immune checkpoints on T lymphocytes have significantly increased the survival rates of patients with advanced melanoma (532). Response to immune checkpoint blockade is influenced by many tumour-intrinsic and -extrinsic factors including PD-L1 expression on tumour cells and immune cells (533, 534), tumour mutational load, which can reflect exposure to environmental mutagens (i.e. UV irraditation in melanoma), tumour-specific defects (i.e. mismatch-repair defects) (101, 444, 535, 536), and immune cell and stromal cell infiltration (420) within the tumour microenvironment (194). Further, tumour-specific downregulation of MHC class I or loss of B2M (101) impede tumour recognition by cytotoxic T cells. JAK1/2 loss-of-function mutations or loss of STAT transcription factors affect the IFNγ signalling pathway and can reduce the efficacy of immune checkpoint blockade therapy (101, 194, 289).

Analysis of cell surface marker expression in 16 immunotherapy resistant melanoma cell lines showed low levels of PD-L1 expression in these cells at baseline, however these were above the level of PD-L1 expression observed in immunotherapy-naïve cells. PD-L1 expression, even at extremely low levels (1-5%), is considered to be predictive of response to immune checkpoint blockade. The marker is not robust, as some patients with PD-L1 negative tumours will respond to PD-1 inhibitors (403, 537), and conversely, patients with PD-L1 positive tumours can fail PD-1 inhibition (538, 539). Similarly, PD-L2 expression has been associated with clinical response to anti-PD-1 immunotherapy (540). PD-L2 is expressed in many tumour types and can be expressed independently of or in association with PD-L1. We have previously confirmed that the expression of these two markers is highly correlated in melanoma cell lines after IFNγ treatment but not at baseline

(see Chapter 2). The co-expression of PD-L1 and PD-L2 may be a better predictor of response considering that both ligands bind to and engage the PD-1 receptor (540).

In this study, we noted that baseline PD-L1 and PD-L2 expression was seen in 25% and 56% of PD-1 PROG cell lines (Table 4.3), respectively and both ligands were induced by IFNγ in most melanoma cell lines. Importantly, compared to immunotherapy naïve cell lines, it was evident that a subset of PD-1 PROG cell lines showed much higher expression and/or induction of PD-L1 and PD-L2, suggesting that overexpression of PD-L1/L2 may help tumour escape from PD-1 blockade therapy. Further, the altered upregulation of PD-L2 in an immune-resistant WMD-084#2 PD-1 PROG cell subclone, supported a distinct role for PD-L2 in mediating melanoma escape from immune cell recognition. However, in co-culture experiments with melanoma and autologous immune cells, we confirmed the potent inhibitory effect of ectopic PD-L1 expression but could not validate a similar role for ectopically expressed PD-L2. This may be due to the assay used, and perhaps IFNγ secretion in a two-day in vitro assay is not sufficient to reveal PD-L2 specific activity. It is also possible that stromal cells and the tumour microenvironment participate in the functional outcomes of PD-L1 and PD-L2 expression. Although co-culture experiments with stromal cells were not in the scope of this thesis, it is interesting to note that PD-1 and PD-L1 inhibitors result in equivalent patient responses and outcomes, suggesting that the principle regulator of tumour cell immunity may be the PD-L1/PD-1 interaction (541) and PD-L2 may have distinct functions (542).

Expression of MHC class I antigen presentation molecules is required for response to immune checkpoint inhibitors (201). In this study, two PD-1 PROG cell lines (SCC13-0156 and SMU-092) showed no expression of HLA-ABC at baseline and after IFNγ exposure, and loss of MHC class I is likely to represent a dominant immunotherapy resistance mechanism in these patients. These two cell lines also showed loss of B2M, and since

B2M is a structural component of the MHC class I complex, loss of B2M is likely to be responsible for loss of HLA-ABC cell surface expression in these two cell lines.

HLA-DR has also been reported as a biomarker of response to anti-PD-1 immunotherapy (201), where positive expression is strongly correlated with response to treatment (201). HLA-DR expression in pre-treatment tumours is associated with enhanced infiltration of CD4+ and CD8+ lymphocytes and is likely to reflect local IFNγ production. In our study, 44% of cell lines did not express HLA-DR at baseline (Table 4.4), possibly indicating an immune-poor tumour, in keeping with previous reports. It is also important to mention that expression of both HLA-ABC and HLA-DR was significantly lower in PD-1 PROG cells compared to immunotherapy naïve cell lines, and decreased expression may affect immune cell recognition of tumour cells, and thus immunotherapy response (543). The mechanism of HLA-ABC downregulation and low HLA-DR expression is currently under investigation.

Immune checkpoint therapy activates immune effector cells resulting in upregulation of IFNγ, and in turn, eradication of tumour cells (544), indicating a crucial role for this cytokine in response to immune checkpoint therapy. IFNγ secreted by immune cells into the tumour microenvironment increases expression of MHC molecules and also the expression of inhibitory checkpoints such as PD-L1 (545-547). In melanoma cells, JAK1/2 loss-of-function mutations leading to loss of IFNγ signalling have been associated with resistance to PD-1 blockade, likely by impacting on PD-L1 and MHC molecule expression (194, 289). Hence, melanoma patients with JAK1/2 loss-of-function mutations would not respond to PD-1 blockade, presumably because diminished IFNγ signalling would impair tumour recognition by immune cells due to loss of MHC molecules, while failure to upregulate PD-L1 on tumour cells will make PD-1 engagement irrelevant (289). Indeed

JAK1/2 mutations may results in lack of T cell infiltration via loss of IFNγ-stimulated expression of T cell chemoattractants including CXCL9, CXCL10, and CXCL11 (289, 548).

In keeping with this, high IFNγ transcriptome signatures in tumours of patients treated with PD-1 checkpoint blockade is associated with better survival (289). In our panel of PD-1 PROG cells, one cell line, SCC16-0016, showed loss of response to IFNγ, with no induction of any target molecules (Table 4.4). Unlike the immunotherapy naïve cell line D22 that had lost IFNGR1 expression due to a point mutation (Chapter 2), SCC16-0016 showed normal expression of IFNGR1. Therefore, the lack of induction of IFNγ target molecules is likely to be due to a defect in the downstream components of the IFNγ signalling pathway and remains to be defined.

In many solid cancers, EMT result in the acquisition of an invasive phenotype. In melanoma, this dynamic and reversible transition from a proliferative to an invasive state is called “phenotype switching” and resembles the EMT process. During this process melanoma cells show increased plasticity, invasiveness, migration, metastasis and poorer prognosis (549).

The receptor tyrosine kinase AXL is important molecule in phenotype switching and metastasis and modulates therapeutic resistance to immune checkpoint blockade-based immunotherapy (345). Inhibition of AXL in mouse models triggers activation and proliferation of tumour infiltrating CD4+ and CD8+ T cells, mainly through the accumulation and activation of CD103+ dendritic cells, which leads to anti-tumour immune effects (409). More importantly, AXL inhibition in combination with PD-1 blockade has been shown to augment the anti-tumour effects of PD-1 blockade and trigger tumour regression in murine models (409). Hugo et al. reported that in tumours from patients resistant to PD-1 blockade, AXL also regulates innate immune cells to reduce the anti-tumour activity induced by anti-PD-1 immunotherapy. Therefore, inhibiting AXL signalling may enhance

both innate and adaptive immunity and increase efficacy of immune checkpoint blockade immunotherapy (409, 524). In our study, AXL expression was detected in 10/16 PD-1 PROG cell lines, both at baseline (Table 4.4) and after exposure to IFNγ. Considering that AXL expression is associated with resistance to immune checkpoint blockade immunotherapy in mouse tumour models and cancer patients (409, 524), AXL-positive PD-1 PROG cell lines may similarly be less responsive to immune checkpoint inhibitor immunotherapy. One cell line (WMD-084) showed no AXL expression at baseline but its expression was induced following exposure to IFNγ suggesting that in this cell line, exposure to IFNγ may induce an immunotherapy resistant phenotype. Interestingly, NGFR expression is also associated with the invasive switch in melanoma (550), although we did not observe any clear association between AXL and NGFR expression, i.e. the SCC16-0016, WMD15-083 #1 and WMD15-083 #2 PD-1 PROG cells had high baseline NGFR expression, but high and low baseline expression of AXL.

The SOX10-MITF-MLANA transcriptional network is also involved in phenotype switching in melanoma. SOX10 expression induces the transcription of MITF which regulates genes involved in the development of melanocytic lineage, including MLANA (99). This network of proteins modulates the differentiation, proliferation and survival of melanocytes (100). MITF expression is important for melanoma survival (100) and transformation (101). It also induces anti apoptotic functions in melanoma through expression of BLC2A1, BCL2 and BIRC7 (100). Low expression of MITF is associated with increased invasiveness and metastasis, and MITF levels are inversely correlated with AXL (99, 549). Seven cell lines in our study showed loss of MITF expression, and these cell lines showed concurrent expression of AXL. Interestingly, all seven cell lines also showed high levels of PD-L2 at baseline. Three of the seven cell lines also had high PD-L1 expression (SMU13-0183 M7, SMU16-0404, SCC15-0534). This suggests possible effect of AXL high/MITF low phenotype on regulation of immunotherapy resistance. Elevated expression of AXL in this

group of cells could affect immunotherapy response by impairing CD4+ and CD8+ T cell infiltration to tumour, whereas high PD-L2 expression could further suppress immune responses and impair immune checkpoint blockade.

As expected, the expression of SOX10, MITF and MLANA was closely correlated. These three markers were co-expressed in 7/16 PD-1 PROGs and 4/16 showed concurrent loss of expression of all three markers. The remaining 5 PD-1 PROGs retained SOX10 in the absence of MLANA and/or MITF. Loss of this differentiation network could negatively impact antigen production and presentation, and therefore, tumour recognition by T cells. Accordingly, the upregulation of MITF in melanoma patients treated with MAPK inhibitors promotes the significant upregulation of MLANA, and both are correlated with an increase in CD8+ T cell infiltration into the tumour microenvironment (551). Thus, MITF and MLANA expression are favorable for immunotherapy (551, 552) and MLANA and SOX10 loss was recently confirmed to impair the effector functions of CD8+ T cells in a genome-wide loss of functions study (397).

In this chapter, we identified several potential mechanisms of immunotherapy resistance in some of the PD-1 PROG cell lines: i) loss of B2M expression, resulting in absence of cell surface HLA-ABC in two cell lines, ii) increased expression of immune inhibitory molecules, including PD-L1 and possibly PD-L2, iii) diminished response to IFNγ stimulation, resulting in loss of induction of at least one target molecule after IFNγ treatment in 10/16 (62%) PD-1 PROG cells, and iv) melanoma de-differentiation.

More in-depth functional work is required to delineate the contribution of these mechanisms to immunotherapy resistance. For example, silencing or overexpression of melanoma de-differentiation markers in our cell models may reveal their functional relevance, and impact on immune-reactivity of tumour cells in coculture assays. Additional markers of de-differentiation such as ZEB1 and E-cadherin, and proliferation and invasion

assays should also be included to validate if melanoma de-differention affected immunotherapy response.

Although we identified potential immunotherapy resistance mechanisms in a subset of our short-term cell lines, mechanisms contributing to immune evasion of the remaining PD-1 PROG cell lines are still undefined. Other potential immunotherapy resistance mechanisms were not explored due to time constraints. These mechanisms include tumour-extrinsic factors, such as immune cell exclusion and exhaustion, or stromal-mediated suppression of immune activity, and tumour-intrinsic alterations in oncogenic and metabolic signaling pathways. Follow-up experiments should be performed as part of future work to address whether these resistance mechanisms contribute to immune evasion in the PD-1 PROG melanomas. For example, transcriptome and exome sequencing of the cell lines may reveal additional mutations or oncogenic pathway activation/inactivation that could contribute to immune evasion, while co-culture assays of the PD-1 PROG cell lines with autologous immune cells may reveal altered immunogenicity and immune-reactivity of these tumour cells. Dysfunction in the tumour microenvironment leading to immune suppression could also be inferred from immunohistochemistry analysis of patient tissue samples, or profiling of tumour dissociates by flow cytometry.

In conclusion, these data highlight that multiple mechanisms of immunotherapy resistance exist and can co-occur in individual melanoma cells. This supports the need for specific individualized therapies to enhance response rates and overcome immunotherapy resistance.

Table 4.4 Summary of findings in 16 PD-1 PROG cell lines

Cell line	HLA-ABC[a]	B2M[a]	HLA-DR[a]	PD-L2[a]	PD-L1[a]	NGFR[a]	AXL[b]	MLANA[b]	MITF[b]	SOX10[b]
SCC11-0270 M2	+	+++	-	-	-	-	-	+	+	+
SCC13-0156	-	-	-	-	-	-	-	+	+	+
SCC15-0111 M3	++	+++	+	-	-	++	+	+	+	+
SCC15-0534	++	+++	-	++	+	+++	+	-	-	-
SCC16-0016[c]	+	++	++	+	-	+++	+	-	+	+
SMU11-0376 M2	++	+++	-	-	-	+	-	+	+	+
SMU11-0376 M4	++	+++	+	-	-	+	-	+	+	+
SMU13-0183 M3	++	+++	-	++	-	+	+	-	-	-
SMU13-0183 M7	++	+++	+	++	+	+	+	-	-	-
SMU15-0404	++	+++	+	++	+	++	+	-	-	-
SMU16-0150	++	+++	+	+	-	+	+	-	-	+
SMU-059	+	n.d.	-	+	-	n.d.	+	-	-	+
SMU-092	-	-	-	-	-	+	-	+	+	+
WMD15-083#1	+	+++	+	+	-	++	+	-	-	+
WMD15-083#2	++	+++	+	++	-	+++	+	-	-	+
WMD-084#1	++	+++	+	-	+	+	-	+	+	+

[a]Baseline cell surface expression is shown. -, expression <1.5; +, expression 1.5-10; ++, expression 10-200; ++, expression >200

[b]Expression based on Western immunostaining

[c]SCC16-0016 cell lines showed no response to IFNγ treatment

Chapter 5
Differential regulation of PD-L1 and PD-L2 expression in melanoma cells

5.1 Introduction

Cancer cells can escape immune attack by utilizing suppressive immune regulators expressed on both immune and cancer cells. Under normal physiological conditions, these suppressive molecules, which are often induced upon T cell activation, prevent the overstimulation of immune responses and protect tissues from autoimmune attack (reviewed in (384). PD-L1 and PD-L2 are key examples of suppressive immune molecules (381), and interaction of these ligands with the PD-1 receptor induces cell death in lymphocytes (378, 553), inhibits effector T cell function and increases the function and development of TRegs leading to the overall suppression of tumour-specific T cell responses (381, 554, 555).

PD-1 (CD279) is a transmembrane protein that belongs to the CD28 immunoglobulin superfamily (377), and is mostly expressed on activated CD4+ T cells and activated CD8+ T cells. PD-1 is also expressed on other immune cell types including B cells, myeloid cells, thymocytes, natural killer (NK) cells, monocytes, DCs and macrophages (375, 376). Persistent expression of PD-1 on T cells can induce an exhausted T cell phenotype leading to loss of CD8+ T cell effector functions including

the inability to produce cytokines such as interleukin-2 (IL-2), interferon-γ (IFNγ), and tumour necrosis factor-α (TNFα) (379, 556).

PD-1 has two main ligands - PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC). These transmembrane glycoproteins belong to the B7-CD28 protein family and they share 40% amino acid homology with each other (375, 377, 557) and 20% homology with two other B7 protein members, B7-1 and B7-2, which act as ligands for CD28 and CTLA-4 (384). PD-L1 and PD-L2 display different affinity and binding kinetics for the PD-1 receptor with PD-L2 showing greater affinity for PD-1 (384). The PD-1 ligands also interact with secondary receptors; PD-L1 binds CD80 (B7-1) and PD-L2 binds RGMb (453). These features suggest that PD-L1 and PD-L2 may compete for PD-1 binding and may have distinct functions that reflect the tissue and cancer type (476).

PD-L1 is mainly induced on tumour cells by IFNγ, but can also be detected on the surface of other cell types including T cells, B cells, myeloid dendritic cells, macrophages, placental trophoblasts, mesenchymal stem cells, myocardial endothelium, cortical thymic epithelial cells and brown adipocytes (375, 377, 432-434) PD-L1 expression has also been reported in human cancers including glioblastoma, melanoma, prostate cancer, colorectal cancer, gastric cancer, lung cancer, ovarian cancer, renal cell carcinoma, multiple myeloma and leukemia (426-428, 430, 431, 476, 558, 559).

Several oncogenic pathways including IFNγ/JAK1/2/STAT, MAPK, ALK/STAT3 and PI3K/AKT/mTOR have been shown to regulate PD-L1 expression (375, 381, 461, 560).In acute myeloid leukemia PD-L1 expression is stimulated by Toll-like receptor activation via the MAPK and MyD88/TRAF6 pathways (561). Binding of hepatocyte growth factor to the receptor tyrosine kinase c-MET can also induce expression of

PD-L1 through the RAS/PI3K/AKT signalling pathway in renal cancer cells (562). In glioma, loss of PTEN protein, which induces the activation of the PI3K/AKT pathway, increased PD-L1 expression (462, 563) but this effect was not observed in melanoma (379), suggesting differential PD-L1 regulatory mechanisms in different cancer types.

In melanoma, expression of PD-L1 is primarily regulated via the IFNγ/JAK/STAT/IRF1 pathway. The IRF1 transcription factor binds to the PD-L1 promoter more potently than the PD-L2 promoter (265). Activation of the MAPK and PI3K/AKT signalling pathways have also been shown to regulate PD-L1 expression in melanoma (519, 564, 565). In the tumour microenvironment, cytokines such as IFNγ, IL-4, IL-10, growth factors including epidermal growth factor (EGF), and bacterial lipopolysaccharide (566), upregulate PD-L1 mRNA and protein expression (463-466), likely by stimulating the oncogenic signalling pathways mentioned above. For example, IFNγ produced by activated NK cells enhanced expression of PD-L1 on tumour cells through activation of JAK1/2 and STAT1 (381). Inhibition of JAK signalling reduced expression of PD-L1 and enhanced tumour regression through NK cell-mediated activity (279). PD-L1 may also be regulated at the post transcriptional level by miRNAs, ubiquitination, lysosomal-mediated degradation, glycosylation, tyrosine or serine/threonine phosphorylation, acetylation and other unknown mechanisms (375).

PD-L2 is typically expressed on antigen presenting cells (375), placental endothelium and medullary thymic epithelial cells (377). In cancer, PD-L2 expression is associated with early stage, smaller tumour size and well-differentiated tumour grade but not with patient survival (476). High expression of PD-L2 in solid tumours has rarely been reported (476). Compared to PD-L1, the regulation of PD-L2 is not well established. IL-4 and IFNγ have both been shown to induce PD-L2 expression on a

wide range of cell types including DCs, macrophages and tumour cells (379, 430, 453). Activation of the transcription factors STAT1, STAT2, STAT3 and IRF1 occurs rapidly in response to IFNγ and IFNß to induce expression of both PD-L1 and PD-L2 (265). IFNγ, which preferentially activates STAT1 in most cells, but also signals via STAT3 in some cells, showed stronger induction of PD-L1 compared to PD-L2 (265) while IFNß, a potent STAT3 activator, showed more potent up regulation of PD-L2 compared to PD-L1 (265). In this chapter, we examined the regulation of PD-L1 and PD-L2 in melanoma. In particular, we focused on the temporal accumulation and stability of total and cell surface-specific expression of both ligands.

5.2 Material and Methods

5.2.1 Cell lines

Eight melanoma cell lines were included in this study; six cell lines were selected from the panel of 39 cell lines described in Chapter 2 (NM177, C060M1, MM418, NM172, SKMel28 and MelAT) and two were selected from the panel of PD-1 PROG cells (SMU15-0404, SMU13-0183 M7).

Cell culture details are described in Chapter 2, Section 2.2.2.

5.2.2 Antibodies and reagents

Mouse anti-STAT1 (1:1000; clone 9H2; Cell Signalling Technology), rabbit anti-phospho-STAT1 (Tyr701) (1:1000; clone D4A7; Cell Signalling Technology), mouse anti-STAT3 (1:1000; clone 124H6; Cell Signalling Technology), rabbit anti-phospho-STAT3 (Tyr705) (1:1000; clone D3A7; Cell Signalling Technology), rabbit anti-PD-L1 (1:1000; clone E1L3N; Cell Signalling Technology), rabbit anti-PD-L2 (1:1000; clone D7U8C; Cell Signalling Technology) and mouse anti-ß-actin (1:6000; Sigma Aldrich) were used for Western blotting.

Mouse anti-CD274/PD-L1 (1:40; clone MIH1; BD Biosciences Franklin Lakes, NJ, USA) conjugated to brilliant violet and two PD-L2 antibodies were used for flow cytometry; mouse anti-CD273/PD-L2 (1:50; clone 24F.10C12; BioLegend; San Diego, CA), mouse anti-CD273/PD-L2 (1:5; clone M1H18; BD Biosciences Franklin Lakes, NJ, USA) both conjugated to allophycocyanin (APC).

Cycloheximide (c7698-1G) was purchased from Sigma Aldrich.

5.2.3 Flow cytometry and Western blotting

Staining was performed in flow cytometry buffer (PBS supplemented with 5% FBS, 10 mM EDTA, and 0.05% sodium azide). Cells (2 × 105) were first stained with a fixable viability dye (Invitrogen, Thermo Fisher Scientific), then incubated for 30 min on ice with anti-CD273/PD-L2 antibody clone 24F.10C12 or anti-CD273/PD-L2 antibody clone M1H18 and anti-CD274/PD-L1 antibody clone MIH1. Cells were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit and stained intracellularly with the same antibodies. Cell surface expression was calculated as (MFI of the antibody-stained sample / MFI of the FMO control), and induction of all markers calculated as expression value after treatment with IFNγ / expression value at baseline.

For protein stability assay, cells were initially treated with 1000 U/ml IFNγ for 24 h, followed exposure to fresh media containing 1000 U/ml IFNγ with or without 50 µg/ml cycloheximide for 4 h, 6 h, 8 h, 16 h or 24 h. Additional flow cytometry details are provided in Chapter 2, Section 2.2.3.

Western blotting was performed as described in Chapter 4, Section 4.2.3. REVERT stain was used as a loading control, except where ß-actin is shown.

5.2.4 RNA interference

For small interfering RNA (siRNA)-mediated knockdown of PD-L2, transfection was carried out using siRNA constructs were obtained from Dharmacon including SMARTpool siGENOME PDCD1LG2 siRNA (Cat# M-018563-00-0005), siGENOME NonTargeting siRNA Pool #1 (Cat# DHA-D-001206-13-05) and DharmaFECT 1 Transfection Reagent (Cat# DHA-T-2001-01). Cells were transfected with 10 nM of

either the targeting or control siRNA using DharmaFECT Transfection Reagent for 72 h.

5.2.5 Statistical analysis

Statistical significance was calculated using GraphPad Prism version 7 (GraphPad software, San Diego, CA, USA). FlowJo software (TreeStar, Ashland, OR, USA) was used for data analyses.

5.3 Results

5.3.1 Total and cell surface-specific expression of PD-L1 and PD-L2

The cell surface expression of PD-L1 and PD-L2 in response to IFNγ was correlated in our panel of 39 melanoma cell lines (Spearman's rank = 0.388, p=0.01; see Chapter 2). We noted, however that some melanoma cell lines responded to IFNγ with disparate expression of PD-L1 and PD-L2. For instance, IFNγ induced PD-L1, but not PD-L2 cell surface expression on MM418 and NM182 cells whereas the PD-1 PROG cell lines SMU15-0404 and SMU13-0183 M7 expressed extremely high PD-L2 relative to PD-L1, post IFNγ stimulation (Figure 5.1). To investigate this phenomenon in greater detail, we selected eight cell lines with concordant and discordant PD-L1 and PD-L2 expression; MelAT and SKMel28 (PD-L1Low/PD-L2High); NM182 and MM418 (PD-L1High/PD-L2Low), SMU15-0404 and SMU13-0183 M7 (PD-1 PROG cells with very high PD-L2 expression) and C060M1 and NM177 cells with concordant PD-L1 and PD-L2 expression (both low or both high, respectively) (Figure 5.1).

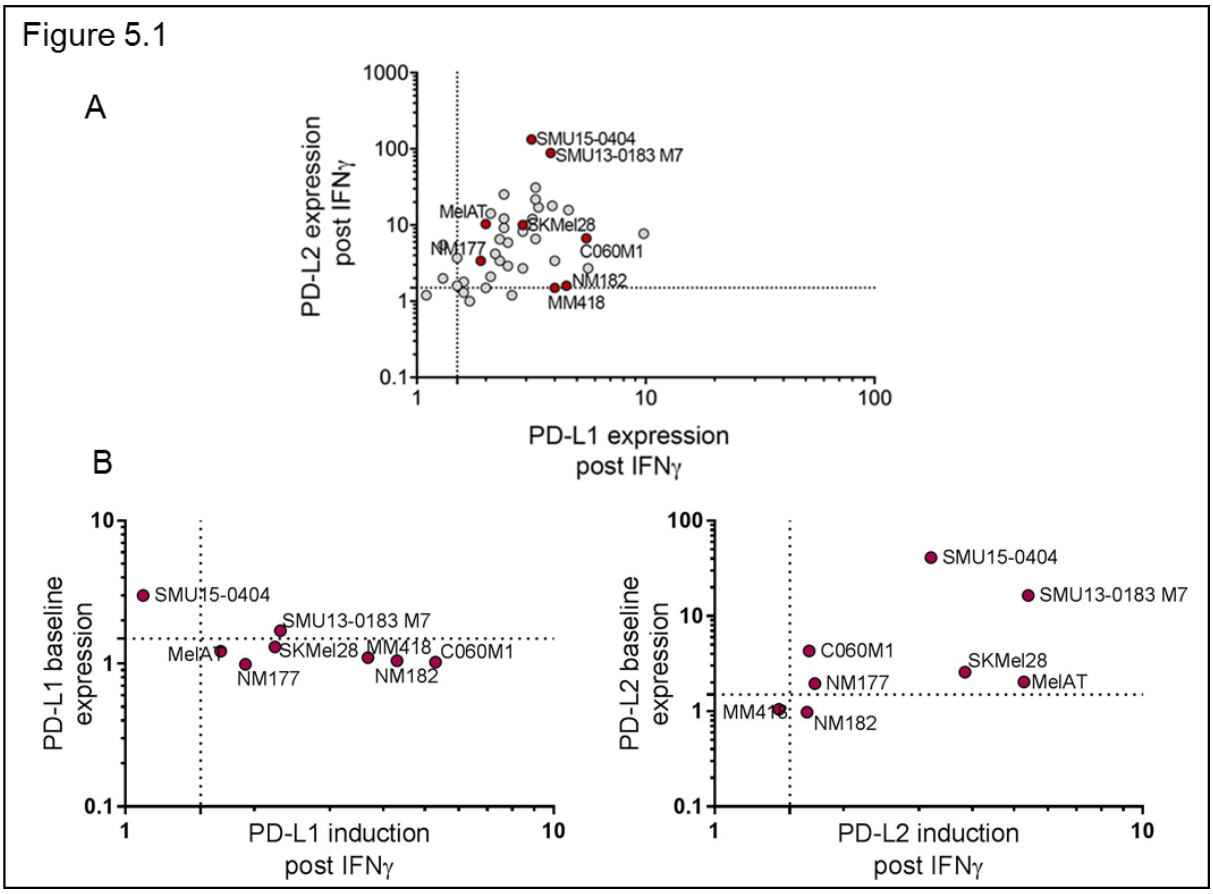

Figure 5.1 Expression (MFI) of the IFNγ-induced PD-L1 and PD-L2 in melanoma cells

(A) Eight melanoma cell lines (labelled in red) were selected for study based on their expression of membrane PD-L1 and PD-L2 (MFI stained sample /MFI FMO control sample) after treatment with IFNγ for 72 h. (B) Fold induction of PD-L1 and PD-L2 (expression post IFNγ / expression at baseline) relative to baseline expression for selected cell lines. Dotted lines indicate a threshold for expression (MFI=1.5).

Total and membrane-specific staining for PD-L1 and PD-L2 was performed in six cell lines [C060M1, MelAT, NM177, NM182, MM418, SKMel28], at baseline and at 4 h, 16 h, 24 h, 48 h and 72 h after treatment with IFNγ. Induced expression of total and membrane PD-L1 was clearly evident by 16 h in all cell lines and plateaued at 24-48 h after exposure to IFNγ in all six cell lines. At 48 h post IFNγ exposure, the amount of total PD-L1 was on average 1.5-fold higher (range 1.3-1.6) than the cell-surface

PD-L1, and the expression of total and cell surface PD-L1 showed equivalent trends during the induction time course (Figure 5.2).

The induction of PD-L2 cell surface expression was similar to PD-L1; substantial induction in expression 16 h post IFNγ exposure. The total PD-L2 protein expression, however, did not track with cell surface PD-L2 expression over the IFNγ time course (Figure 5.2). Of four cell lines showing increased cell surface PD-L2 expression at 16 h post IFNγ treatment (C060M1, MelAT, NM177, SKMel28), minimal changes in the level of total PD-L2 was observed (Figure 5.2). Importantly, we noted that the PD-L1 levels declined at 72 h post IFNγ treatment in the PD-L1low/PD-L2high SKMel28 and MelAT cell lines (Figure 5.2).

The PD-L1^{High}/PD-L2^{Low} NM182 and MM418 cell lines showed minimal induction of PD-L2 over the 72 h time course, indicating a general failure in PD-L2 responsiveness.

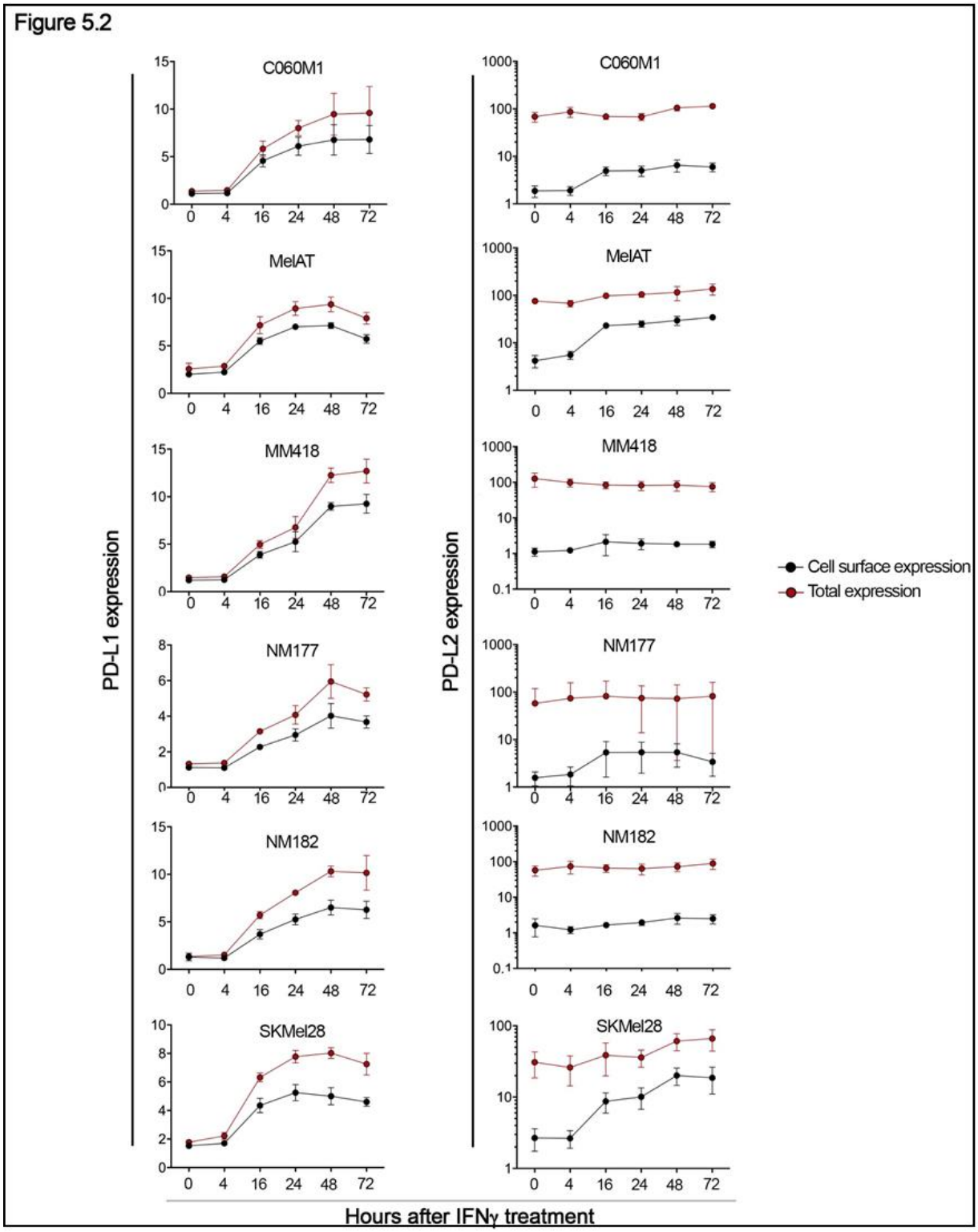

Figure 5.2 Total and membrane expression of PD-L1 and PD-L2 following IFNγ stimulation

Total and membrane expression of PD-L1 and PD-L2 was calculated as (MFI stained sample /MFI FMO control sample) at baseline and 4 h, 16 h, 24 h, 48 h and 72 h after exposure to IFNγ. Results show mean of at least three biological replicates with standard deviation.

5.3.2 Analysis of cell surface and total PD-L2 expression

As shown in Figure 5.2, total PD-L2 expression did not reflect cell surface PD-L2 expression and thus we re-examined PD-L2 expression using different PD-L2 antibodies by flow cytometry and western immunoblotting. We analyzed a series of melanoma cell lines with variable levels of IFNγ-induced PD-L2 cell surface expression and included the immortalized Jurkat T cell line as a negative control, as this cell line has been reported not to express PD-L2 (381).

The accumulation of total and cell membrane PD-L2 was examined at 72 h post IFNγ treatment using the M1H18 PD-L2 antibody clone. This antibody produced cell surface expression values that were consistently lower than PD-L2 expression values observed for the original PD-L2 clone 24F.10C12 (Table 5.1). Importantly, both PD-L2 antibodies produced similar induction values of cell surface PD-L2 (IFNγ expression of PD-L2 / baseline expression PD-L2) for each cell line (Table 5.1). In contrast, when evaluating total PD-L2 expression with these two antibodies, we did not observe consistent induction levels, and the original 24F.10C12 antibody clone also produced total PD-L2 values that did not reflect the cell surface expression of PD-L2 (Figure 5.2; Table 5.1). For instance, although we did not detect membrane PD-L2 in the MM418 and Jurkat cells, the 24F.10C12 PD-L2 antibody clone detected substantial levels of total PD-L2 in both cell lines (Table 5.1). The M1H18 clone, however, produced identical induction levels for cell surface and total PD-L2 for the five cell lines and these were similar to the induction of membrane PD-L2 observed with the 24F.10C12 PD-L2 antibody (Table 5.1). Taken together, these data strongly suggest that clone 24F.10C12 is not specific for intracellular PD-L2.

Table 5.1 Flow cytometric analysis of PD-L2 expression with independent antibodies

Cell Line	PD-L2 (clone 24F.10C12)						PD-L2 (clone M1H18)					
	Baseline expression		Expression post IFNγ		Induction		Baseline expression		Expression post IFNγ		Induction	
	M	T	M	T	M	T	M	T	M	T	M	T
SMU15-0404	47	115	155	316	3	3	23	48	69	145	3	3
SMU13-0183 M7	22	79	64	161	3	2	11	16	31	54	3	3
MM418	1	46	1	43	1	1	1	2	1	2	1	1
MelAT	2	56	10	61	5	1	2	3	5	8	3	3
SKMel28	4	46	20	81	5	2	3	5	9	17	3	3
Jurkat	1	38	n.d.	n.d.	n.d.	n.d.	1	3	n.d.	n.d.	n.d.	n.d.

M, membrane PD-L2 expression; T, total PD-L2 expression (i.e. membrane and intracellular); n.d. not determined

Induction = MFI expression post IFNγ / MFI baseline expression; Expression = MFI stained / MFI FMO control

We also examined the expression of total PD-L2 using western immunoblotting in a series of cell lines using a third PD-L2 antibody (clone D7U8C) that was suitable for Western blotting. We detected a predominant band at 40 kDa in all cell lines using this antibody clone, even in the two cell lines with no detectable membrane PD-L2 (MM418 and NM182). Further, the PD-L2 band intensity did not reflect the flow cytometry cell membrane expression data, nor did we detect consistent evidence of IFNγ-mediated protein induction by Western blotting (Figure 5.3). Thus, the Western blotting data did not reflect our flow cytometry-based data and thus we did not pursue Western blotting analysis of PD-L2 further.

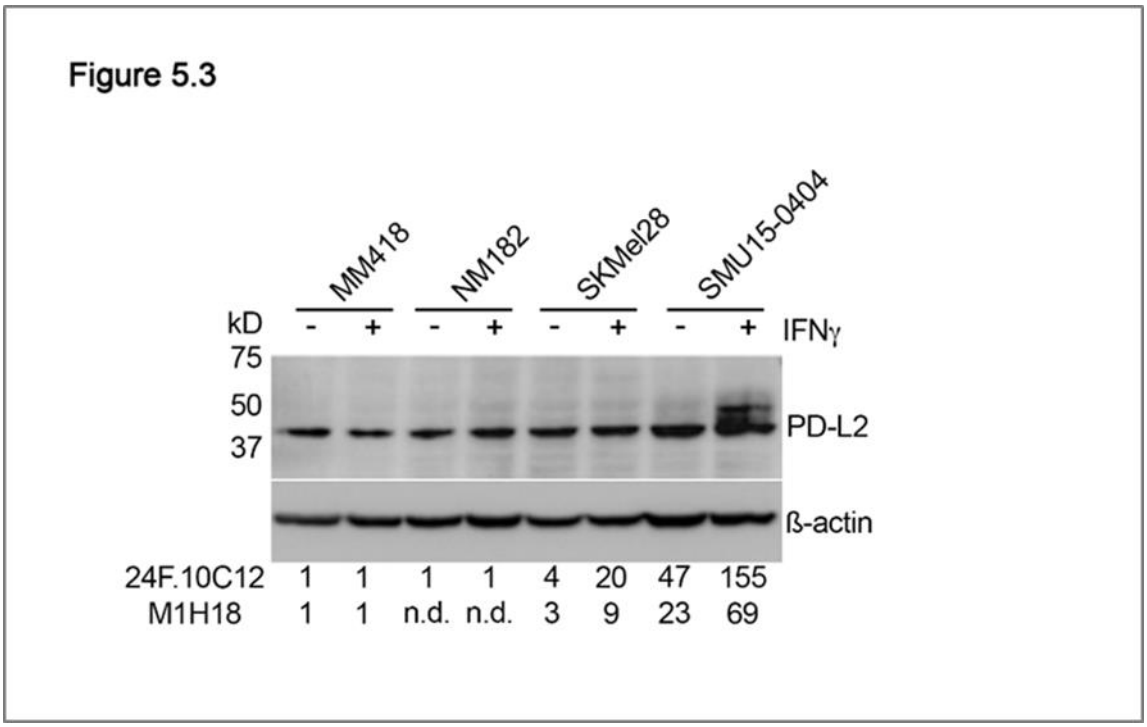

Figure 5.3 Western blotting of PD-L2

PD-L2 protein expression at baseline and in response to IFNγ treatment was detected using the PD-L2 antibody clone D7U8C. ß-actin was used as the protein loading control. The membrane expression of PD-L2 was determined by flow cytometry with the indicated PD-L2 antibodies shown below the protein blots. This western blot was kindly performed by Ms Ashleigh Stewart.

5.3.3 Knockdown of PD-L2 expression by siRNA

We also examined PD-L2 expression after PD-L2 silencing using siRNA constructs in the SMU15-0404 cell line that showed high PD-L2 levels by flow cytometry (Table 5.1). Expression levels after siRNA transfection was assessed by flow cytometry using PD-L2 antibodies 24F.10C12 and M1H18. Both antibodies showed a significant decrease in the level of membrane PD-L2 to approximately 10% of the control siRNA-transfected cells. Although total PD-L2 levels were also diminished in the PD-L2 siRNA-transfected cells, approximately 30% PD-L2 expression remained detectable post silencing with both antibodies (Table 5.2; Figure 5.4). Given the discrepant results in total PD-L2 expression observed between the different antibody clones and detection systems (flow cytometry and western blotting), we did not have confidence in quantitating the intracellular levels of PD-L2, and we restricted our analyses to membrane expression of PD-L1 and PD-L2.

Table 5.2 Membrane and total expression PD-L2 using two antibodies

	Control siRNA		PD-L2 siRNA	
PD-L2 antibody	Membrane	Total	Membrane	Total
24F.10C12	28.4	98.4	2.8	31.7
M1H18	13.8	27.1	1.8	7.3

Cell membrane and total PD-L2 staining was performed on the SMU15-0404 cells at baseline. Cells were transfected with control or PD-L2 specific siRNAs and examined for PD-L2 expression 72 h post siRNA transfection.

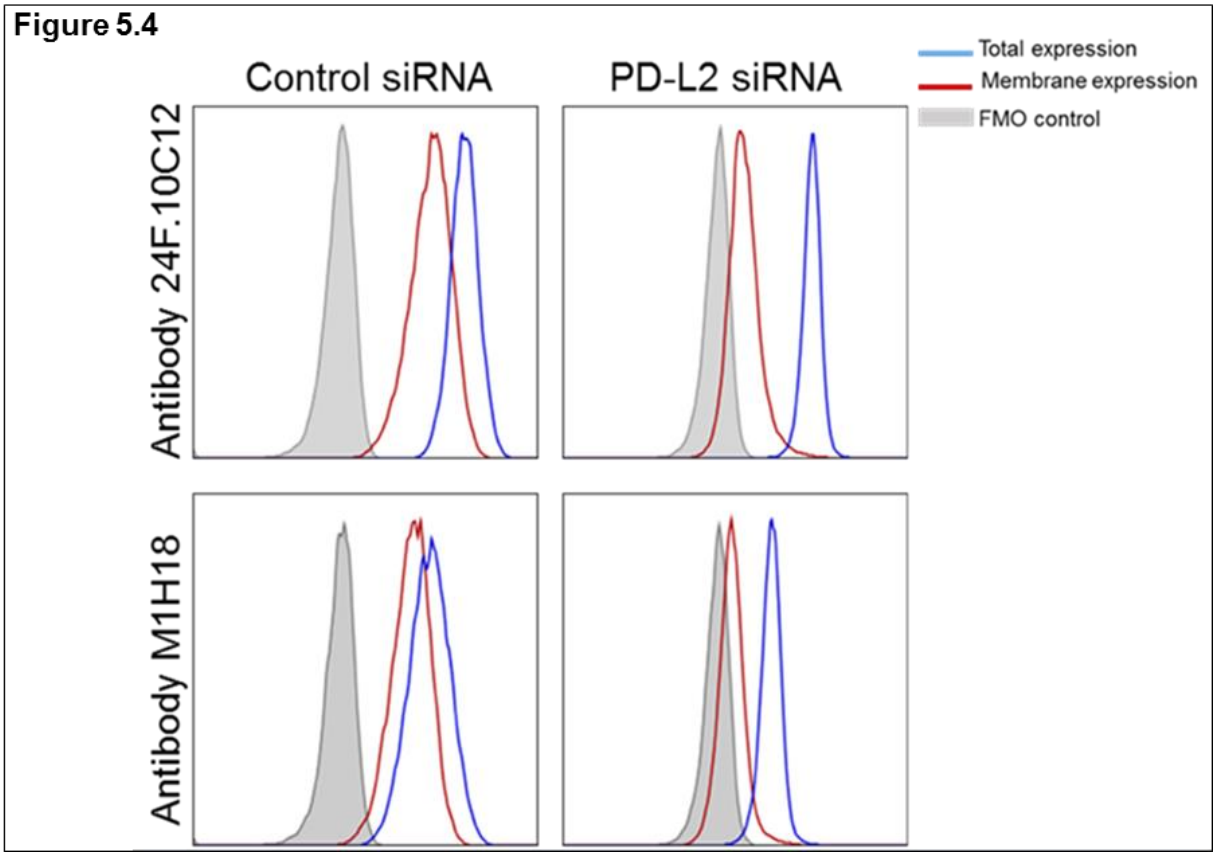

Figure 5.4 Representative histograms for SMU15-0404 cells transfected with control siRNA or PD-L2 specific siRNAs

SMU15-0404 melanoma cells were transfected with control or PD-L2 specific siRNAs for 72 h. Flow cytometry analysis, using the two indicated antibodies, was used to detect cell membrane alone or total (intracellular plus membrane) PD-L2.

5.3.4 Stability of membrane expression of PD-L1 and PD-L2

In this section we examined the temporal stability of cell surface PD-L1 and PD-L2 in four cell lines that expressed both proteins, including two PD-1 PROG melanoma cell lines, SMU15-0404 and SMU13-1087 M7. Cells were exposed to the protein translation inhibitor, cycloheximide 24 h with IFNγ after an initial 24 h treatment with IFNγ. Cell surface PD-L1 and PD-L2 expression was examined over time (0 h, 4 h, 6 h, 8 h, 16 h and 24 h post cycloheximide treatment). In all cell lines the expression of PD-L2 steadily decreased over the 24 h time course with the addition of

cycloheximide, and this included the two PD-1 PROG cell lines with high PD-L2 membrane expression. In contrast PD-L1 protein expression remained stable in three of the melanoma cell lines following cycloheximide exposure (Figure 5.5). In the PD-1 PROG cell line, SMU15-0404, PD-L1 membrane accumulation consistently increased in the presence of IFNγ and cycloheximide treatment (Figure 5.5). These data indicate that PD-L1 is more stable than PD-L2 in these melanoma cell models, and that PD-L1 stability may vary in melanoma (Figure 5.5).

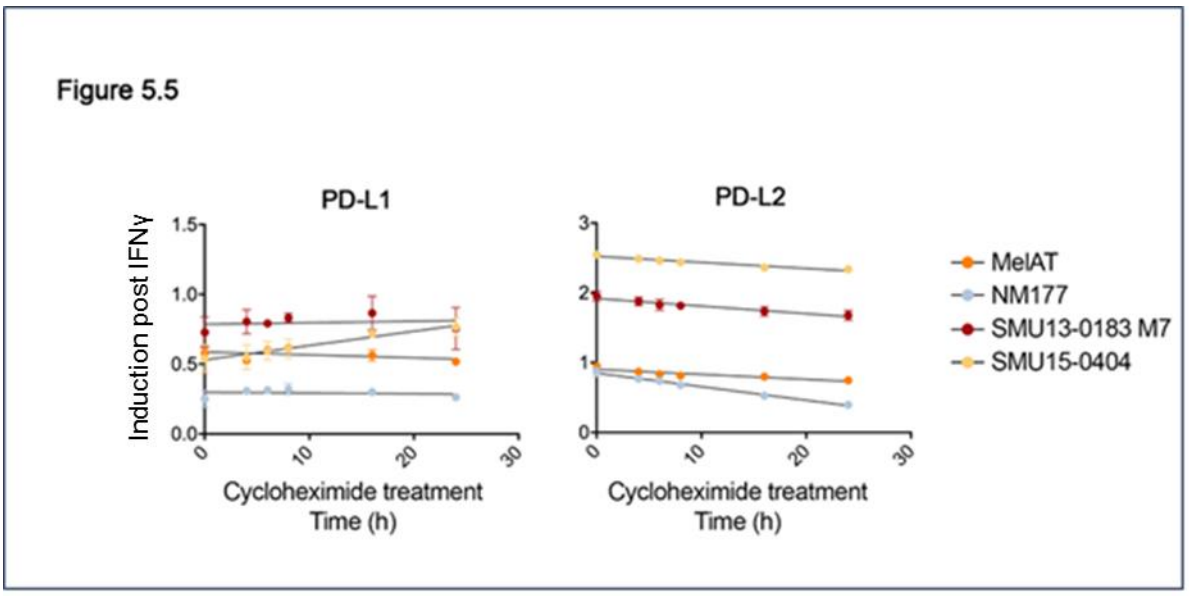

Figure 5.5 Stability of membrane PD-L1 and PD-L2

Membrane induction of PD-L1 and PD-L2 (expression after IFNγ treatment / expression at baseline) in melanoma cells following treatment with cycloheximide. Data represent mean with standard deviation of three independent experiments and linear regression lines of replicates are shown.

5.3.5 Expression of STAT1 and STAT3 in melanoma cells

To further examine potential mechanisms contributing to the differential expression of PD-L1 and PD-L2 in the melanoma cell lines, we performed Western blotting for two key downstream effectors of the IFNγ signalling pathway, STAT1 and STAT3.

Expression of phosphorylated STAT1Y701 and total STAT1 was elevated after treatment with IFNγ compared to baseline in four cell lines tested (NM177, MelAT, SMU15-0404, and SMU13-0183 M7). Phosphorylated STAT3Y705 and total STAT3 showed minor increases in response to IFNγ, although it is worth noting that STAT3 was constitutively phosphorylated in these melanoma cells. Cell lines with high levels of membrane PD-L2 as determined by flow cytometry (i.e. SMU15-0404 and SMU13-0183 M7), did not show consistently elevated total or phosphorylated levels of STAT1 and STAT3 expression compared to cell lines with low membrane PD-L2 (NM177 and MelAT) (Figure 5.6).

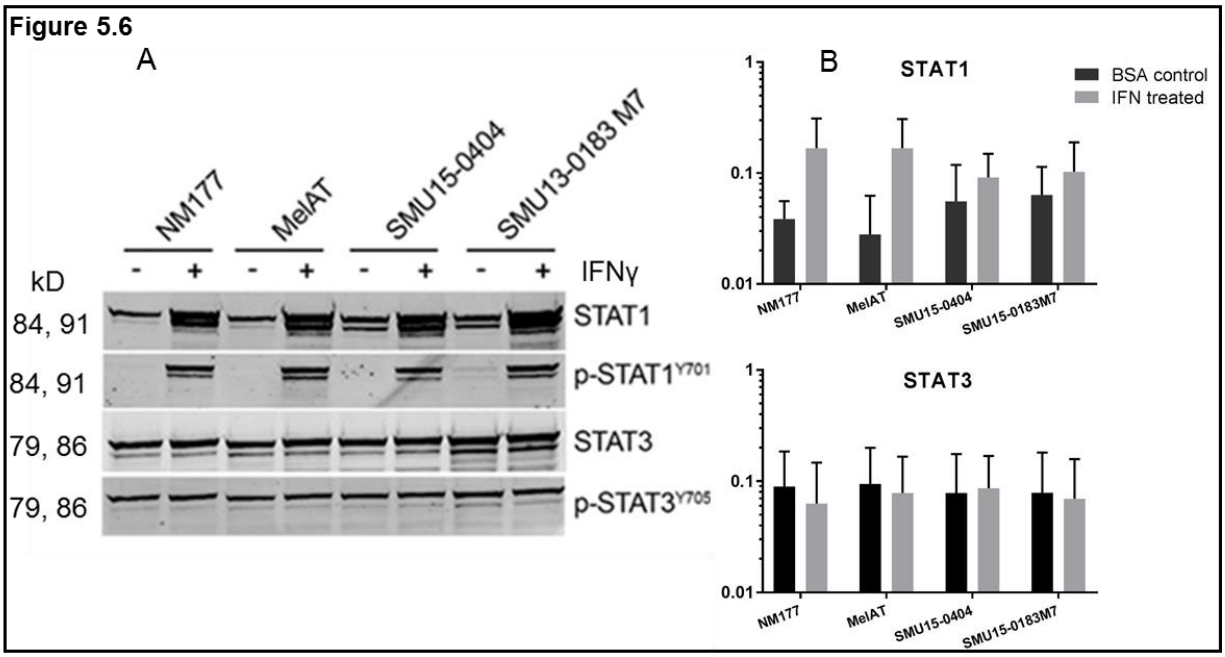

Figure 5.6 Expression and activation of STAT1 and STAT3 in response to IFNγ treatment

(A) Expression of total and phosphorylated STAT1 and STAT3 were determined by Western blotting at baseline (-) and after treatment with IFNγ for 72 h (+). (B) Densitometry was performed for STAT1 and STAT3. Data represent mean with standard deviation for four independent biological experiments.

5.4 Discussion

The binding of PD-L1 and PD-L2 to their cognate receptor PD-1 is an important immune suppressive mechanism and recent data suggests that these ligands may have distinct activities and independent regulatory networks (431). For instance, only PD-L2 induces local cytokine production to promote tumour cell growth (567), and unlike PD-L1, PD-L2 expression is limited to antigen-presenting cells, such as macrophages and myeloid DCs, and non-hematopoietic tissues, such as lung (431, 567). Further, although PD-L1 and PD-L2 expression on melanoma cells reflects the degree of immune cell infiltration, including CD3+, CD8+, PD-1+ T cells and TRegs, this association is weaker for PD-L2 expression compared to PD-L1 expression (377).

Our data confirm that a substantial proportion of PD-L1 is expressed at the cell surface post IFNγ exposure, although intracellular PD-L1, which was also induced by IFNγ, was evident in melanoma. Cytoplasmic PD-L1 has also been reported in ovarian cancer cells (568) and NSCLC (569). Intracellular PD-L1 may simply act as a reservoir for the membrane protein, or it may perform additional functions that are distinct from cell membrane PD-L1. For example, intracellular PD-L1 can induce MTORC1 signalling in mouse melanoma and ovarian cancer cells, leading to inhibition of autophagy and conferring resistance to MTOR inhibitors (568).

In this study, we detected high levels of intracellular PD-L2 in all melanoma cell lines tested. The total level of PD-L2 did not reflect the level of membrane-specific PD-L2 expression, however, and showed minimal induction in response to IFNγ treatment. Although these initial data were indicative of an intracellular PD-L2 reservoir, we were unable to confirm these results in complementary experiments, including with the use of additional antibodies, the analysis of PD-L2 negative control cell models

and in PD-L2 siRNA experiments. We observed lower total PD-L2 expression with a second antibody clone (clone M1H18) in our flow cytometry studies, and western blotting results were not comparable with our flow cytometry data. Moreover, the original PD-L2 antibody showed low membrane PD-L2 but high intracellular PD-L2 in the PD-L2-null Jurkat cell line. Collectively, our data suggest that the PD-L2 antibody clone 24F.10C12 shows non-specific binding intracellularly. To help confirm the subcellular distribution of PD-L1 and PD-L2, future experiments will examine ectopically expressed PD-L1 and PD-L2 proteins tagged with small epitope tags such as MYC and FLAG.

We also explored the potential influence of PD-L1 and PD-L2 protein stability on their regulation. In these experiments, melanoma cells were treated with the protein translation inhibitor, cycloheximide after 24 h IFNγ treatment and at the same time as the addition of IFNγ. We chose to include IFNγ in these experiments, as PD-L1 expression is very low at baseline. The membrane expression of PD-L1 and PD-L2 was monitored over a 24 h time course (0 h, 4 h, 6 h, 8 h, 16 h and 24 h). PD-L1 membrane expression remained constant over the 24 h cycloheximide treatment, suggesting that PD-L1 protein has very low turnover rate in melanoma cells. The PD-1 PROG cell line, SMU15-0404, actually showed IFNγ-mediated stimulation of PD-L1 in the presence of cycloheximide, and it will be interesting to explore the mechanism underlying this protein increase and compare the stability of PD-L1 in the larger panel of PD-1 PROG cell lines. Although, these data are only based on a single PD-1 PROG cell model, it is tempting to speculate that increased PD-L1 stability may contribute to the melanoma resistance to PD-1 based immune checkpoint inhibitor therapy. It is also worth noting that although we did not detect PD-L1 protein turnover within 24 h of cycloheximide treatment, PD-L1 levels did decrease at 72 h post IFNγ

treatment in some melanoma cell models, and additional work is needed to explore the differences in temporal regulation of PD-L1 and PD-L2.

Changes to PD-L1 stability can occur via various post translational modifications including glycosylation, phosphorylation, and de-ubiquitination of this molecule (457). For example, in breast cancer cells, EGF has been shown to stabilize PD-L1. In these cells, glycosylation of PD-L1 is another mechanism for stabilizing PD-L1 through inhibition of 26S proteasome-mediated protein degradation (467). Another factor involved in PD-L1 stabilization is CMTM6 which is a transmembrane glycoprotein that prevents lysosomal degradation of PD-L1 and reduce its ubiquitination leading to enhanced PD-L1 stability (472).

In contrast to PD-L1, PD-L2 membrane expression gradually decreased over the 24h cycloheximide time course. Thus, PD-L2 has a more rapid turnover compared to PD-L1 in melanoma cells. The variation in PD-L1 and PD-L2 turnover may also reflect the stability of key regulators, and these ligands are regulated via related, but distinct interferon signalling cascades. In particular, IFNγ stimulation is associated with the upregulation of JAK1/JAK2/STAT1/IRF1 while IFNβ is associated with STAT2/STAT3/IRF1/9 upregulation. Binding of STAT1 to the PD-L1 promoter has important role in PD-L1 regulation whereas STAT3 binding to the PD-L2 promoter appears to play a dominant regulatory role (265).

To investigate whether the specific STAT transcription factors contribute to the differential expression of PD-L1 and PD-L2 in melanoma cell lines, we examined the expression and phosphorylation of STAT1 and STAT3 by Western blotting. The total and phosphorylated levels of STAT1 increased after IFNγ treatment in all cell lines compared to their baseline levels. STAT3 accumulation and phosphorylation also increased with IFNγ treatment, although this was less evident because STAT3 was

constitutively activated in our melanoma cell models. Our findings reflect those recently reported in another study that showed IFNγ stimulation leading to the phosphorylation of JAK1/JAK2 followed by phosphorylation and activation of STAT1 in most cells and STAT3 in some cells (265). In the same study, the authors further demonstrated that PD-L2 expression is regulated through IFNß and IFNγ leading to STAT3 binding to the PD-L2 promoter. In our study, the levels of STAT3 and STAT1 activation did not completely reflect levels of PD-L2. For instance, although both SMU15-0404 and SMU13-0183 M7 cells expressed high PD-L2, only the latter showed evidence of elevated STAT3 expression.

This work highlights that the mechanisms regulating PD-L1 and PD-L2 expression and stability are not fully understood (375). Understanding the regulation of PD-L1 and PD-L2 in melanoma is particularly timely, considering their role as predictive biomarkers and potential resistance effectors of immunotherapy (375). A thorough understanding of PD-L1 and PD-L2 regulation may also reveal novel treatment approaches that can circumvent immunotherapy resistance.

Chapter 6
Conclusion

The aim of immunotherapy is to induce the immune system so that it recognises and destroys cancer cells. Traditionally, vaccination with tumour-specific antigens, or passive transfer of modified immune effector cells, such as adoptive T cell transfer have been used to induce adaptive immunity (570). Immune checkpoint blockade is a relatively new therapy that can reinvigorate anti-tumour immune activity by targeting immune checkpoints on T cells, cancer cells and/or antigen-presenting cells.

The biological function of immune checkpoints is to regulate the immune response in order to ensure balance between immune activity and self-tolerance and minimize tissue damage (342). The expression of immune checkpoints on cancer cells shifts the balance towards immune tolerance and suppresses anti-cancer immunity by downregulating T cell activation (571, 572). Immune checkpoint blockade can reinstate anti-tumour T cell activity and is FDA-approved for multiple cancers including melanoma, renal cell carcinoma, non-small cell lung cancer, Hodgkin's lymphoma, urothelial carcinoma, hepatocellular carcinoma, gastric and gastroesophageal carcinoma and head and neck squamous cell carcinoma (571, 572). Approximately 35% to 60% of patients with advanced melanoma have a RECIST response to immune checkpoint blockade and approximately 10% of these patients will have durable and complete responses (389, 390, 392). Despite these remarkable results, however, innate and acquired resistance to immune checkpoint blockade has been demonstrated in a large proportion of patients.

The efficacy of immune checkpoint blockade is influenced by tumour-intrinsic and -extrinsic factors, including the level of cytokines within the tumour microenvironment and the expression of the immune checkpoints on both tumour and immune cells. IFNγ and TNFα are abundantly expressed in the tumour microenvironment and signatures reflecting IFNγ response are strongly associated with response to PD-1 based therapies (262, 573-576). Consequently, loss of response to IFNγ, via mutations in the IFNγ receptor IFNGR or alterations in IFNγ signalling pathway regulators, such as JAK1/2 and STAT1 confer immunotherapy resistance in melanoma (162, 163, 194, 478, 577).

In this study of immunotherapy naïve and PD-1 PROG melanoma cell lines, we found complete loss of response to IFNγ, defined as a lack of induction of all target molecules tested, in only a small proportion of melanoma cells. In fact, we observed loss of IFNγ response in only two cell lines, an immunotherapy-naïve melanoma cell line (D22M1, Chapter 2) and a PD-1 PROG cell line (SCC16-0016, Chapter 4). For D22M1, whole exome sequencing revealed a damaging missense mutation resulting in a P44R substitution in the extracellular portion of the IFNGR1, preventing cell surface expression off the receptor. This mutation has not been reported previously. In contrast, cell surface expression of IFNGR1 was unaltered in SCC16-0016 cells, suggesting a defect in the downstream components of the IFNγ signalling pathway. Whole exome sequencing of SCC16-0016 did not identify a clear genetic mechanism underlying IFNγ non-responsiveness and further analysis of the IFNγ signalling cascade is being undertaken.

Although the complete loss of response to IFNγ was uncommon in our large panel of melanoma cells, we did observe heterogeneity of response to IFNγ. In particular, 67% of immunotherapy-naïve melanoma and 63% of PD-1 PROG cell lines showed

no induction of one or more markers in response to IFNγ stimulation. The impact of heterogeneous IFNγ signalling on immune checkpoint inhibitor responses remains to be determined, but these data, reinforce the complexity of IFNγ signalling. They also support recent data showing that PD-L1 and PD-L2 regulation via IFNγ rely on variable downstream effectors (i.e. JAK–STAT1/2/3–IRF1 predominantly induce PD-L1 expression whereas the JAK–STAT3–IRF1 cascade preferentially upregulates PD-L2 expression).

A second critical cytokine in immune activity regulation is TNFα, and we found that TNFα was a weaker inducer of immune markers in melanoma cells compared to IFNγ. TNFα was less effective at upregulating PD-L1, PD-L2, HLA-ABC and HLA-DR, but induced higher levels of the neural crest marker NGFR compared to IFNγ. High expression of NGFR is associated with the melanoma de-differentiation (413), a process that leads to the loss of melanocyte lineage antigen expression. Thus, the balance of TNFα and IFNγ expression within the microenvironment may impact the response of melanoma to immune checkpoint inhibitors, with a TNFα-enriched environment leading to loss of antigen expression and possible escape from immune cell recognition. Indeed, melanomas acquire resistance to adoptive T cell transfer therapies via TNFα-induced loss of melanocytic antigen expression (149).

As mentioned above, the expression of melanocytic lineage antigens is important for immune recognition, and immune cells isolated from melanoma patients frequently recognise these antigens (149). Importantly, we noted that expression of the melanocyte differentiation antigen MLANA was lost at baseline in almost 56% of immunotherapy naïve and PD-1 PROG cell lines, and this was often associated with other markers of de-differentiation including loss of MITF (the master regulator of melanocyte differentiation) expression, overexpression of the receptor tyrosine

kinase AXL (99) and/or upregulation of NGFR. A recent report defined a multi-step differentiation process in melanoma, with undifferentiated melanoma displaying an $NGFR^{low}/AXL^{high}/MITFl^{ow}$ expression pattern, and de-differentiated neural crest-like melanoma marked by an $NGFR^{high}/AXL^{high}/MITF^{low}$ expression pattern (578). Both, phenotypes show loss of MLANA and MITF expression and display resistance to BRAF/MEK and immune checkpoint inhibitors (99, 579). Therefore, potential therapies aimed at blocking or reversing the de-differentiation of melanoma are currently being explored. De-differentiated melanoma cell models displayed sensitivity to iron-dependent oxidative stress and compounds that inhibited the detoxification of iron-dependent lipid reactive oxygen species induced ferroptosis, an iron-dependent form of programmed cell death. Ferroptosis was selectively induced in de-differentiated melanoma cell models (578). These data suggest that first line therapies that combine ferroptosis-inducing drugs with molecular targeted or immune therapies may prove effective in circumventing de-differentiation-mediated acquired resistance.

It is also worth noting that 8/16 PD-1 PROG cell lines with loss of MLANA, showed high PD-L2 expression. This raises the possibility that high PD-L2 expression is associated with melanoma cell de-differentiation or these two mechanisms co-operate to promote PD-1 inhibitor resistance.

The processing and presentation of antigens is a critical step in immune cell recognition (417, 580), and predictably, defects in MHC molecules or their structural component, B2M has been found in immune checkpoint inhibitor resistant melanoma. For instance, loss of B2M was identified in two patients who progressed on PD-1 based therapy (193, 194, 359, 482). In our study, we found that complete loss of antigen presentation was uncommon. One immunotherapy-naïve cell line (SMU15-

0217, Chapter 2) and two PD-1 PROG cells (SCC13-0156 and SMU-092, Chapter 4) displayed no cell surface HLA-ABC expression and this was associated with loss of B2M expression. Further, immunotherapy resistance melanoma cells showed significantly lower expression of HLA-ABC and HLA-DR compared to immunotherapy naïve cell lines (Chapter 2 and Chapter 4). These data confirm that down-regulation of MHC class I and II molecule expression may be common contributors to immunotherapy resistance.

Another important difference between immunotherapy-naïve and immunotherapy-resistant melanoma cells was the higher level of baseline and IFNγ-induced expression of both PD-L1 and PD-L2 in immunotherapy resistant melanoma cells, with PD-L2 expression being particularly high in 9/16 of PD-1 PROG cells. Although PD-L1 expression on melanoma cells in vivo generally correlates with a good response to PD-1 blockade in melanoma patients (539), as little as 1% PD-L1 positivity is used to predict response (390). It is therefore possible that the over-expression of tumour-associated PD-L1 and/or PD-L2 expression may be sufficient to overcome PD-1 inhibition.

The differential overexpression of PD-L1 and PD-L2 in some of our PD-1 PROG cell lines was also of interest as these immune checkpoint inhibitors show variable expression patterns, have different binding partners and may have variable roles in modulating immune activity. PD-L2, for instance, is typically expressed on antigen-presenting cells (375), whereas PD-L1 is broadly expressed on tumour cells, T cells, B cells, dendritic cells, macrophages, mesenchymal stem cells, epithelial, endothelial cells, brown adipocytes (375, 432). In our study, 25% of PD-1 PROG cells showed PD-L1 expression and 56% showed expression of PD-L2 at baseline. Six cell lines with loss of PD-L1 expression showed high expression of PD-L2 and one cell line

with high PD-L1 expression showed loss of PD-L2 expression. In two cell lines (SCC11-0270 and WMD15-083 #1) IFNγ induced expression of PD-L1 without inducing PD-L2 and in three cell lines (SMU13-0183 M3, SMU15-0404 and WMD-084) IFNγ induced PD-L2 while not inducing PD-L1.

It has been shown that PD-L1 expression on the membrane reflects total PD-L1 expression, (568) and we also found that cell surface PD-L1 reflected the total pool of PD-L1. Unfortunately, we could not make definitive conclusions on intracellular PD-L2 accumulation as the antibodies tested were non-specific. Nevertheless, the turnover rate of cell surface PD-L1 was slow compared to PD-L2 turnover, indicating higher PD-L1 stability in melanoma cells. PD-L1 is stabilized via different modifications including de-ubiquitination (457) and de-glycosylation (467). The chemokine-like factor-like (CKLF) MARVEL transmembrane domain containing family member 6 (CMTM6) and its homolog CMTM4 can stabilize PD-L1 by suppressing its ubiquitination (471). Another factor, COP9 signalosome 5 (CSN5), which is induced by TNFα also enhances stabilization of PD-L1 via de-ubiquitination (473). In breast cancer, GSK3β interacts with PD-L1 and induces phosphorylation-dependent proteasome degradation of PD-L1. EGF stabilizes PD-L1 via GSK3β inactivation (467). Additional work is required to confirm the higher stability PD-L1 and the mechanisms regulating PD-L1 and PD-L2 turnover in melanoma.

The poor response of uveal melanoma to immune checkpoint inhibitors (493) was also of particular interest in this study. It has been reported that PD-L1 expression is decreased in uveal compared to cutaneous melanoma, and this provides a rationale for the lack of response to PD-1 inhibitor therapy in uveal melanoma patients (496). This study also found less PD-1 expressing TILs in uveal compared to cutaneous melanoma specimens and this could result in lower IFNγ production and a

concomitant decrease in PD-L1 expression (496). However, our data show that uveal melanoma cells display significantly diminished response to IFNγ, with lower PD-L1 induction post IFNγ stimulation. These results were supported by our analysis of TCGA data, showing reduced IFNγ transcriptome signatures in uveal compared to cutaneous melanoma (Chapter 2). Thus, the poor response of uveal melanoma to IFNγ, compared to cutaneous melanoma (Chapter 2), could explain the poor response of uveal melanoma patients to immune checkpoint inhibitor immunotherapy (494) and also suggests the possibility of using an in vitro IFNγ-induction test as a potential predictor of immunotherapy response.

In this thesis, *in vitro* analysis of melanoma cells and their responses to critical cytokines has provided valuable data on the regulation of immune effectors in melanoma, highlighting the important contribution of antigen processing and presentation, and de-differentiation, in melanoma response to PD-1 based immunotherapies. Although we identified some commonly-reported resistance mechanisms, these studies are by no means complete, and should be further expanded to include functional assays that can validate the impact of specific resistance mechanisms on melanoma response to PD-1 based immunotherapies. For example, overexpression or silencing of specific antigen presentation molecules or melanoma de-differentiation markers may help confirm their contribution to melanoma immune evasion. However, given the time constraints, these assays could not be performed within the duration of this thesis. Another limitation of these studies is the use of melanoma cell lines, which may not truly represent the physiological processes occurring in tumours. Inclusion of more complex cell models, such as those incorporating immune and stromal cells, patient tissue samples or murine melanoma models will help validate the identified resistance mechanisms in a more physiological setting. It is worth noting, however, that he melanoma-intrinsic

mechanisms identified in vitro have also been identified in vivo , confirming the relevance of cell models, at least for cell autonomous mechanisms.

The regulation of the PD-L1 and PD-L2 immune checkpoints in melanoma cells is an area requiring further investigation, as their stability, cellular localization and induction in response to immune-cell derived cytokines are potential modulators of response to PD-1 blockade. Future work is needed to uncover the mechanisms regulating PD-L1 and PD-L2 expression, including a more precise investigation into the stability, localization and temporal expression patterns. Such understanding will be essential for the rational design of next-generation immunotherapies.

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