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Characterising PD-L1 expression in circulating melanoma and non-small cell lung cancer cells

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Characterising PD-L1 expression in circulating melanoma and non-small cell lung cancer cells

This thesis is presented in partial fulfilment of the degree of
Master of Science (Medical Science)

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Supervisors:
Associate Professor Elin Gray
Professor Melanie Ziman

School of Medical & Health Sciences
Edith Cowan University
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Abstract

Incidence rates for both melanoma and non-small cell lung cancer (NSCLC) have risen in recent decades. While advanced cases of both diseases have previously demonstrated low survivability, novel therapies such as immune checkpoint inhibitors, have significantly improved the outcome of patients suffering from these cancers. Recent clinical trials have led to the United States Food and Drug Administration (FDA) approving the use of antibodies targeting the PD-1 immune checkpoint for both melanoma and NSCLC. Anti-PD-1 antibodies have been seen to illicit responses in up to 40% of patients, however, particularly for melanoma, there is a lack of biomarkers to select for patients likely to respond.

PD-L1 expression in tumour tissue is the most studied biomarker of response to anti-PD-1 therapy in melanoma patients and is also the biomarker currently in use as a companion diagnostic test for NSCLC patients. It is believed that circulating tumour cells (CTCs) that break away from the tumour and enter the bloodstream, would share the same immune escape mechanisms as the parent tumour, and so would express PD-L1 in the same way as the tumour. Therefore, we postulate that these cells provide an accessible tumour sample for analysis of PD-L1 expression.

A previous study by our group used multi-marker flow cytometry to evaluated PD-L1 expression on CTCs from melanoma patients commencing anti-PD-L1 therapy. Here, the PD-L1 expression on CTC from the latter study was compared to PD-L1 expression in matched tumour tissues. Moreover, this study describes the establishment of methodologies for immunocytochemistry analysis and scoring of PD-L1 expression on melanoma CTCs and on carcinomas CTCs, including NSCLC. Cell lines representing negative, low and high PD-L1 expression were identified to serve as controls, again for both melanoma and carcinomas. To further evaluate our methods, CTCs were extracted from the blood samples of patients with melanoma and NSCLC using microfluidic devices and immunostained to investigate the expression of PD-L1. Finally, comparison between CTC and tumour tissue PD-L1 expression was carried out in a small number of cases.
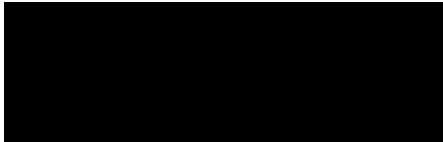
Overall, this study successfully developed immunocytochemistry protocols to effectively identify and score PD-L1 expression on both melanoma and carcinoma CTCs, thus providing a basis for further work to evaluate the clinical potential of CTCs.

Declaration

I certify that this thesis does not, to the best of my knowledge and belief:

i. Incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;

ii. Contain any material previously published or written by another person except where due reference is made in the text of this thesis; or

iii. Contain any defamatory material.

Signed:

MSc candidate

Isaac Spencer

Acknowledgements

Statement of contribution of others

Isaac Spencer designed and conducted experiments, analysed the results and wrote the thesis.

For the manuscript 'Is the Blood an Alternative for Programmed Death Ligand 1 Assessment in Non-Small Cell Lung Cancer'. Emmanuel Acheampong contributed to the conception of the study, data curation and writing of the manuscript. A/Professor Elin Gray contributed to the conception of the study and the review and editing of the manuscript. Weitao Lin, Michael Morici and Professor Melanie Ziman contributed to the review and edited of the manuscript.

For the manuscript 'PD-L1 expression on circulating tumour cells may be predictive of response to pembrolizumab in advanced melanoma: results from a pilot study'. Professor Adnan Khattak contributed to the conception of the study, recruited patients, collected data, analysed data and wrote the manuscript. Professor Michael Millward contributed to the conception of the study, recruited patients, collected data, and assisted in writing the manuscript. Professor Melanie Ziman contributed to the conception of the study and assisted in writing the manuscript. A/Professor Elin Gray contributed to the conception of the study, collected data, analysed data and assisted in writing the manuscript. Dr Tarek Meniawy recruited patients, collected data and assisted in writing the manuscript. Anna Reid, James Freeman, Michelle Pereira, Ashleigh McEvoy, Johnny Lo, Markus Frank and Ali Didan collected data and assisted in writing the manuscript.

Professor Michael Millward, Professor Adnan Khattak, Dr Afaf Abed, Dr Samantha Bowyer and Dr Tarek Meniawy recruited patients into the study, supervised collection of blood samples and procurement of clinical data. Dr Ashleigh McEvoy assisted with the procurement of clinical data and patient recruitment. Paula Van-Miert assisted with the development of the flow cytometry and immunocytochemistry panels as well as processing of patient blood samples. Michael Morici assisted with the development of the immunocytochemistry panels and processing of patient blood samples. Co-supervisor Professor Melanie Ziman provided guidance and supervision throughout the study. A/Professor Elin Gray, the principal supervisor of the project, provided guidance on experimental design, data interpretation and protocol development, supervision throughout the study, and reviewed the writing of the thesis.

Table of contents

List of Figures

List of Terms

CD16: Cluster of Differentiation 16

CD45: Cluster of Differentiation 45

CK: Cytokeratin

CTC: Circulating Tumour Cell

CTLA-4: Cytotoxic T-Lymphocyte-Associated protein 4

EMT: Epithelial to Mesenchymal Transition

EpCAM: Epithelial Cell Adhesion Molecule

FDA: Food and Drug Administration (United States)

HRP: Horseradish Peroxidase

ICI: Immune Checkpoint Inhibitor

IF: Immunofluorescence

IFN-γ: Interferon Gamma

IHC: Immunohistochemistry

NDS: Normal Donkey Serum

NRS: Normal Rabbit Serum

NSCLC: Non-Small Cell Lung Cancer

PD-1: Programmed cell Death protein 1

PD-L1: Programmed Death Ligand 1

PE: Phycoerythrin

PFA: Paraformaldehyde

TCR: T-Cell Receptor

TMB: Tumour Mutational Burden

TPS: Tumour Proportion Score

TSA: Tyramide Signal Amplification

WBC: White Blood Cell

1. CHAPTER 1: General Introduction and Literature Review

Section 1.6.1 includes sections from the following publication, of which I am a joint first author:

Acheampong, E.*, **Spencer, I.***, Lin, W., Ziman, M., Millward, M., & Gray, E. (2019). Is the Blood an Alternative for Programmed Cell Death Ligand 1 Assessment in Non-Small Cell Lung Cancer? *Cancers (Basel), 11*(7). doi:10.3390/cancers11070920. *Contributed equally

Section 1.6.2 includes sections from the following publication, of which I am an author:

Khattak, M. A., Reid, A., Freeman, J., Pereira, M., McEvoy, A., Lo, J., Frank, M.H., Meniawy, T., Didan, A., **Spencer, I.**, Amanuel, B., Millward, M., Ziman, M., & Gray, E. (2019). PD-L1 Expression on Circulating Tumour Cells May Be Predictive of Response to Pembrolizumab in Advanced Melanoma: Results from a Pilot Study. *Oncologist*. doi:10.1634/theoncologist.2019-0557

1.1. Introduction

Incidence rates for lung cancer and melanoma have increased substantially over the past several decades (Garbe and Leiter, 2009; Molina et al., 2008). Five-year survival rates for NSCLC are still less than 5% and until recently, it was common for metastatic melanoma to be fatal within 18 months (Garbe and Leiter, 2009; Molina et al., 2008). However, treatment options for both advanced NSCLC and metastatic melanoma have improved dramatically with the advent of novel therapies, including checkpoint inhibitors such as anti-PD-1 antibodies (Luke et al., 2017; Somasundaram et al., 2016).

Anti-PD-1 antibodies have been extensively studied in clinical trials to determine their efficacy (Brahmer et al., 2010; Luke et al., 2017; Somasundaram et al., 2016; Topalian et al., 2012). Though these antibodies have been demonstrated to have high response rates, they also have significant side effects (Larkin et al., 2015). This, combined with the high cost of treatment, has led to attempts to find biomarkers of response to treatment (Jessurun et al., 2017). The only biomarker currently in clinical use to determine the suitability of treatment with anti-PD-1 therapy is the evaluation by IHC of PD-L1 expression in NSCLC tumour tissue. The IHC analysis of PD-L1 expression in this was is used to qualify patients for first line therapy with pembrolizumab (Teixido et al., 2018).

Circulating tumour cells (CTCs) are capable of expressing the same immune escape mechanisms as the tumour that they arose from (Aya-Bonilla et al., 2019). Though there has been a significant increase in PD-L1 assessment on NSCLC CTCs in recent years, these results are not conclusive and there have been a limited number of comparisons to expression in tumour tissue (Adams et al., 2017; Dhar et al., 2018; Guibert et al., 2018; Ilié et al., 2017). Meanwhile, there has only been a single study assessing PD-L1 expression on melanoma CTCs relative to treatment response and comparing it to expression in tumour tissue (Khattak et al., 2019). It may therefore be possible to use CTC expression of PD-L1 from a liquid biopsy as a predictive biomarker of response to therapy or as a replacement companion diagnostic test for tumour tissue expression.

1.2. Immune checkpoint blockade: Anti-PD-1 therapies

1.2.1. Immune checkpoint molecules

Immune checkpoint molecules regulate the immune system to maintain self-tolerance and control the duration and level of immune response (Pardoll, 2012). There are several known immune checkpoint molecules, which can be categorised into two primary groups, stimulatory and inhibitory checkpoint molecules. Of the inhibitory checkpoint molecules there are currently 11 known, A2AR, B7-H3, B7-H4, BTLA, CTLA-4, IDO, KIR, LAG3, PD-1, TIM-3 and VISTA (some of which are included in Figure 1) (Dangaj et al., 2013; Derre et al., 2010; Huang et al., 2004; Kolar et al., 2009; Leitner et al., 2009; Leone et al., 2015; Lines et al., 2014; Prendergast et al., 2014; Topalian et al., 2012; Zhu et al., 2011). Inhibitory immune checkpoint molecules are integral in the functioning of the healthy immune system and have recently been of particular interest due their involvement in cancer immune escape. As a result, a variety of therapeutic agents have been developed and studied to block or reactivate their signalling to promote immune cell activation (Pardoll, 2012; Postow et al., 2015; Wei et al., 2018).
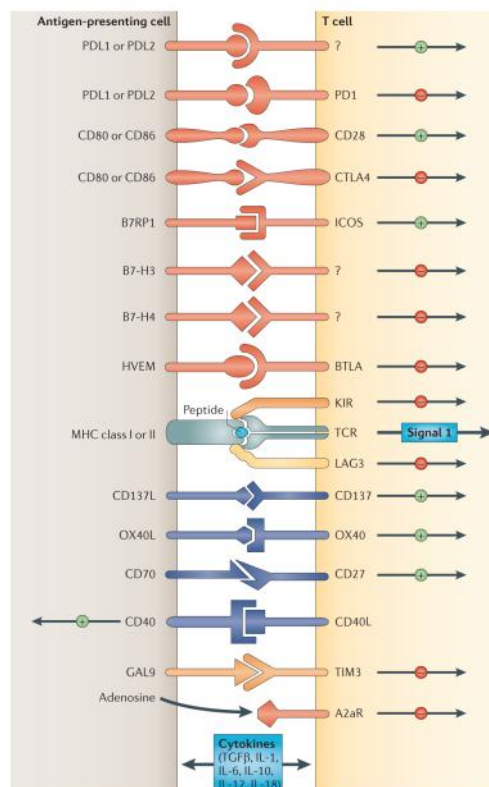
Figure 1. Depiction of various ligand-receptor interactions between T cells and antigen-presenting cells that regulate T cell responses, including multiple immune checkpoint molecules (Pardoll, 2012).

1.2.2. Mechanism of PD-1 signalling

Of the 11 known inhibitory immune checkpoint molecules, this study will focus on PD-1/PD-L1 axis given its demonstrated clinical relevance for the treatment of cancer (Le et al., 2015; Motzer et al., 2015; Nghiem et al., 2016; Powles et al., 2014; Topalian et al., 2012) . PD-1 was first discovered to be an inhibitory immune checkpoint molecule in 1999, though its means of action were not known (Nishimura et al., 1999). It was later found that PD-L1, also known as B7-H1, was a ligand for PD-1 (Freeman et al., 2000). The binding and interaction between PD-1 and PD-L1 has been identified as a normal part of immune regulation that protects native tissue from autoimmune damage (Barber et al., 2006; Freeman et al., 2000). PD-1 is expressed on T cells, B cells and myeloid cells and high levels of expression, compared to other CD28 molecules, suggests that PD-1 regulates a wide range of immune responses (Liang et al., 2003; Okazaki and Honjo, 2007).

PD-1 has two ligands, PD-L1 and PD-L2; both are transmembrane glycoproteins that interact with PD-1 to affect the immune system through subsequent PD-1 signalling as neither PD-L1 or PD-L2 possess a means of signal transduction (Okazaki and Honjo, 2007). PD-L1 and PD-L2 can be found in a plethora of different tissues including lymphoid, heart and lung tissues (Liang et al., 2003). As PD-L1 is expressed in both lymphoid and non-lymphoid tissues, it is believed that interaction between PD-1 and PD-L1 could regulate immune responses in both secondary lymphoid organs and target organs (Okazaki and Honjo, 2007). Recent findings by Zhao et al. in 2019 demonstrated that presence of PD-L1 can also reduce cell surface expression of CD80, the ligand for cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), on antigen-presenting cells. This interaction allows PD-L1 to exert immunostimulatory effects through the repression of the CTLA-4 immune checkpoint (Zhao et al., 2019).

Interaction between PD-1 and PD-L1 suppresses aberrant immune responses that damage host tissue. However, despite PD-1 signalling, some normal healthy responses produced by the immune system are not affected, such as that required for effective anti-bacterial immune responses (Okazaki and Honjo, 2007).

Stimulation of T cell receptors activates the phosphatidylinositol 3-kinase (PI3K) signalling cascade (Parry et al., 2005). This cascade involves the activation of the Akt pathway, which is responsible for mediating the cell growth and proliferation of T cells as well as cytokine

production, among other effects (Manning and Cantley, 2007). The binding of PD-L1 to PD-1 receptors on activated effector T cells results in a signal to attract phosphatase SHP-2, which can lead to the inactivation of the PI3K signalling cascade (Figure 2) (Chemnitz et al., 2004; Parry et al., 2005). Akt deactivation, as a downstream result of PD-1 signalling, has been established as a casual mediator of cell death and thus reduces both T cell levels and activation (Luo et al., 2003; Parry et al., 2005). The gross result of these events, initiated by PD-1 signalling, is the inhibition of T cell activation and proliferation, blockage of cytokine production and general obstruction of immune processes that would otherwise lead to the destruction the target cell (Chen and Mellman, 2013).

Figure 2. *PD-1 signalling. Ligation of T-cell receptor and PD-1 leads to tyrosine phosphorylation (P) of the immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) of PD-1. Binding of the ITSM by SHP-1 or SHP-2 results in the dephosphorylation of proximal signalling molecules and augmentation of PTEN expression. This effectively attenuates the activation of the PI3K and Akt pathways. PD-1 signalling may result in decreased T-cell proliferation, survival, protein synthesis and IL-2 production. (Red arrows and text indicate consequence of PD-1 mediated signalling) Adapted from (Francisco et al., 2010).*

PD-1 stimulation produces a signal that is vital for the regulation of the immune system by host cells, however, genetic mutations can affect this function. Over thirty different single nucleotide polymorphisms have been identified in the human *PDCD1* gene (which encodes the PD-1 protein) (Okazaki and Honjo, 2007). These single nucleotide polymorphisms in the human *PDCD1* gene have been associated with a variety of diseases including systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes, multiple sclerosis, ankylosing spondylitis, myocardial infarction and allergies (Bennet et al., 2006; James et al., 2005; Kroner et al., 2005; Lee et al., 2006; Nielsen et al., 2003; Prokunina et al., 2002; Prokunina et al., 2004). These studies demonstrate the importance of the PD-1 immune checkpoint and why some of these ailments are seen as immune related adverse events when anti-PD-1 therapy is administered (Bennet et al., 2006; James et al., 2005; Kroner et al., 2005; Lee et al., 2006; Nielsen et al., 2003; Prokunina et al., 2002; Prokunina et al., 2004).

1.2.3. Immune checkpoint activation and tumour immune escape

In 2002, PD-L1 was found to be expressed on the surface of several tumours (Dong et al., 2002) . Knowledge of the function of PD-1 signalling in healthy tissues led to the suggestion that PD-L1 expression on these tumours bolstered their ability to evade the immune system (Dong et al., 2002). There are two primary hypotheses as to how tumours initially begin expressing PD-L1; innate response and adaptive response. The innate response hypothesis suggests that tumours begin to express PD-L1 as a side effect of other signalling happening in the cells (Pardoll, 2012). In contrast, the second hypothesis of adaptive response suggests that cancer cells express PD-L1 as a protective mechanism after invasion by lymphocytes allowing tumour growth (Taube et al., 2012). This immune control is antigen specific blocking anti-cancer T cells while the host's immune system continues to function correctly for any other antigens (Ribas, 2015a). This mechanism was first used to describe how the production of interferons by T cells upon recognition of their cognate antigen results in the reactive expression of the ligand of PD-1 (PD-L1) by cancer cells and the inactivation of PD-1 expressing T cells (Pardoll, 2012). Secretion of interferon-γ (IFN-γ) by cytotoxic T lymphocytes is known to up-regulate PD-L1 expression in tumour cells from mouse models of various cancer types (Abiko et al., 2015; Blank et al., 2004). This interaction has also been observed in cell lines derived from human tumours, confirming that IFN-γ induces PD-L1 up-regulation in human cancers (Chen et al., 2012; Gao et al., 2018; Gowrishankar et al., 2015).

1.2.4. Immune checkpoint inhibitors

Antibodies targeting immune checkpoint molecules can serve as a means to alter the cell mediated immune response, primarily to enable the targeting of cancerous cells. They function by binding to immune checkpoint molecules or receptors so as to inhibit signalling that would otherwise prevent T cell activation and response. T cell activation allows proliferation, increase cytokine production and other immune processes that lead to the destruction the target cell (Chen and Mellman, 2013).

Anti-PD-1 therapies target and bind to PD-1 receptors, effectively blocking the receptor from binding to either of its ligands, thus preventing immune suppression (Figure 3) (Chen and Mellman, 2013). To date, PD-1/PD-L1 inhibitors have been approved for the treatment of a variety of cancers including melanoma, NSCLC, renal cell carcinoma, bladder cancer, colorectal cancer and squamous cell carcinoma of the head and neck, among some others (Diaz et al., 2017; Le et al., 2015; Motzer et al., 2015; Nghiem et al., 2016; Powles et al., 2014; Topalian et al., 2012). Approved treatments nivolumab and pembrolizumab are both monoclonal antibodies that function in essentially the same way by binding to the PD-1 receptor and disabling its interaction with PD-L1 (Postow et al., 2015). Anti-PD-L1 antibodies, such as atezolizumab, block the same pathway as anti-PD-1 antibodies but exert slightly different effects (Butte et al., 2007). PD-L1 does not only interact with the PD-1 receptor, the molecule also interacts with B7-1 as a secondary means to exert negative signals on T cells (Butte et al., 2007). PD-L1 antibodies also differ from PD-1 antibodies in that they do not inhibit the interaction of PD-1 and PD-L2. However, the importance of this interaction in relation to T cell activity is relatively unknown (Postow et al., 2015).
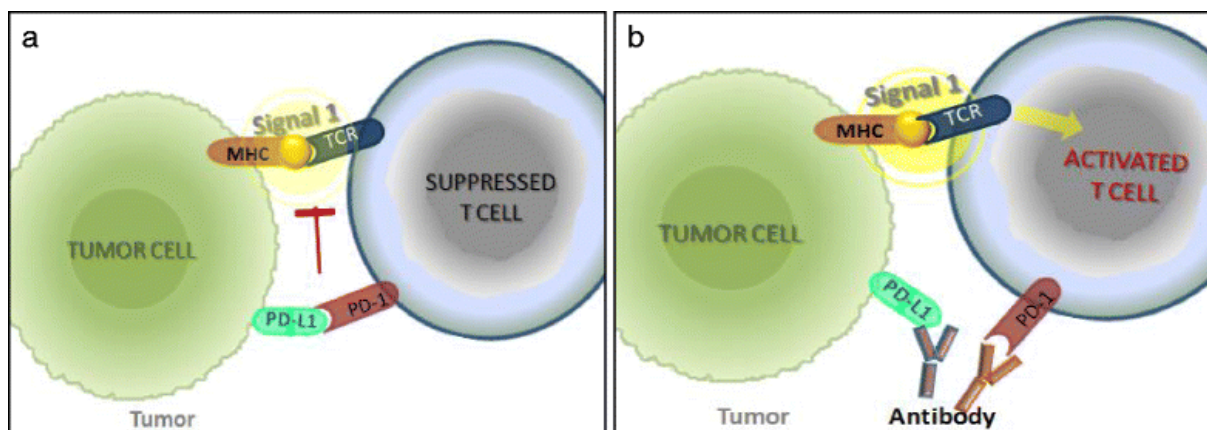
Figure 3. T cell activation in the tumour microenvironment. *a*. PD-1 and PD-L1 interaction activate signalling to inhibit T cell activity, blocking anti-tumour responses. *b*. Antibodies targeting either PD-

1 or PD-L1 block the interaction and allow T cell activation, producing an anti-tumour response (Farkona et al., 2016; Ribas, 2015b).

1.3. Currently approved anti-PD-1 therapies

Several large, randomised, phase III studies conducted on advanced-stage melanoma patients that have been completed in recent years demonstrated therapeutic outcomes in patients treated with anti-PD-1 antibodies (Figure 4). The success of these studies led to the approval of anti-PD-1 antibodies, alone and in combination with anti-CTLA4, by the United States Food and Drug Administration (FDA) for treating melanoma (Hodi et al., 2015; Luke et al., 2017). There are currently two anti-PD-1 antibodies approved for single use in melanoma patients, nivolumab and pembrolizumab (Postow et al., 2015).

Nivolumab was first approved for the treatment of advanced melanoma in 2014, based on the results of CheckMate 066. CheckMate 066 was a phase 3 clinical trial which compared nivolumab to dacarbazine in untreated, unresectable or metastatic melanoma (Robert et al., 2015b). Patents participating in CheckMate 066 were treated with either nivolumab or dacarbazine until disease progression or unacceptable toxicity. At the latest report, a minimum follow-up of 38.4 months for the nivolumab group provided a median overall survival (OS) of 37.5 months, while a minimum follow-up of 38.5 months for the dacarbazine group provided a median OS of 11.2 months. The three year OS rate for nivolumab was also considerably higher than that for dacarbazine, being 51.2% and 21.6% respectively (Ascierto et al., 2019). The median progression free survival (PFS) for the nivolumab and dacarbazine groups were 5.1 months and 2.2 months respectively. Much like OS and PFS, the response rates for the nivolumab group were considerably higher than those for the dacarbazine group. The objective response rate (ORR) for the nivolumab group was 42.9%, with a median duration of response not being reached (38.2-NR). Conversely, the ORR for the dacarbazine group was 14.4%, with a median duration of response being 6 months (3.9-24.3 months) (Ascierto et al., 2019). All of the results from CheckMate 066 conclusively demonstrated the superiority of nivolumab to dacarbazine in untreated, unresectable or metastatic melanoma. Since this first FDA approval, nivolumab has been approved in combination with ipilimumab for treating advanced melanoma (CheckMate 069) (Hodi et al., 2015) and alone as an adjuvant therapy in patients with completely resected melanoma with lymph node involvement or metastatic disease (CheckMate 238) (Weber et al., 2017).

Pembrolizumab was also first approved for the treatment of advanced melanoma in 2014, based on the results of KEYNOTE-002. KEYNOTE-002 was a phase 2 clinical trial which compared pembrolizumab to chemotherapy in patients with advanced melanoma (Ribas et al., 2015). Patients participating in KEYNOTE-002 were randomly assigned to three groups to be treated with either pembrolizumab 2 mg/kg or 10mg/kg every 3 weeks or investigator-choice chemotherapy (paclitaxel plus carboplatin, paclitaxel, carboplatin, dacarbazine, or oral temozolomide) until disease progression or unacceptable toxicity. The 9-month PFS was 24% and 29% for the pembrolizumab 2 mg/kg or 10mg/kg respectively, and 8% for the investigator-choice chemotherapy (Ribas et al., 2015). However, pembrolizumab treatment did not provide a statistically significant improvement in the median OS when compared to chemotherapy. The median OS for the pembrolizumab 2 mg/kg or 10mg/kg groups was 13.4 (11.0-16.4) and 14.7 months (11.3-19.3) respectively, versus 11.0 months (8.9-13.8) for the chemotherapy group. Finally, the ORR for both pembrolizumab 2 mg/kg and 10mg/kg was significantly higher than for chemotherapy, being 22%, 28% and 4% respectively (Hamid et al., 2017). The results of KEYNOTE-002 demonstrated the significant improvements in PFS for advanced melanoma patients when treated with pembrolizumab versus treatment with chemotherapy. Since this first FDA approval of pembrolizumab for the treatment of melanoma, it has received an expanded indication for treating advanced melanoma patients, now being available as a first line treatment. This expanded indication for pembrolizumab was approved based on the results of KEYNOTE-006 (Schachter et al., 2017). FDA approval for pembrolizumab was also further expanded to include adjuvant treatment of melanoma patients with involvement of lymph nodes following complete resection due to the results from KEYNOTE-054 (Eggermont et al., 2018).

For NSCLC, much like in melanoma, immune checkpoint blockade has been shown as an effective treatment. Since 2010 there have been a number of anti-PD-1 studies involving or focusing on NSCLC patients (Figure 4) (Somasundaram et al., 2016). These studies resulted in the approval of PD-1 checkpoint blockade antibodies nivolumab and pembrolizumab to treat NSCLC.

Nivolumab was first approved for the treatment of NSCLC in 2015 based on the results from CheckMate 017. CheckMate 017 was a phase 3 clinical trial which compared nivolumab to docetaxel in patients with previously treated advanced or metastatic squamous NSCLC (Brahmer et al., 2015). Patients participating in CheckMate 017 were treated with either

nivolumab or docetaxel until progression or unacceptable toxicity. The study reported a minimum follow up of 11 months, with a median OS of 9.2 months in the nivolumab group and 6 months in the docetaxel group. The OS for the nivolumab group was significantly higher after one year, being 42% versus the 24% OS for the docetaxel group. Median PFS was also higher for the nivolumab group, being 3.5 months compared to 2.8 months for the docetaxel group. The ORR was significantly higher for the nivolumab group, with 20% of patients responding compared to the 9% response rate for the docetaxel group. Finally, the median duration of response was not reached for the nivolumab group, being 2.9 to 20.5+ months, compared to the significantly shorter 8.4 month median for the docetaxel group (1.4 to 15.2+) (Brahmer et al., 2015). Following this first approval of nivolumab for the treatment of NSCLC, the FDA expanded the approval to include non-squamous NSCLC based on the results from CheckMate 057 (Borghaei et al., 2015).

Pembrolizumab was first approved for the treatment of NSCLC in 2015 based on the results from KEYNOTE-001 and KEYNOTE-010. KEYNOTE-001 was a phase 1 clinical trial assessing pembrolizumab in patients with locally advanced or metastatic carcinoma, melanoma or NSCLC (Robert et al., 2014). KEYNOTE-010 was a phase 2 clinical trial comparing pembrolizumab to docetaxel in previously treated participants with NSCLC (Herbst et al., 2016). Patients participating in KEYNOTE-010 were randomly assigned to one of three groups to be treated with either pembrolizumab 2 mg/kg or 10mg/kg every 3 weeks or docetaxel, until disease progression or unacceptable toxicity. The median OS for the pembrolizumab 2 mg/kg or 10mg/kg groups was higher than for the docetaxel group, being 10.4 (9.4 – 11.9), 12.7 (10.0 – 17.3) and 8.5 months (7.5 – 9.8) respectively. PFS was not significantly different between docetaxel and either the pembrolizumab 2 mg/kg or 10mg/kg groups for the total patient population. The ORR for patients treated with pembrolizumab was double that of those treated with docetaxel, being 18% for both dosage groups compared to 9% for the docetaxel group. Patients participating in KEYNOTE-010 also had tumour tissue assessed for PD-L1 expression using the 22C3 pharmDx immunohistochemistry assay. Patients were split into two sub-groups for further assessment, those with PD-L1 expression in at least 50% of tumour cells vs those with less than 50% of tumour cells expressing PD-L1. When performing the same analyses for the ≥ 50% tumour PD-L1 group as were done for the total population, there were larger differences in OS, PFS and ORR between the pembrolizumab treated groups and the

docetaxel group. These results demonstrated that response to pembrolizumab was improved for patients with ≥ 50% of tumour cells expressing PD-L1 (Herbst et al., 2016).

Since the first approval of pembrolizumab for the treatment of NSCLC, there have been multiple expansions of the approved treatment. These include expansions in approval as a first line treatment based on the results of KEYNOTE-24 (Reck et al., 2016) and KEYNOTE-042 (Mok et al., 2019). Pembrolizumab has also been approved in combination with pemetrexed and carboplatin, pemetrexed and platinum chemotherapy or carboplatin and either paclitaxel/nab-paclitaxel based on the results of KEYNOTE-021 (Langer et al., 2016), KEYNOTE-189 (Sheela et al., 2018) and KEYNOTE-407 (Paz-Ares et al., 2018) respectively.
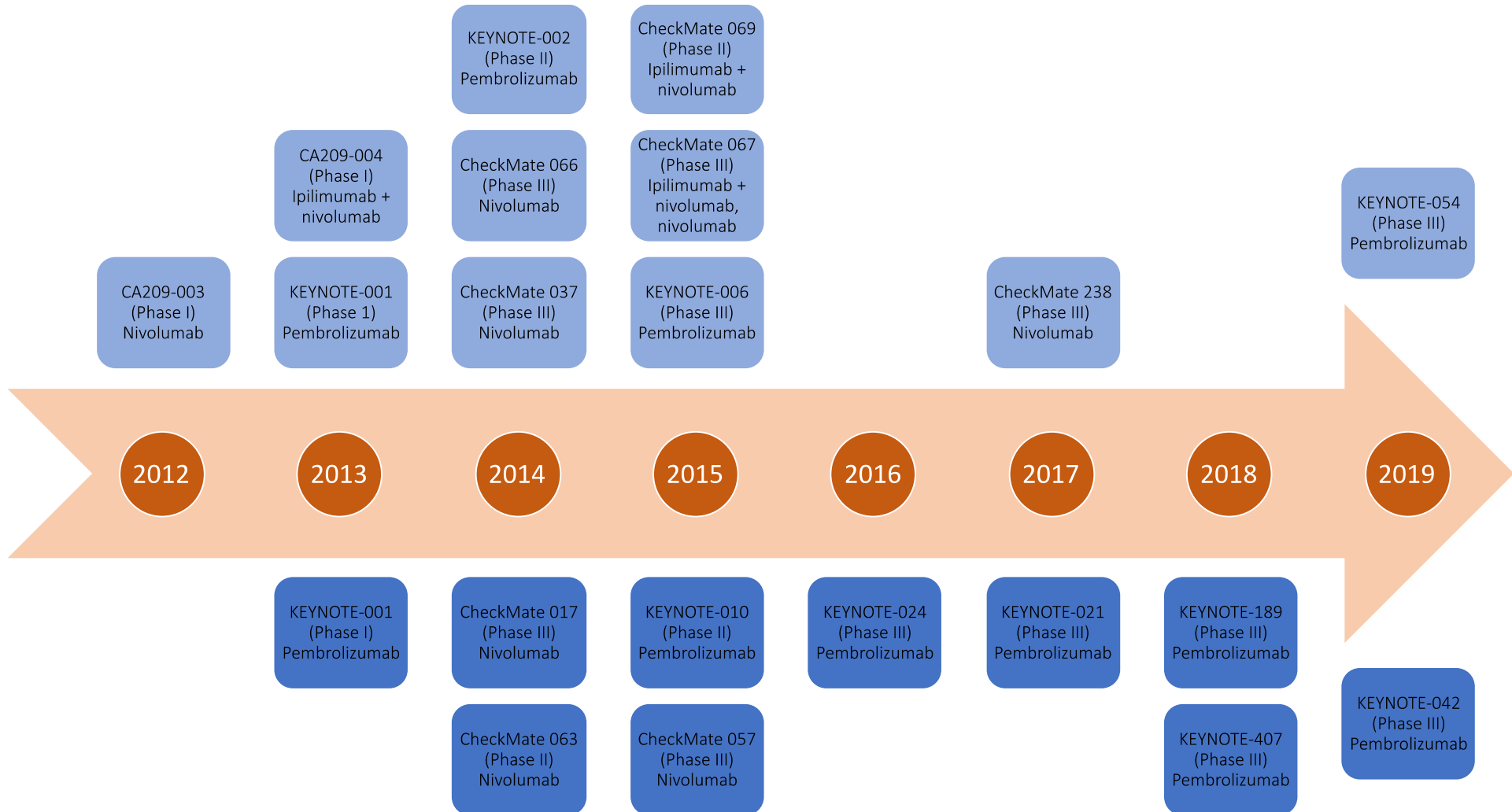
Figure 4. *Timeline charting the seminal, practice-changing clinical trials of anti-PD-1 antibodies in advanced-stage melanoma (top) and NSCLC (bottom).*

There have also been a number of clinical trials involving melanoma and NSCLC patients assessing anti-PD-L1 antibodies. Several early clinical trials using anti-PD-L1 antibodies, such as NCT01375842, which evaluated safety, tolerability and pharmacokinetics of atezolizumab in participants with locally advanced or metastatic solid tumours, demonstrated the efficacy of atezolizumab (Herbst et al., 2014). NCT01375842 demonstrated that atezolizumab produced an ORR of 26 % in melanoma patients and 21% in NSCLC patients (Herbst et al., 2014). Based on the following OAK trial, atezolizumab was approved as a treatment for multiple cancers, including NSCLC provided other treatment options have previously failed (Rittmeyer et al., 2017).

Other anti-PD-L1 antibodies have reached clinical development in recent years, including the monoclonal antibody MDX-11-5 which was shown to be capable of producing durable responses in a variety of cancers (Brahmer et al., 2012). The phase 1 clinical trial NCT00729664 demonstrated that MDX-11-5 had an ORR of 17% with a 24-week PFS of 42% for melanoma patients, while NSCLC patients had an ORR of 10% with a 24 week PFS of 31% (Brahmer et al., 2012). There are, however, no anti-PD-L1 antibodies that have been approved for the treatment of melanoma (Postow et al., 2015).

1.3.1. Side effects

Coinciding with their potential to cause tumour regression, immune checkpoint inhibitors may also lead to a multitude of adverse effects, including autoimmune responses (Eigentler et al., 2016; Phan et al., 2003). Immune checkpoint inhibitors are distinctly different in the type of toxicity that they produce when compared to cytotoxic or targeted agents (Robert et al., 2011). Immune related adverse events are the main form of toxicity for immune checkpoint inhibitors several cancer types, with up to 90% of anti-CTLA-4 treated patients, and up to 70% of anti-PD-1 treated patients being affected by adverse events (Hodi et al., 2010; Topalian et al., 2012). Immune related adverse events are graded on a scale of one to five using the Common Terminology Criteria for Adverse Events, with grades one through five being classified as mild, moderate, severe, life threatening and death related to toxicity respectively (Institute, 2009). Skin manifestations are the most common adverse effects related to CTLA-4, PD-1 and PD-L1 immune checkpoint blockade in melanoma patients (Figure 5); histological analysis of skin from adversely affected patients often shows deep

perivascular lymphocytic infiltration in the dermis with CD4+ and CD8+ cells in close proximity to melanocytes (Jaber et al., 2006).
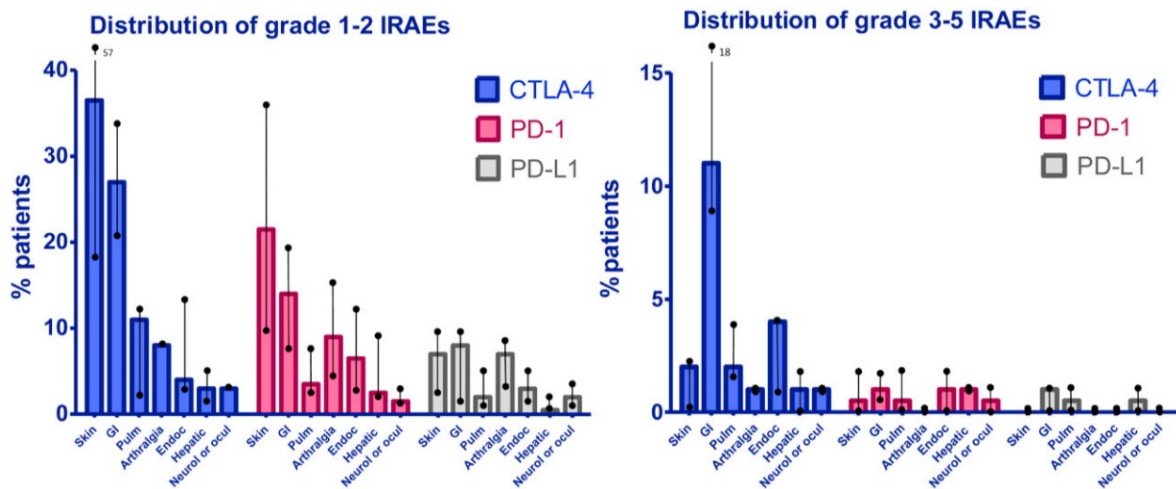
Figure 5. Distribution of grade I-II and grade III-V immune related adverse events for all tumour types in the main clinical trials using anti-CTLA-4, anti-PD-1 or anti-PD-L1 as single therapies. Bar graphs depict the percent of patients experiencing adverse events with values quoted for the median (range). Adapted from (Michot et al., 2016).

The most common adverse effects associated with anti-PD-1 treatment include diarrhoea, rash, pruritus and fatigue (Michot et al., 2016; Robert et al., 2015b). Other, less common but potentially more serious, adverse effects that can be produced by immune responses associated with anti-PD-1 therapy include, but are not limited to, hypothyroidism, hyperthyroidism and colitis (Robert et al., 2015b). Severe or prolonged treatment-related adverse effects result in discontinuation of treatment with anti-PD-1 antibodies despite active response in four to twenty-five percent of patients depending on the treatment regime (Robert et al., 2017; Robert et al., 2015b).

While the combination of anti-PD-1 and anti-CTLA-4 therapies has a higher objective response rate than either alone as a monotherapy in melanoma patients, the incidence of high grade immune related adverse events also increases (Figure 6). In the Checkmate 067 phase III clinical trial the majority of patients experienced some level of treatment related adverse event, with 82.1%, 86.2% and 95.5% of patients affected from the nivolumab, ipilimumab and nivolumab + ipilimumab groups respectively (Larkin et al., 2015). The number of grade 3 or 4 treatment related adverse events was also raised, effecting 55% of the nivolumab + ipilimumab group compared to 16.3% and 27.3% of the nivolumab and ipilimumab groups respectively. Finally, cessation of treatment due to severe treatment related adverse events occurred in 7.7%, 14.8% and 36.4% of patients from the nivolumab,

ipilimumab and nivolumab + ipilimumab groups respectively (Larkin et al., 2015). These results show that combination therapy increases the severity of immune related adverse events for all tumour types.
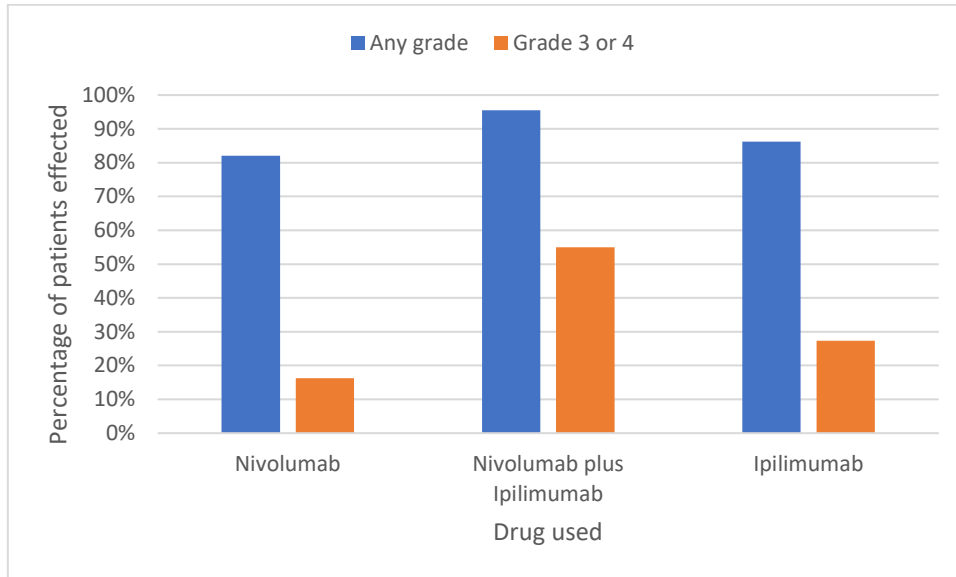
Figure 6. *Treatment related adverse events associated with nivolumab, nivolumab plus ipilimumab and ipilimumab treatments in melanoma patients, adapted from (Larkin et al., 2015).*

Much like other immune checkpoint inhibitors, treatment related adverse events are experienced by a number of patients undergoing anti-PD-L1 therapy. The OAK clinical trial that led to the approval of Atezolizumab for treatment of NSCLC patients, among others, reported that 64% of patients (390 of 609) experienced treatment related adverse events. Far fewer patients, 15% (90 of 609), experienced grade 3-4 treatment related adverse events (Rittmeyer et al., 2017).

1.4. Biomarkers of response to anti-PD-1 therapy

1.4.1. Biomarkers in research

As stated above, immune checkpoint inhibitors have rapidly become a key treatment option in advanced stage melanoma and NSCLC patients. Anti-PD-1 therapy in particular has shown significant promise, affecting a durable response in 26% - 40% of patients (Ribas et al., 2016; Robert et al., 2015a; Robert et al., 2015b; Topalian et al., 2014). However, toxicities and adverse events associated with the treatment, combined with the response rate, have resulted in the search for a biomarker to identify responders prior to or early during treatment.

The identification of potential biomarkers in melanoma patients has seen moderate success when assessing tumour tissue through a variety of means. A slew of studies have displayed varying degrees of correlation between response and potential biomarkers including high mutational load (Hugo et al., 2016), high neoantigen load (McGranahan et al., 2016), immune gene expression signature (Hugo et al., 2016) and high levels of CD8+ T cell infiltrate (Weber et al., 2013) among others (Figure 7). Circulating proteins demonstrating diagnostic and prognostic value include S100B, C reactive protein and melanoma-inhibiting activity protein (Vereecken et al., 2012). Blood-based biomarkers showing correlation with response to anti-PD-1 therapy include T cell receptor diversity (Tumeh et al., 2014), Bim-expressing T cells (Dronca et al., 2016) and plasma soluble PD-L1 (Zhou et al., 2017). PD-L1 expression in tumour tissue is currently the most studied potential biomarker, and also the most predictive biomarker, of response to anti-PD-1 therapy in advanced stage melanoma (Jessurun et al., 2017).
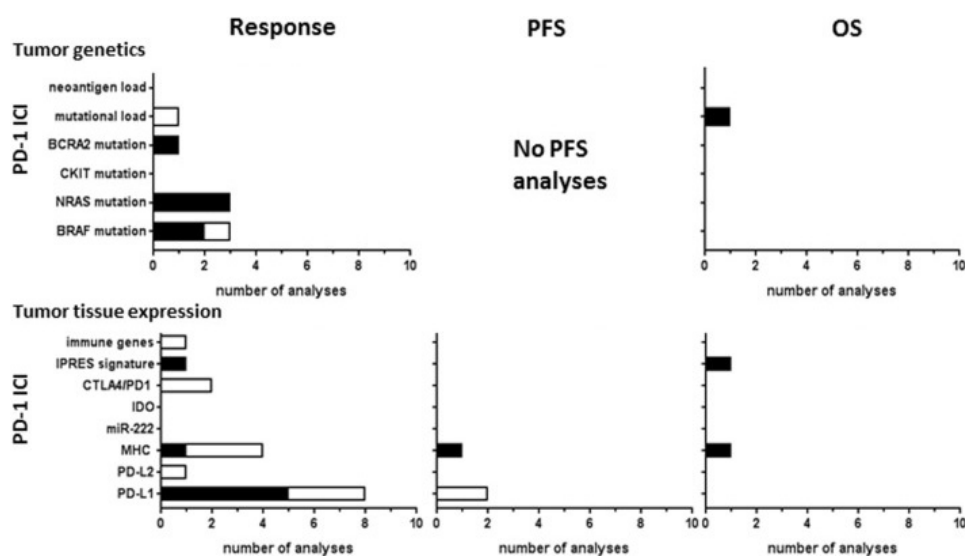
Figure 7. Biomarkers of response to PD-1 immune checkpoint inhibitor (ICI) in melanoma tumour tissue. Graphs show the number of studies reported per biomarker type for correlations with clinical response, progression-free survival (PFS), or overall survival (OS) upon PD-1 ICI therapy. Black bars indicate significant correlation, white bars indicate non-significant correlation. Adapted from Jessurun et al 2017 (Jessurun et al., 2017).

For NSCLC, unlike melanoma, there are multiple other treatment options that may offer prolonged clinical benefit (Carmichael et al., 2018). Other potential first line treatments that are often considered for treating NSCLC include chemotherapy (Schiller et al., 2002), epidermal growth factor receptor blockers (Shepherd et al., 2005), vascular endothelial growth factor inhibitors (Reck et al., 2009) and ALK blockers (Camidge et al., 2012). Therefore, it is critical to choose therapies to which the patient is likely to respond. This has

led to a significant number of studies assessing potential biomarkers of response to anti-PD-1 treatment to enable identification of NSCLC responders that can receive anti-PD-1 as a first-line treatment. The potential biomarkers being studied in NSCLC include antigen-specific T cells (Dhodapkar et al., 2013), the mutational load of the tumour (Rizvi et al., 2015) and loss of mutation-associated neoantigens (Anagnostou et al., 2017). However, PD-L1 expression in tumour tissue is the only biomarker for response to anti-PD-1 therapy that has been approved by the FDA as a companion or diagnostic test to decide treatment with pembrolizumab as first line therapy (Teixido et al., 2018).

1.4.2. PD-L1 as a biomarker

The first evidence suggesting the potential of PD-L1 as a predictive biomarker of response to anti-PD-1 therapy was produced by the first human phase I nivolumab trial, conducted in patients with varying solid tumour types. The results of this study showed that patients with PD-L1 expression at the cell surface were likely to respond to anti-PD-1 therapy whereas all patients that were not positive for PD-L1 expression at the cell surface failed to respond to treatment (Brahmer et al., 2010). However, the sample size of the study was small, a total of 9 biopsies were tested for PD-L1 expression, and so this result would require further research before it could be validated. Several subsequent trials have assessed PD-L1 expression in tumour biopsies, producing similar results and supporting the potential of PD-L1 as a predictive biomarker. However, none of these follow up clinical trials have managed to demonstrate a response rate as high as the initial trial. One of these follow up clinical trials was the phase I study of nivolumab in subjects with advanced or recurrent malignancies, which demonstrated that PD-L1 expression in tumour biopsies correlated with clinical benefit to the anti-PD-1 therapy but did not significantly correlate with objective response (Topalian et al., 2012).

Several other studies assessing PD-L1 expression in the tumour tissue of melanoma patients have linked expression to treatment response. However, these studies have been inconsistent in demonstrating a significant correlation between expression and treatment response (Daud et al., 2016; Kaunitz et al., 2017; Topalian et al., 2012). A study of pembrolizumab in participants with progressive locally advanced or metastatic carcinoma, melanoma, or NSCLC also demonstrated that melanoma patients with higher levels of PD-L1 expression were more likely to respond to treatment. PD-L1 expression was scored on a

0-5 scale by the percentage of cells staining positive, 0 being 0% and 5 being ≥ 66% (Figure 8). Objective response rates to pembrolizumab were calculated individually for each group as 8%, 12%, 21%, 40%, 57% and 50%, for patients with scores 0-5 respectively (Daud et al., 2016).
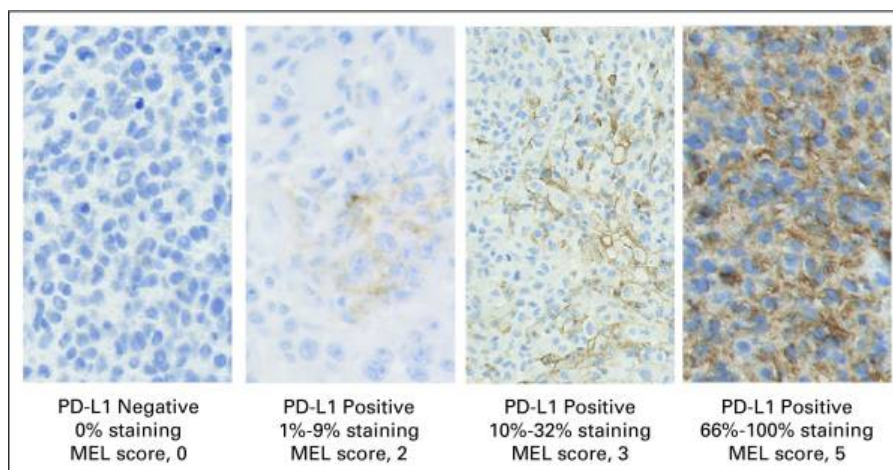
Figure 8. Photomicrographs depicting immunohistochemical staining of PD-L1 (22C3 antibody) in melanoma (MEL) samples. PD-L1 staining seen as brown chromogen, whereas blue is haematoxylin counterstain (Daud et al., 2016).

PD-L1 expression is highly variable between separate melanoma subtypes, whilst following a general trend within the subtypes. This has been demonstrated by a study using immunocytochemistry to stain for PD-L1 in patients with rare melanoma subtypes. This study found that PD-L1 is more highly expressed in chronic sun damaged melanomas such as lentigo maligna, which has a higher response rate to anti-PD-1 therapy, and has lower expression in uveal melanoma, which has lower response rate to anti-PD-1 therapy, when comparing each to cutaneous melanoma (Kaunitz et al., 2017). The tumour mutational burden (TMB) of each of these melanoma subtypes also parallels the PD-L1 expression and response rate to anti-PD-1 therapy, with chronic sun damage melanomas having the highest TMB and uveal having the lowest (Kaunitz et al., 2017). This study is significant as the results provide a potential factor contributing to the different objective response rates for each melanoma subtype. The general conclusion reached by these studies is that PD-L1 expression in tumour tissue does correlate with response rate, with the caveat that it is still possible for some PD-L1 negative patients to respond to treatment (Daud et al., 2016; Powles et al., 2014; Topalian et al., 2012).

PD-L1 expression in NSCLC tumour tissue has been studied extensively to determine its potential as a biomarker for anti-PD-1 therapies. Although PD-L1 expression in tumour

tissue was integral to the approval of pembrolizumab as a first line treatment for NSCLC, 15% of PD-L1 negative patients achieve an objective response to treatment (Garon et al., 2015). Nonetheless, pembrolizumab is only approved by the FDA and Australian Therapeutic Goods Administration as a first line treatment in EGFR and ALK negative patients given a positive result from a companion diagnostic test assessing PD-L1 expression, specifically staining with the 22C3 pharmDx assay (Teixido et al., 2018). A positive result from the 22C3 pharmDx assay was previously listed as a tumour proportion score of ≥ 50% (Pai-Scherf et al., 2017), with this tumour proportion score being observed in 35% of patients (Garon et al., 2015). The 22C3 monoclonal antibody was approved as the companion diagnostic test for pembrolizumab due to the results of two large phase III clinical trials, KEYNOTE-010 and KEYNOTE-024 (Herbst et al., 2016; Reck et al., 2016). KEYNOTE-010 and KEYNOTE-024 studied responses to pembrolizumab in PD-L1 positive patients, based on the 22C3 pharmDx assay, as a first line treatment (KEYNOTE-024) and a second line treatment (KEYNOTE-010). Both clinical trials found that pembrolizumab prolonged overall survival with minimal risk, establishing the antibody as a suitable new treatment option (Herbst et al., 2016; Reck et al., 2016). However, since then, a positive result from the 22C3 pharmDx assay has been adjusted to ≥ 1% based on the results from KEYNOTE-042 (Lopes et al., 2018). KEYNOTE-042 was an open-label phase 3 study comparing pembrolizumab versus platinum-based chemotherapy as first-line therapy for advanced/metastatic NSCLC with a PD-L1 tumour proportion score (TPS) ≥ 1%. The results of KEYNOTE-042 demonstrated that pembrolizumab provided higher overall survival than platinum-based chemotherapy for all patients with a TPS ≥ 1% (Lopes et al., 2018).

The FDA has also approved 28.8 pharmDx as a diagnostic test for PD-L1 expression, for treatment with nivolumab as a second line therapy following platinum-based chemotherapy of NSCLC, although it has been listed as a nonessential complementary diagnostic test (Kazandjian et al., 2016). The Checkmate 057 clinical trial that led to the approval of nivolumab as a second line therapy found that PD-L1 expression, as determined by 28-8 pharmDx, was an appropriate biomarker (Kazandjian et al., 2016). The approval and utilisation of PD-L1 assays as diagnostic tests by the FDA demonstrates that PD-L1 expression in tumour tissue is a directly relevant biomarker for anti-PD-1 therapy in NSCLC.

1.5. Circulating tumour cells

CTCs were first observed and described in 1869 by Australian clinician Thomas Ashworth (Ashworth, 1869). Since then, a multitude of clinical studies involving CTCs have been conducted thanks to advances in the detection and isolation of the cells in more recent years (Adams et al., 2017; Alix-Panabieres and Pantel, 2014; Dhar et al., 2018; Guibert et al., 2018; Ilié et al., 2017; Joosse et al., 2015; Krebs et al., 2010). CTCs are found in miniscule concentrations in the blood, there generally being between 1 – 10 cells in 10mL of blood, but this can be cancer-type dependant (Alix-Panabieres and Pantel, 2014). CTCs are thought to arise by detaching of cancer cells from the tumour mass and entering the blood stream. CTCs can leave the bloodstream and enter organs or tissues, leading to the development of new metastatic tumours (Figure 9) (Friedl and Alexander, 2011; Nguyen et al., 2009). Not all CTCs develop into metastases, a significant number are destroyed by the immune system or fail to survive without interaction with the extracellular and surrounding cellular matrix (Cohen et al., 2008). It is also unclear what proportion of CTCs have the capacity to seed new tumours (metastatic potential) based on whether they display cancer stem cell properties (Krebs et al., 2010).
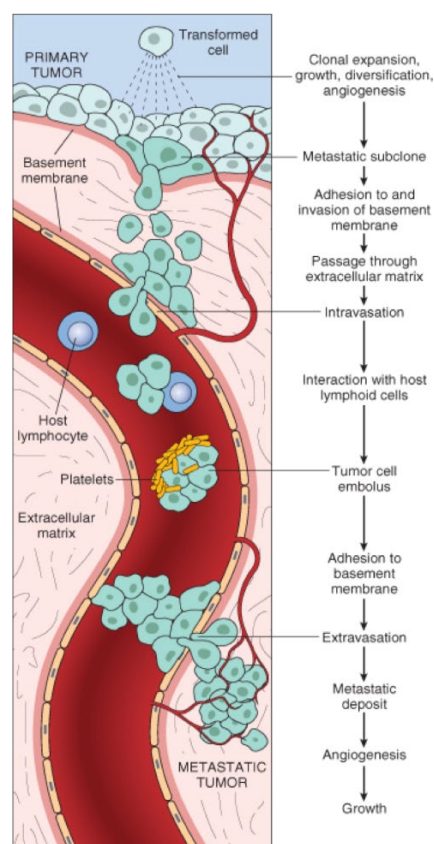
Figure 9. CTC extravasation and intravasation of blood vessels to form a metastasis (Cotran, 1991).

1.5.1. CTC detection and enrichment techniques

The incredibly low number of CTCs, when compared to other blood cells, poses a significant challenge when trying to isolate them from the blood. Techniques to isolate CTCs therefore must be capable of differentiating them from other haematogenous cells. Isolation is achieved by utilising techniques that identify the differences between CTCs and haematogenous cells, such as physical or biological properties exhibited by the cells (Joosse et al., 2015). The primary biological property utilised for CTC isolation is the expression of proteins on the cell surface. In contrast, there are several physical properties that may be used to isolate CTCs including but not limited to cell size, cell density and the electric charge of the cell (Yu et al., 2011). Therefore, there are two general methods used for the isolation of CTCs, label-dependent methods and label-free methods (Joosse et al., 2015; Marsavela et al., 2018).

Label dependent methods isolate CTCs from the blood by using antibodies that target tumour-associated antigens or common leukocyte antigens such as CD45. Immunomagnetic systems target unique CTC cell surface antigens with antibodies bound to magnetic beads. The beads, along with the attached cell, can then be isolated by exposing the mixture to a magnetic field (Alix-Panabieres and Pantel, 2014). An example of this type of system is the FDA approved CellSearch system, which utilises magnetic beads that target epithelial cell adhesion molecule (EpCAM) to isolate CTCs from epithelial tumours such as NSCLC (Pantel et al., 2009). There are a variety of CTC isolation systems that target EpCAM, including CTC-chip (Sequist et al., 2009), herringbone-chip (Stott et al., 2010) and MagSweeper (Talasaz et al., 2009). All of these systems utilise EpCAM to target and isolate CTCs but use different means to do so (Alix-Panabieres and Pantel, 2014).

Whilst EpCAM targeting systems are suitable for a variety of carcinomas such as NSCLC, they are not effective for the isolation of CTCs for non-epithelial cancers like melanoma. Label-dependent melanoma CTC isolation therefore requires the use different cell surface markers, or the use of negative selection techniques. There has been difficulty in determining the best markers for the isolation of melanoma CTCs due to their heterogeneity (Gray et al., 2015). There are several markers that have been used individually to isolate melanoma CTCs including MCSP, MCAM, ABCB5 and CD271 (Freeman et al., 2012; Gray et al., 2015; Khoja et al., 2014). However, it has been demonstrated that utilising multiple markers can improve the sensitivity when compared to individual marker assays (Freeman et al., 2012). On the other hand, negative selection isolates CTCs by removing leukocytes

through the targeting of CD45, leaving CTCs and a background of white blood cells (WBCs) (Joshi et al., 2014).

Label-free methods provide another option for CTC isolation by separating cells based on their physical properties. These techniques often target cells based on their size, deformability and density as CTCs are predominantly larger, stiffer and less dense than leukocytes (Alix-Panabieres and Pantel, 2014; Aya-Bonilla et al., 2017). As with label-dependent isolation methods, there are a variety of different label-free isolation systems that use different techniques to separate the CTCs form the blood. Two of such systems for the label-free detection of CTCs are the ClearCell FX system (Clearbridge BioMedics) and the Parsortix system (Angle plc).

The ClearCell FX system is a microfluidic device that utilises a spiral chip to isolate CTCs based on size using centrifugal forces. When pumped through the spiral chip smaller cells migrate to the outer wall while larger cells, such as CTCs, move to the inner wall. The separate cell colonies are collected into different outlets after passing through the spiral, providing isolated CTCs (Figure 10) (Chudasama et al., 2016; Hou et al., 2013).
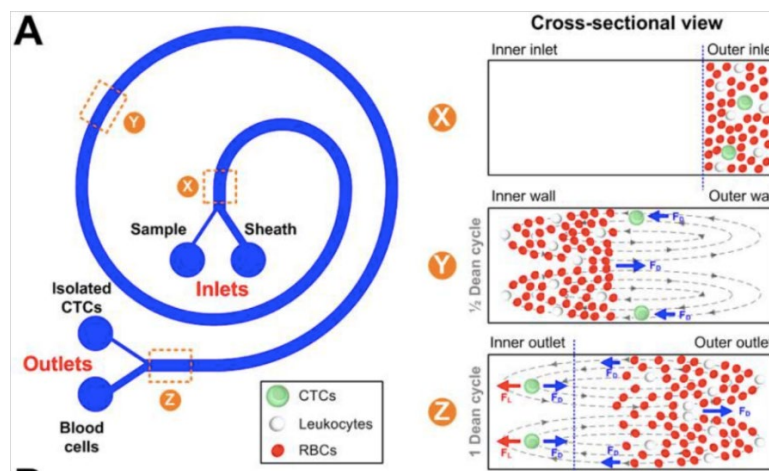
Figure 10. *Diagram depicting the inner workings of a ClearCell machine, Adapted from (Hou et al., 2013).*

The Parsortix system is a microfluidic device that separates CTCs using a series of steps over which cells are forced, leading to a final gap of 10μm. Leukocytes and erythrocytes pass through the gap and are flushed out of the system while the larger, rigid CTCs are captured and harvested at the end of the process (Figure 11) (Xu et al., 2015).
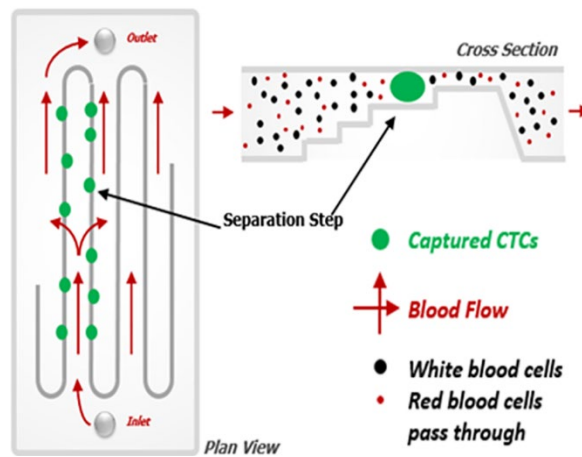
Figure 11. *Diagram depicting the inner workings of the Parsortix machine (Xu et al., 2015).*

No matter which method of CTC enrichment is used, there will still be a number of leukocytes remaining in the sample. It is therefore necessary to be able to detect CTCs and differentiate them from leukocytes, allowing the identification and visualisation of individual CTCs (Pantel and Alix-Panabieres, 2012). As with the isolation techniques, there are a variety of different methods that can be used to distinguish CTCs from leukocytes. Cells are often stained via immunocytochemistry techniques, using antibodies known to be specific to markers expressed on cancer cells. A cocktail of melanoma markers can be used to identify melanoma CTCs (Aya-Bonilla et al., 2017), while cytokeratin and EpCAM can be used to identify CTCs from carcinomas such as NSCLC (Adams et al., 2017; Kallergi et al., 2018; Nicolazzo et al., 2016). Cells are considered to be CTCs if they positively stain for tissue of origin, tumour-cell specific markers while remaining negative for CD45, which should only be found on leukocytes and granulocytes (Joosse et al., 2015; Khoja et al., 2014).

1.6. Expression of PD-L1 on CTCs

As stated above, PD-L1 expression in tumour biopsies is correlated with response to anti-PD-1 therapy. However, to identify PD-L1 on patient tissue, biopsies are taken from a single tumour, at a single time point. This means that biopsies are not able to account for changes in the tumour over time, or heterogeneity between tumours. Both of these problems could potentially be addressed by analysing the expression of PD-L1 on CTCs. Due to the fact that they arise from tumours, it is believed that CTCs are likely to share the same immune escape mechanisms, such as PD-L1 expression (Nicolazzo et al., 2016). Moreover, since CTCs may arise from any or all current tumours in a patient at any one time, they are more likely to be representative of the tumours at a given time point (Figure 12).
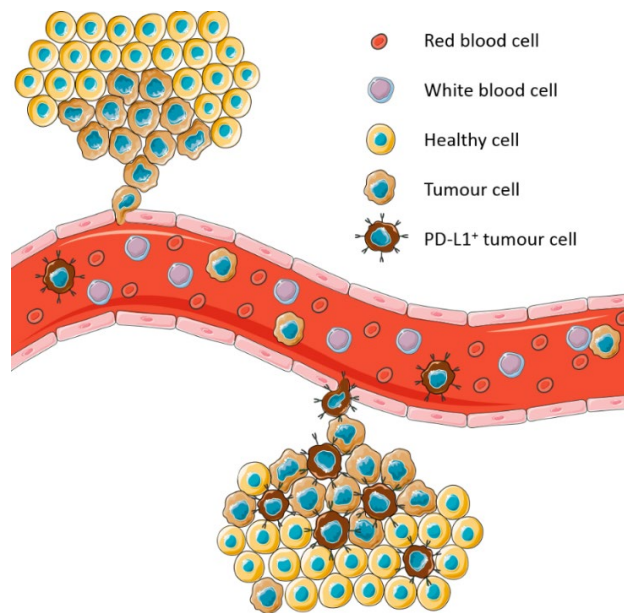
Figure 12. Model of expression of programmed cell death ligand-1 (PD-L1) on circulating tumour cells (CTCs) derived from heterogeneous tumours. CTCs represent the sum of all tumours in a patient at the time of blood collection.

1.6.1. PD-L1 expression on NSCLC CTCs

A total of nine studies addressing the expression of PD-L1 on CTCs have been published to date. Among these nine publications, the number of patients with CTCs (referred herein as CTC detection rate) varied between 22% and 100% at both baseline and after front-line therapy. Several different methods were employed for the isolation and detection of CTCs, making it difficult to compare the results between studies. Three studies employed the size of epithelial tumour cells (ISET) platform (Guibert et al., 2018; Ilié et al., 2017; Kallergi et al., 2018), while all other studies employed different types of methodologies such as the CellSearch System (Nicolazzo et al., 2016), the Epic CTC platform (Boffa et al., 2017), Vortex high throughput (HT) technology (Dhar et al., 2018), graphene oxide (GO) Chip (Wang et al., 2019), spiral microfluidic technology (Kulasinghe et al., 2019), and the CellSieve Microfiltration Assay (Adams et al., 2017) (Table 1). Despite differences in CTC detection rates and methodologies, these studies indicate that the detection of CTCs provides potential for predictive assessment in advanced NSCLC, in agreement with previous literature (Normanno et al., 2016).

All studies reported on the presence of PD-L1 expressing CTCs in NSCLC patients, with detection rates ranging between 2.0% and 96.8%. Three studies evaluated PD-L1+ CTC in patients undergoing anti-PD-1 therapy (Dhar et al., 2018; Guibert et al., 2018; Nicolazzo et al., 2016), with only two of them evaluating whether PD-L1 expression on CTCs can serve as

a predictive biomarker of response (Dhar et al., 2018; Guibert et al., 2018). Four studies evaluated the dynamics of PD-L1 expression on CTCs prior to treatment and after treatment initiation (Adams et al., 2017; Kallergi et al., 2018; Nicolazzo et al., 2016). However, only two studies compared the expression of PD-L1 on CTCs with that on matching tumour tissue (Guibert et al., 2018; Ilié et al., 2017). All but one study was single centred, conducted in non-metastatic NSCLC patients with cohort sizes varying from a total of 13 to 112 NSCLC patients (Table 1).

Table 1. *Prospective studies evaluating programmed cell death ligand-1 (PD-L1) on circulating tumour cells (CTCs) in NSCLC.*

Reference (Sample Size)	Detection Method	Therapy	Stage	Tumour Tested for PD-L1 Expression	Time Point of Blood Draw/ Method of Staining	Criteria for CTC Identification	Detection Rates of CTCs	Detection Rates of PD-L1+ CTCs	Anti-PD-L1 Antibody	Prognostic/Predictive (Cut-Off Value)
Nicolazzo et al., 2016 (Nicolazzo et al., 2016) (n = 24)	CellSearch	Nivolumab	Stage IV NSCLC	No	Baseline 3 months 6 months IF	CK+/EPCAM+/DAPI+ and PD-L1±	20/24 (83.0%) 10/15 (66.7%) 10/10 100.0%	19/20 (95.0%) 10/15 (66.7%) 10/10 (100%)	130021 (R&D system)	NE
*Boffa et al., 2017 (Boffa et al., 2017) (n = 112)	Epic Sciences CTC detection plates	Treatment-naïve	Stage I–IV NSCLC	No	Treatment naïve IF	CK+/CD45−/DAPI+ /PD-L1+	112/112 (100%)	26/112 (23.0%)	EIL3N (Cell Signalling Technology)	Prognostic (>1.1 PD-L1+ CTC/mL) [HR = 3.85 (1.64–9.09); p = 0.002]
Ilie et al., 2017 (Ilié et al., 2017) (n = 106)	ISET platform; Rare cells	99-Chemotherapy naïve and 7-neoadjuvant chemotherapy	Stage III–IV	Yes	Baseline IF	Circulating non-haematological cells with malignant features (CNHC-MF]	80/106 (75%)	6/71 (8.0%)	SP142 (Ventana)	Not Prognostic (no PD-L1+ CTC/mL) PFS: HR: 1.452, (0.82–2.58), and stage-adjusted HR: 1.36, (0.77–2.42)]. OS: HR: 1.55, (0.7–3.470), and stage-adjusted HR: 1.42, (0.63–3.31)].
Adams et al., 2017 (Adams et al., 2017) (n = 41)	Cell Sieve Microfiltration Assay	Chemotherapy Radiotherapy	Stage I–IV NSCLC	Yes	Baseline (T0) Post-induction of Radiotherapy (T1) IF	CK+/EPCAM+/DAPI+	35/41 (85.0%) 41/41 (100%)	35/41 (85.0%) 41/41 100%	130021 (R&D system) for CTCs 22C3 and 28-8 (DAKO pharmDX) for tissue biopsies	Not Prognostic (≥2 PD-L1+ CTC/mL) At T0, PFS: HR: 1.8, p = 0.305 At T1, PFS: HR: 0.7, p = 0.581
Guibert et al., 2018 (Guibert et al., 2018) (n = 96)	ISET platform; Rare cells	Nivolumab	Stage IV NSCLC	Yes	Baseline Post cycle 1 IF	DAPI+/CD45−/Cytomorphometric malignant features	89/96 (93.0%) 23/24 (95.8%)	74/89 (83.0%) 23/23 (100%)	EIL3N (Cell Signalling Technology)	Not Predictive At ≥ 1% PD-L1+ CTC/mL; HR = 1.21(0.64–2.27), p = 0.55 At ≥ 5% PD-L1+ CTC/mL; HR = 1.05(0.59–1.88), p = 0.55] At ≥ 5% PD-L1+ CTC/mL; HR = 0.75(0.45–1.25), p = 0.27
Dhar et al., 2018 (Dhar et al., 2018) (n = 21)	Vortex HT Technology	Nivolumab/Pembrolizumab	Stage IV NSCLC	Yes	Baseline IF	CK+/DAPI+/CD45− or CK+/DAP+/CD45−/cytopathological features	14/31 (45.2%)	12/14 (85.7%)	4059 (ProSci Inc.)	Not predictive (≥2 PD-L1+ CTC/mL) PFS: HR = 0.83 (0.24–2.84, p = 0.764)
Kallergi et al., 2018 (Kallergi et al., 2018) (n = 30)	ISET platform; Rare cells	Chemotherapy-naïve	Stage IV NSCLC	No	Baseline Post cycle 1 Giemsa / Baseline Post cycle 1 IF	CK+/CD45−/PD-L1+	28/30 (93.3%) 9/11 (81.8%) / 17/30 (56/7%) 8/11 (72.7%)	8/30 (26.7%) 5/11 (45.5%)	EH 12.2H7 (Biolegend) B7-H1/PD-L1/CD274 (Novus Biological)	NE
Wang et al., 2019 (Wang et al., 2019) (n = 13)	GO chip	5-radiation 8-chemoradiation	Stage I–III	No	Baseline (visit 1) Visit 2 Visit 3 IF	CK+/CD45−/DAPI+	13/13 (100%) 12/13 (92.3%) 13/13(100%)	6/13 (46.2%) 10/13 (76.9%) 6/13 (46.2%)	329702 (BioLegend)	PFS analysed (≥5% PD-L1+ CTC/mL) Log rank p = 0.017 HR not provided
Kulasinghe et al., 2019 (Kulasinghe et al., 2019) (n = 35)	Spiral Microfluidic Technology	Treatment-naïve	Stage III–IV	No	Baseline IF	CK+/CD45−/DAPI+/ PD-L1+	18/35 (51.4%)	10/18 (55.6%)	28-8 (Abcam)	NE

IF: immunofluorescence, NSCLC: non-small cell lung cancer, HR: hazard ratio, NE: prognostic or predictive significance not evaluated, ISET: size of epithelial tumour cells, EpCAM: epithelial cell adhesion molecules, CK: cytokeratin, PFS: progression free survival. * prospective multi-institutional study.

Boffa *et al*. described the only multi-institutional prospective study reported to date evaluating PD-L1 expression on CTCs in 112 treatment naïve NSCLC patients, the largest sample size analysed to date. PD-L1$^+$ CTCs were detectable in the peripheral blood of 23% of NSCLC patients assessed prior to therapy. While most of the circulating cells identified in this study met the consensus criteria for CTCs—expression of epithelial protein, absence of CD45 expression, and an intact nucleus—many PD-L1$^+$ CD45$^-$ cells in patient samples contained both a nuclear morphology distinct from surrounding WBCs and other CTCs and with expression of CK below the analytical cut-offs of the assay. These cells were not observed in healthy controls and have not been genetically confirmed to be of malignant origin; therefore, the authors refrain from labelling them as "CTCs" and adopted the nomenclature circulating cells associated with malignancy (CCAMs). Within the PD-L1$^+$ CCAM population (47 cells from 26 NSCLC patients), two distinct subpopulations were noted based on differential expression of cytokeratin (CK). Nineteen (40%) cells were positive for CK [PD-L1$^+$ CK$^+$], whereas 60% were negative for CK [PD-L1$^+$ CK$^-$].

Ilie *et al*. investigated the utility of CTCs as a non-invasive biomarker to evaluate PD-L1 status in 106 advanced NSCLC patients. CTCs were detected in 75% of patients. PD-L1 staining was assessed in 71 samples that showed >1 CTCs, but only 8% of NSCLC patients exhibited PD-L1$^+$ CTCs (Ilié et al., 2017).

Adams *et al*. tracked PD-L1 expression in circulating tumour and stromal cells in 41 stage I–IV NSCLC patients undergoing radiotherapy. CTCs were identified in 85% and 100% of patient samples prior to (T0) and after radiotherapy (T1), respectively. Three different patterns of the expression of PD-L1 between T0 and T1 were observed in 35 patients who were assessable for both time points. Eleven patients (32%) showed a rise in PD-L1 expression scores from T0 to T1, 18 patients (51%) had no/low PD-L1 expression, and 6 patients (17%) had persistently medium/high PD-L1 at the two-time points. Of note, the authors characterised the two most common CTC subtypes undergoing epithelial-to-mesenchymal transition (EMTCTC); the prognostically relevant pathological definable CTCs (PDCTC); and a subtype of circulating stromal cells, cancer-associated macrophage-like cells (CAMLs). CAMLs were the most prevalent at both times points (at baseline and after induction of treatment) followed by EMTCTCs and PDTCTCs. Both CAMLs and EMTCTCs have been well proven as cancer-specific biomarkers; therefore, data obtained from the study demonstrated that combining CAMLs

and EMTCTCs significantly expands the capacity to characterise cellular biomarkers such as PD-L1 in blood-based diagnosis (Adams et al., 2017).

Guibert *et al*. monitored the expression of PD-L1 on CTCs among 96 NSCLC patients treated with nivolumab. CTCs were detected in 93% of patients' samples at baseline, and they found that 83% of the patients had PD-L1$^+$ CTCs on at least 1% of CTCs prior to therapy. At the time of progression, 95% of analysed CTC-positive patients had PD-L1$^+$ CTCs, of whom 83% had more than 10% of CTCs as PD-L1$^+$, compared with 68% of pre-treatment CTCs (Guibert et al., 2018).

Dhar *et al*. evaluated PD-L1 expression on CTCs among patients receiving nivolumab therapy prior to or during treatment. The study evaluated CTCs from 31 samples collected from 22 patients. They observed that 96.8% of the patient samples had at least 1 CTC and 48.4% of samples had at least 10 CTCs. Using a cut-off criterion of 1.32 CTCs/mL of blood, based on the analysis of 10 healthy controls, they concluded that 14 of 31 patient samples were positive for CTCs. Among patient samples with CTCs, 85.7% had one or more PD-L1$^+$ CTCs (Dhar et al., 2018).

Nicolazzo *et al*. evaluated the presence of PD-L1$^+$ CTCs longitudinally, at three-month intervals during therapy in 24 metastatic NSCLC patients. All patients analysed underwent anti-PD-1 therapy through an expanded access program for nivolumab. CTCs were detected in 83% patients and PD-L1$^+$ CTCs were detected in 19 of 20 patients with detectable CTCs at baseline. Ten out of 15 patients with detectable CTCs after three months from starting therapy had PD-L1 expressing CTCs, and 5 of 10 patients with detectable CTCs after six months of treatment had PD-L1$^+$ CTCs. It was observed that both baseline and at three months after treatment initiation, almost all CTCs expressed PD-L1, irrespective of clinic-pathological characteristics (Nicolazzo et al., 2016).

Kallergi *et al*. investigated PD-L1 expression on CTCs isolated from 30 NSCLC patients treated with chemotherapy. CTCs were evaluated by Giemsa staining and immunofluorescence (IF). Giemsa staining revealed that 28 of 30 patients and 8 of 11 patients had detectable CTCs before and after their third chemotherapy cycle, respectively, while IF could detect CTCs in 17 of 30 (56.7%) chemo-naïve patients and in 8 of 11 patients after the third chemotherapy cycle. The rate of PD-L1$^+$ CTC detection in the group of patients was 26.7% at baseline and 45% after three cycles of front-line chemotherapy in the whole patient group (Kallergi et al., 2018).

Wang *et al.* monitored the dynamic changes of the expression of PD-L1 in CTC in 13 non-metastatic NSCLC patients treated with radiotherapy or chemoradiation. CTCs were detected in all patients throughout the course of treatment and PD-L1$^+$ CTCs were found in 25 out of 38 samples collected with an average of 4.5 cells/mL. All patients who received radiation only had significantly increased numbers of PD-L1$^+$ CTCs during treatment and, similarly, 7 out of 8 non-metastatic NSCLC patients showed increased numbers of PD-L1$^+$ CTCs with concurrent chemotherapy (Wang et al., 2019).

Kulasinghe *et al.* phenotypically characterised CTCs in 35 stage IV NSCLC patients for clinically actionable targets. The authors identified CTCs or CTC clusters in 74% of the patients. Using the presence of at least one PD-L1 positive cell as the cut-off for PD-L1 positivity, 10 out of 18 NSCLC patients expressed PD-L1 in CTCs (Kulasinghe et al., 2019).

Altogether, these studies demonstrate that analysis of PD-L1 on CTCs is feasible and could be detected prior to and after frontline therapy. We should underscore that two studies (Adams et al., 2017; Wang et al., 2019) reported that PD-L1 expression is significantly increased in patients after radiotherapy, which is consistent with reports from preclinical studies (Deng et al., 2014; Dovedi et al., 2014).

1.6.1.1. PD-L1 expression on CTCs as prognostic marker in NSCLC

Several studies have reported that PD-L1 expression in tumour tissue is associated with either shorter or longer survival time in NSCLC patients (Pan et al., 2015; Velcheti et al., 2014; Wu et al., 2015; Zhang et al., 2016). The prognostic value of PD-L1$^+$ CTCs in NSCLC was analysed in four of the nine studies identified (Table 1) (Adams et al., 2017; Boffa et al., 2017; Ilié et al., 2017; Wang et al., 2019). Only two studies demonstrate a statistically significant difference in survival probabilities between patients with PD-L1$^+$ CTCs (Boffa et al., 2017; Wang et al., 2019).

Boffa *et al.* reported that NSCLC patients with >1.1 PD-L1$^+$ CTCs/mL of blood at diagnosis had worse two-year survival compared with those with ≤1.1 PD-L1$^+$ CTCs/mL (31.2% vs. 78.8%, *p* = 0.002). A multivariable Cox proportional hazard model controlling for staging indicated that the number of PD-L1$^+$ CTCs/mL is a significant independent predictor of mortality (HR = 3.85; 95% CI, 1.64–9.09; *p* = 0.002) (Boffa et al., 2017). On the other hand, Wang *et al.* showed that patients with >5% PD-L1 expression on CTCs had significantly shorter PFS compared with PD-L1 negative patients (median 7.1 months vs. median not reached: *p* = 0.017). However, no

Cox regression analysis was performed nor multivariate analyses to control for other predictors of progression (Wang et al., 2019).

It is important to note that none of the four studies analysed patients treated with anti-PD-1/PD-L1 therapy.

1.6.1.2. PD-L1 expression on CTCs as a predictive marker in NSCLC

Three of the studies highlighted here explored PD-L1 expression on CTCs in association with the response to PD-1 inhibitors (Dhar et al., 2018; Guibert et al., 2018; Nicolazzo et al., 2016). Only Guibert *et al.* correlated PD-L1+ CTCs with the response to PD-1 inhibitor, nivolumab in a large cohort of NSCLC patients (n = 96). The presence of PD-L1+ CTCs prior to treatment at different thresholds had no statistically significant impact on PFS. Opposite to what would be expected, patients with >1% PD-L1+ CTCs at baseline were frequently non-responders compared with patients who had PD-L1− CTCs (47/69 (68%) vs. 6/15 (40%), p = 0.04) (Guibert et al., 2018). Similarly, Dhar *et al.* analysed the association between PD-L1 expression and PFS in 17 patients prior to starting treatment. They found that having ≥2 PD-L1+ CTCs was not a predictor of response or clinical benefit, though this small cohort included patients treated with nivolumab as well as pembrolizumab (Dhar et al., 2018). Nicolazzo *et al.* did not specifically address the predictive significance of PD-L1 in CTCs in their study. Nevertheless, PD-L1+ CTCs were identified in 19 patients at baseline, and only 5 of them (26%) achieved clinical benefit after six months of treatment. Thus, PD-L1+ CTCs were not demonstrated to be a predictor of response to therapy (Nicolazzo et al., 2016).

Overall, no report to date has shown evidence on the predictive value for PD-L1+ CTCs for response to anti-PD1 agents.

1.6.1.3. Correlation between PD-L1 expression on tumour biopsy tissue and CTCs in NSCLC patients

Four studies explored the correlation between the expression of PD-L1 on CTCs and its expression in tumour tissue biopsies (Adams et al., 2017; Dhar et al., 2018; Guibert et al., 2018; Ilié et al., 2017). The largest reported study to date (n = 71) was conducted by Ilié *et al.*, who noted 93% concordance between PD-L1 expression on CTCs and matched tumour tissues (Ilié et al., 2017). In contrast, Guibert *et al*. found no statistically significant correlation between the expression of PD-L1 on archived tissue and CTCs (n = 66), with the observed rate

of concordance being 45% (Guibert et al., 2018). The other two studies only presented descriptive data indicating some concordance between PD-L1 expression on CTCs and matched tumour tissue. However, they had a limited sample size (n = 9 and n = 4), which prevented appropriate statistical analysis from being performed (Adams et al., 2017; Dhar et al., 2018).

Generally, these studies demonstrated the feasibility of comparing PD-L1 expression on CTCs and matched tumours; however, the findings from these studies are not directly comparable because of the different anti-PD-L1 monoclonal antibodies used between CTCs and matched tumours, and different antibodies used between studies. Adams *et al*. used three anti-PD-L1 monoclonal antibodies for staining, with clone 130021 (R&D Systems, Minneapolis, MN, USA) being used for CTCs, while matched tumours were stained with either the anti-PD-L1 clone 28.8 (DAKO) or the clone 22C3 (DAKO). Similarly, Dhar *et al*. used two different antibodies, with an anti-PD-L1 antibody (ProSci Inc Ref# 4059, Poway, CA, USA) being used to stain CTCs and the anti-PD-L1 clone SP142 (Ventana) being used for matched tissues. Ilié *et al*. used an anti-PD-L1 monoclonal antibody clone SP142 for PD-L1 staining both on CTCs and matched tumour tissue, while Guibert *et al*. used the anti-PD-L1 rabbit monoclonal antibody clone E1L3N (Cell Signalling Technology, Danvers, MS, USA). The use of different antibodies between studies may explain the discrepancies in results.

Four different PD-L1 immunohistochemistry (IHC) assays (PD-L1 assays (22C3, 28-8, SP142, SP263) have been approved by the United States Food and Drug Administration as companion diagnostic tests for tissue staining. Reports from phase I of the blueprint study (BPI) by Hirsh *et al*., 2017 revealed that three of the four antibodies (22C3, 28-8, SP263) demonstrate similar analytical performance for tumour cell staining, whereas the fourth (SP142) provides significantly lower staining for tumour proportion score (Hirsch et al., 2017). Recently, results obtained from phase II of the blueprint PD-L1 IHC assay (BP2) study using real-life clinical lung cancer samples affirmed the result of BP1 and consolidated the evidence for interchangeability of three different assays (22C3, 28-8, and SP263) (Tsao et al., 2018). Compared with the above-mentioned studies, these findings demonstrate that the best approach for comparing PD-L1 IHC testing is to perform the test with assays that have undergone rigorous analytical and clinical validation. Similar studies will be needed to develop standardised methods for PD-L1 assessment on CTCs.

1.6.2. Expression of PD-L1 on melanoma CTCs

To date there have been only two published studies assessing PD-L1 expression on CTCs in melanoma patients (Khattak et al., 2019; Po et al., 2019). Po *et al*. enriched for melanoma CTCs from 9mL of blood using immunomagnetic beads coated with either MCAM, MCSP or both, and detected them using immunocytochemistry (Po et al., 2019). Po *et al*. utilised a combination of Melan-A, gp100 and S100 antibodies to identify melanoma CTCs, CD45 to exclude WBCs and the E1L3N antibody clone for PD-L1 expression. MCAM conjugated beads isolated CTCs from 10/16 patients, MCSP conjugated beads isolated CTCs from 13/16 patients and combined MCAM & MCSP conjugated beads isolated CTCs from 14/16 patients (Po et al., 2019). MCAM beads isolated fewer CTCs than both MCSP and MCAM/MCSP combination beads, with the median CTC counts being 2.5, 9 and 16 respectively per patient (Po et al., 2019). PD-L1 was assessed on 13 patients with detectable CTCs, PD-L1 positive CTCs were identified in 5/13 patients with the percentage of PD-L1 expressing CTCs ranging from 1.5% to 60% (Po et al., 2019).

Khattak *et al.* did not perform CTC enrichment. Instead peripheral blood mononuclear cells (PBMCs) from 8mL of blood were then stained with a multimarker antibody panel targeting MCAM, MCSP, ABCB5, CD271 and RANK to identify tumour cells, CD45 and CD34 to identify WBCs and circulating endothelial cells, and PD-L1 (Khattak et al., 2019). CTCs were detected in 25/40 patients with the CTC number ranging from 7 to 291 cells. PD-L1 was identified on CTCs from 16/25 patients with detectable CTCs with the percentage of PD-L1 expressing CTCs ranging from 1% to 89% (Figure 15) (Khattak et al., 2019).

1.6.2.1.   Clinical benefit of PD-L1 expression on CTCs in melanoma

The study published by Khattak *et al*. is the only report to date to assess the clinical benefit of PD-L1 expression on CTCs in melanoma patients. For patients with detectable CTCs, those with PD-L1 expressing CTCs had a significantly longer progression free survival compared to those with only PD-L1 negative CTCs (HR 0.162, 95% CI 0.042-0.631, *P* = 0.009, Figure 13a). The median PFS for the PD-L1 negative CTCs group was 5.5 (5.2-5.8) months, while median PFS was not reached for the group with PD-L1 positive CTCs. The 12-month PFS rates were 81% vs. 22% in the PD-L1 positive vs. PD-L1 negative CTCs groups, respectively (*P* = 0.034). Interim overall survival analysis did not reveal statistically significant differences between the

groups, although survival rates were lower in patients with PD-L1$^-$ CTCs (Figure 13b), with median OS not reached in the group with PD-L1$^+$ CTCs (Khattak et al., 2019).

Multivariate Cox regression analysis controlling for age, sex, line of therapy, disease stage, BRAF status, ECOG status, neutrophil-to-lymphocyte ratio (NLR) and presence of liver metastases confirmed that CTC PD-L1 positivity is an independent predictive biomarker of PFS (HR 0.11, 95% CI 0.01-0.81, *P = 0.03*) (Khattak et al., 2019).
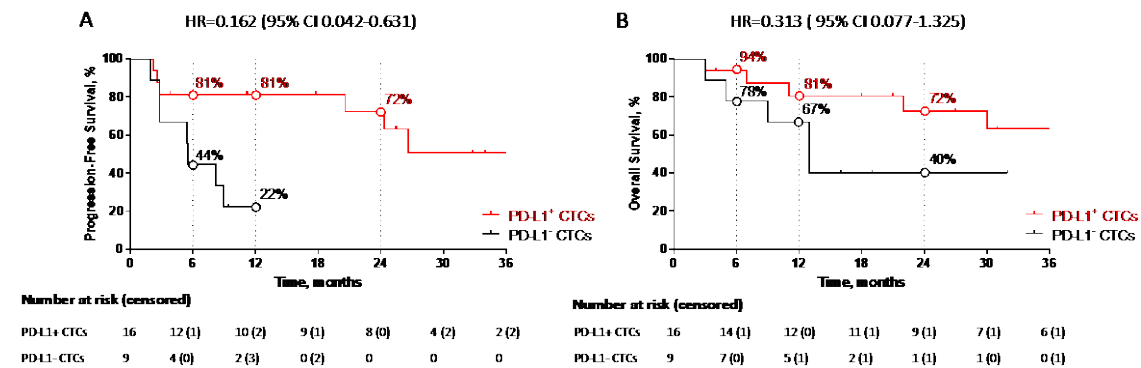
Figure 13*. Kaplan-Meier plots of progression-free survival and overall survival according to PD-L1 expression on CTCs prior to treatment initiation. (A): Progression-free survival. (B): Overall survival. Abbreviations: CI, confidence interval; CTC, circulating tumour cell; HR, hazard ratio (Khattak et al., 2019).*

1.6.2.2.   PD-L1 expression on CTCs as a predictive marker in melanoma

The study by Khattak *et al*. in 2019 is also the only published study to assess CTC PD-L1 expression as a predictive marker in melanoma patients. Patients in the study were treated with pembrolizumab monotherapy, with 21 responders and 19 non-responders. The total number of CTCs was similar and did not significantly differ between responders and non-responders, and there were no differences in PFS or OS between patients with detectable and non-detectable CTCs. However, the number of PD-L1 positive CTCs was significantly higher in responders (*P=0.005,* Figure 14). Amongst patients with detectable CTCs, patients with PD-L1 positive CTCs were eight times more likely to be responders compared to patients with no PD-L1 positive CTCs [OR 8.67 (95% CI 1.19-342.96), *P = 0.017*) (Khattak et al., 2019).

Comparison of CTCs detected at baseline and after 6-12 weeks post treatment initiation (follow up), showed that the total number of CTCs, as well as the proportion of CTC expressing PD-L1, decreased upon treatment in most responders, and increased or remained the same in most non-responders (Khattak et al., 2019).

Khattak *et al.* demonstrated that PD-L1 expression on CTCs is an independent predictor of response and prolonged PFS in melanoma patients treated with pembrolizumab. This study provided a proof of concept that detection of PD-L1 status through liquid biopsy can provide clinically relevant information (Khattak et al., 2019).
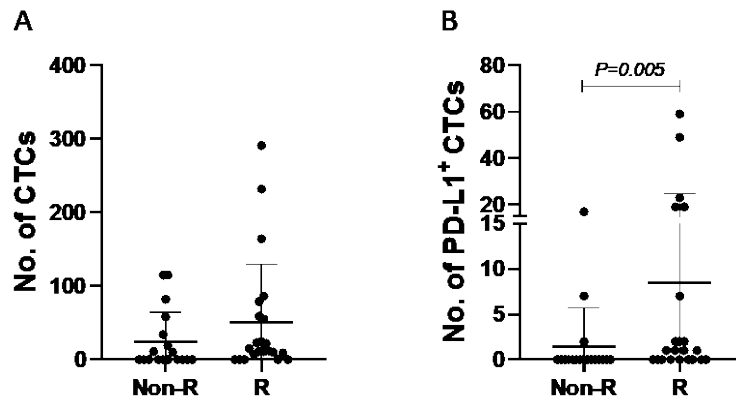
Figure 14. Comparison of total and PD-L1+ CTCs in responders and non-responders to pembrolizumab treatment. (A): Total CTCs. (B): PD-L1+ CTCs. Abbreviations: CTC, circulating tumour cell; R, responder.

1.7. Theoretical framework

Immune checkpoint inhibitors, in particular anti-PD-1 antibodies, are a relatively new treatment option that have significantly altered clinical management of late stage melanoma and NSCLC patients. These new treatments are highly successful in a subset of patients, however, there are a number of patients that do not respond. Moreover, treatment with these antibodies are associated with high grade adverse events (Yuan et al., 2016). The combination of anti-PD-1 and anti-CTLA4 therapies has frequently been used as a means to increase effectiveness (Ascierto et al., 2017; Gide et al., 2019). Clinical trials have also tested anti-PD-1 therapy in combination with other checkpoint inhibitors such as anti-Tim-3 and anti-LAG3 (Zou et al., 2016), and co-stimulatory factors such as glucocorticoid-induced TNFR family related gene, CD134 and CD137 (Hellmann et al., 2016; Perez-Ruiz et al., 2017). Where these combinations are not FDA approved, anti-PD-1 therapy in combination with chemotherapy has been approved by the FDA to treat NSCLC patients (Langer et al., 2016; Paz-Ares et al., 2018; Sheela et al., 2018). However, combination therapies are often more expensive and more toxic when compared to monotherapy, and so should be avoided if monotherapy could be found to be effective.

PD-L1 is one of the biomarkers that can potentially be used to predict response to treatment with anti-PD-1 antibodies. PD-L1 binds to PD-1 and this interaction acts to suppress the immune system and so provides an evasion mechanism to tumours that express the protein (Yuan et al., 2016). CTCs can be obtained without invasive surgery, display tumour specific markers such as PD-L1, can be used to monitor disease progression in real time and can be evaluated after failure of first line treatment (Carter et al., 2017; Nicolazzo et al., 2016; Scher et al., 2016). While a limited number of studies have been published detailing PD-L1 expression on CTCS in NSCLC patients, even fewer studies have been published detailing PD-L1 on melanoma CTCs. Only a single study compared CTC PD-L1 expression and tumour PD-L1 expression for melanoma and a limited number have been published for NSCLC (Adams et al., 2017; Dhar et al., 2018; Guibert et al., 2018; Ilié et al., 2017; Khattak et al., 2019). This information would enable more efficient treatment selection for melanoma patients and would provide details of dynamic PD-L1 expression levels closer to treatment initiation for both NSCLC and melanoma patients.

This study established a method to characterise PD-L1 expression in CTCs using fluorescence immunocytochemistry and investigated PD-L1 expression in CTCs relative to tumour biopsies from NSCLC and melanoma patients. These results will enable further studies that ascertain the relation between PD-L1 expression in CTCs and patient response to immunomodulatory antibodies targeting the PD-1 immune checkpoint.

1.7.1. Hypothesis

PD-L1 expression on melanoma and NSCLC CTCs can be identified and evaluated by immunocytochemistry.

1.7.2. Specific Aims

1. Compare PD-L1 expression on melanoma CTCs, assessed by flow cytometry, to matched tissue samples;

A cohort of melanoma patients from an earlier study had PD-L1 assessed on CTCs using flow cytometry. Matched tissue samples for this cohort of melanoma patients were obtained, stained by immunohistochemistry and given a PD-L1 score. Tissue PD-L1 scores were then compared to the CTC PD-L1 data.

2. Develop an immunocytochemistry assay to evaluate PD-L1 expression in melanoma CTCs;

The 28.8, 22C3 and 130021 PD-L1 antibodies were assessed for PD-L1 detection to identify the most suitable candidate for incorporation into an immunocytochemistry panel. Cell lines to be used as positive (high and low PD-L1 expression) and negative controls were then identified using flow cytometry and used in the development of a multimarker immunocytochemistry panel to identify PD-L1 expression on melanoma CTCs. The final protocol was then validated in a small cohort of melanoma patients.

3. Develop an immunocytochemistry assay to evaluate PD-L1 expression in carcinoma CTCs;

Carcinoma cell lines to be used as positive (high and low PD-L1 expression) and negative controls were identified using first by flow cytometry and then used in the development of a multimarker immunocytochemistry panel to identify PD-L1 expression on carcinoma CTCs. The protocol was then validated in a small cohort of non-small cell lung cancer patients. PD-L1 expression on NSCLC CTCs was then compared to expression in matched tissue samples.

2. CHAPTER 2: Materials and Methods

2.1. Aim 1: Compare PD-L1 expression on melanoma CTCs, assessed by flow cytometry, to matched tissue samples

2.1.1. Comparing tumour and CTC PD-L1 expression

Immunohistochemistry for PD-L1 expression was performed in a Dako ASL48 Autostainer as described previously (Ye et al., 2019), using the PD-L1 IHC 22C3 pharmDx (Dako, Carpinteria, CA) and approved by the US FDA for use in NSCLC (Herbst et al., 2016). Verification of successful reaction on each slide was assessed with tonsil and placenta as external tissue controls. PD-L1 expression was assessed based on the Tumour Proportion Score (TPS) by an experienced pathologist (Dr Benhur Amanuel). Only viable tumour cells were assessed, positivity is defined as any perceptible linear cell membrane staining (partial or complete), the score reflects percentage of positive tumour cells, and any associated immune cells are excluded from scoring.

A two-sided Fisher's exact test was performed to compare response relative to PD-L1 expression on tumour tissue and to PD-L1 expression on CTCs or tumour tissue.

2.2. Aim 2: Develop an immunocytochemistry assay to evaluate PD-L1 expression in melanoma CTCs

2.2.1. Cell lines

Melanoma cell lines (MP41, SKMEL28, A2058, UACC62, WM164 & WM793) were maintained in cell culture media as a monolayer at 37 °C and 5% CO_2. Cell lines were cultured in either DMEM (ThermoFisher Scientific, Waltham, United States of America) or RPMI 1640 (ThermoFisher Scientific), each containing 10% - 20% foetal bovine serum (ThermoFisher Scientific) as required. In preparation for flow cytometry or immunofluorescent staining, cells were grown until 80% confluent.

To increase PD-L1 expression, SKMEL28 cells were incubated with varying concentrations (10 to 100ng/ml) of IFN-γ for 24 hours to induce PD-L1 expression.

2.2.2. PD-L1 expression assessment by flow cytometry

The level of PD-L1 expression for each cell line was first assessed by flow cytometry using the rabbit anti-human PD-L1 antibody clone 28.8 (Abcam, Cambridge, United Kingdom).

Cells were harvested from flasks using Trypsin/EDTA (ThermoFisher Scientific) and washed in phosphate buffered solution (PBS) once before counting. A total 1×10^4 cells were suspended in 100μL of FACS staining buffer (1% bovine serum albumin (BSA)/10% normal donkey serum (NDS) in PBS) containing the anti-PD-L1 antibody (clone 28.8, Abcam) diluted 1/100 for 30 minutes. The cells were then washed once with 0.5% BSA/PBS before resuspending in FACS staining buffer containing a secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor® 488 (Abcam) diluted 1/500 for 30 minutes. The cells were again washed once in 0.5% BSA/PBS before being resuspended in 100μL of FACS staining buffer and analysed using a Gallios Flow Cytometer (Beckman Coulter, Brea, United States of America).

Table 2. Antibodies used for immunocytochemistry staining.

Antibody	Host Species	Conjugate	Clone	Antigen location	Supplier (CTLG #)	Dilution	Use
gp100	Rabbit	n/a	EPR4864	Cytosol	Abcam (ab137062)	1/50	Melanoma CTC identification
MLANA	Rabbit	n/a	EP1422Y	Cytosol	Abcam (ab51061)	1/50	Melanoma CTC identification
S100	Rabbit	n/a	EP1576Y	Cytosol	Abcam (ab52642)	1/500	Melanoma CTC identification
PD-L1	Rabbit	AF647	28.8	Membrane	Abcam (ab209960)	1/100	PD-L1 expression
PD-L1	Mouse	n/a	22C3	Membrane	DAKO (M365329-2)	1/100	PD-L1 expression
PD-L1	Mouse	FITC	130021	Membrane	R&D Systems (FAB1561G)	1/100	PD-L1 expression
Anti-rabbit IgG	Donkey	AF488	n/a	n/a	ThermoFisher Scientific (A-21206)	1/500	Melanoma CTC identification
CD16	Mouse	PE	3G8	Membrane	BioLegend (302008)	1/50	WBC identification
CD45	Mouse	PE	HI30	Membrane	BD Biosciences (555483)	1/50	WBC identification
CK3-6H5	Mouse	FITC	CK3-6H5	Cytoskeleton	Miltenyi Biotech (130-118-964)	1/50	NSCLC CTC identification
C11	Mouse	AF488	C11	Cytoskeleton	Cell Signalling Technology (4523S)	1/100	NSCLC CTC identification
AE1/AE3	Mouse	AF488	AE1/AE3	Cytoskeleton	ThermoFisher Scientific (53-9003-80)	1/200	NSCLC CTC identification
EpCAM	Mouse	FITC	VU-1D9	Membrane	ThermoFisher Scientific (MA1-10197)	1/100	NSCLC CTC identification
Vimentin	Mouse	AF647	V9	Cytoskeleton	Abcam (ab195878)	1/1000	NSCLC CTC identification
PD-L1	Rabbit	n/a	28.8	Membrane	Abcam (ab205921)	1/400	PD-L1 expression

2.2.3. WBC isolation from healthy volunteers

Healthy donors signed consent forms, previously approved by the Human Research Ethics Committees at ECU (No.11543 and No.21261). The blood was drawn by phlebotomists into vacutainer tubes, containing EDTA anticoagulant (Becton-Dickinson, Franklin Lakes, United States of America). The first 6ml of blood was collected in SST tubes for serum collection, doubling as a discard tube to avoid epithelial contamination.

Red blood cells (RBCs) were lysed using RBC lysing buffer (140mM NH_4Cl + 17mM Tris in ddH_2O, pH 7.65) at a 1:5 volume ratio with continuous gentle mixing for 10 minutes at room temperature. The lysed RBCs were removed by centrifugation at 300g for 5 minutes. Following this, the nucleated cell fraction was washed once with PBS and finally resuspend in PBS. Cells were then counted and diluted into 10,000 cell fractions.

2.2.4. Spiking of cancer cell lines

Cell lines were spiked into WBC fractions in order to mimic cancer cells that could be present after CTC enrichment. For all immunocytochemistry spiking experiments involving WBCs, approximately 500 cells (from whichever cell line was required) were spiked into WBCs. Spiking melanoma cells involved diluting to the appropriate concentration of cells and mixing the fraction with a 10,000 WBC fraction, obtained from a healthy volunteer as described in 2.2.3.

2.2.5. Melanoma immunocytochemistry

Cells (from CTC isolation/cell line spiked into WBC) were immediately fixed in 4% PFA for 10 minutes. After that, cells were cytospun (Cytospin™ 4, Thermo Fisher Scientific) onto glass slides at 2000g for 5 minutes at medium acceleration. Cells were then dried and stored in a desiccator at 4°C or progressed straight to staining.

Cells attached to the slides were incubated in blocking buffer (10% NDS/ 10% Glycine/ 5% Human FcR block/ 3% BSA/ 0.2% Triton X-100 (TX) in PBS) for 15 minutes before incubating in staining buffer (10% NDS/ 3% BSA/ 0.2% TX in PBS) containing the diluted antibody mix (see Table 2 for details on antibody dilutions).

For the final melanoma CTCs staining protocol was adapted from Aya-Bonilla et al. (Aya-Bonilla et al., 2017), gp100 (clone EPR4864, Abcam), MLANA (clone EP1422Y, Abcam), S100 (clone EP1576Y, Abcam), CD16 conjugated to phycoerythrin (PE) (clone 3G8, BioLegend, San

Diego, United States of America) and CD45 conjugated to PE (clone HI30, BD Biosciences, Franklin Lakes, United States of America) were incubated for 1 hour. Following this incubation, cells were washed three times for 5 minutes each in 1% BSA/PBS before incubating in stain buffer containing donkey anti-rabbit IgG antibody conjugated to Alexa Fluor® 488 dye (ThermoFisher Scientific) for 15 minutes. Cells were once again washed three times for 5 minutes each in 1% BSA in PBS before the final incubation in 10% normal rabbit serum (NRS)/10% NDS/ 3% BSA/ 0.2% TX in PBS containing PD-L1 conjugated to Alexa Fluor® 647 (clone 28.8, Abcam) for 1 hour.

Finally, cells were washed three times for 5 minutes each in 1% BSA in PBS, washed once in PBS for 5 minutes, dried and mounted with Fluoromount Gold plus DAPI (ThermoFisher Scientific).

2.2.6. Patient recruitment

Patients beginning treatment for metastatic melanoma were recruited by Professor Michael Millward (Sir Charles Gairdner Hospital, WA), Dr Adnan Khattak (Fiona Stanley Hospital, WA) and Dr Tarek Meniawy (Sir Charles Gairdner Hospital, St John of God Hospital, WA) prior to commencing therapy. Procedures were approved by Human Research Ethics Committees at Edith Cowan University (No. 18957) and Sir Charles Gairdner Hospital (No. 2013-246). Clinical data such as disease stage, prior therapy and forthcoming therapy was collected for all patients. At least 9mL of blood was collected from each patient into EDTA tubes for CTC analysis. Samples were processed within 24 hours of blood collection.

2.2.7. CTC enrichment and immunofluorescent staining

Blood samples (9 mL) were enriched from CTCs using the Parsortix system (Angle plc, Guildford, United Kingdom) as described previously (Aya-Bonilla et al., 2019). Enriched fractions were processed in accordance with the immunofluorescence staining method described for melanoma patients in section 2.2.5. Patient samples were periodically processed in tandem with high, medium and low PD-L1–expressing cell line controls to ensure consistent results.

2.2.8. CTC identification and PD-L1 scoring

Stained slides were scanned using an automated inverted Eclipse Ti-E Nikon® fluorescence microscope to visualise immunofluorescent staining and analysis was performed using the NIS-Elements High Content Analysis software, version 4.2.01. Slides were scanned using; a DAPI filter with excitation and emission (Ex/Em) wavelengths of 395/460 nm with an exposure time of 300ms, a FITC only filter (Ex/Em = 470/535 nm) with an exposure time of 700ms and 2x2 binning, a TRITC filter (EX/Em = 555/607 nm) with an exposure time of 500ms and 2x2 binning, and a Cy5 filter (640/720 nm) with an exposure time of 1s and 2x2 binning. Look up tables, adjusted based on control slides, were used to remove background fluorescence and adjust displayed intensity appropriately.

CTCs were identified among nucleated cell populations based on the size/morphology of cell, nuclear features and expression of melanoma markers in the absence of blood-lineage CD16 or CD45 expression. Detectable PD-L1 expression was scored as high or low based on the negative control and low and high positive controls.

2.3. Aim 3: Develop an immunocytochemistry assay to evaluate PD-L1 expression in carcinoma CTCs

2.3.1. Cell lines

Cell lines (MCF7, MDA-MB-231 & LNCaP) were maintained in cell culture media as a monolayer at 37 °C and 5% CO_2. Cell lines were cultured in either DMEM (ThermoFisher Scientific) or RPMI 1640 (ThermoFisher Scientific), each containing 10% - 20% foetal bovine serum (ThermoFisher Scientific) as required. In preparation for flow cytometry or immunofluorescent staining, cells were grown until 80% confluent. Some MCF7 cells were incubated with 100ng/ml IFN-γ for 24 hours to induce PD-L1 expression.

2.3.2. PD-L1 expression assessment by Flow cytometry

The level of PD-L1 expression for each cell line was first assessed by flow cytometry using a Gallios Flow Cytometer (Beckman Coulter) with the 28.8 PD-L1 antibody clone (Abcam). Cells were harvested from flasks using trypsin before being washed in phosphate buffered solution (PBS). $1x10^4$ cells were suspended in 100μL of stain buffer (1% bovine serum albumin (BSA)/10% normal donkey serum (NDS) in PBS) containing PD-L1 (clone 28.8, Abcam) diluted 1/100 for 30 minutes. The cells were then washed once with 0.5% BSA in PBS before

resuspending in stain buffer containing a secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor® 488 (Abcam) diluted 1/500 for 30 minutes. The cells were once again washed once in 0.5% BSA in PBS before being resuspended in 100μL of stain buffer and analysed using a Gallios Flow Cytometer (Beckman Coulter). Fluorescence values obtained for each cell line were then compared to form a relative scale which was utilised to identify high, low and negative PD-L1 expressing cell lines which were subsequently used as controls. PD-L1 expression levels for these control cell lines was then validated by immunocytochemistry to ensure expression readings were consistent between methods.

2.3.3. WBC isolation from healthy volunteers

Healthy donors signed consent forms, previously approved by the Human Research Ethics Committees at ECU (No.11543 and No.21261). The blood was drawn by phlebotomists into vacutainer tubes, containing EDTA anticoagulant (Becton-Dickinson). The first 6ml of blood was collected in SST tubes for serum collection, doubling as a discard tube to avoid epithelial contamination.

Red blood cells (RBCs) were lysed using RBC lysing buffer (140mM NH_4Cl + 17mM Tris in ddH_2O, pH 7.65) at a 1:5 volume ratio with continuous gentle mixing for 10 minutes at room temperature. The lysed RBCs were removed by centrifugation at 300g for 5 minutes. Following this, the nucleated cell fraction was washed once with PBS and finally resuspend in PBS. Cells were then counted and diluted into 10,000 cell fractions.

2.3.4. Spiking of cancer cell lines

Cell lines were spiked into WBC fractions in order to mimic the cell population present after enrichment. For all immunocytochemistry spiking experiments involving WBCs, approximately 500 cells (from whichever cell line was required) were spiked into WBCs. Spiking carcinoma cells involved diluting to the appropriate concentration of cells and mixing the fraction with a 10,000 WBC fraction, obtained from a healthy volunteer as described in 2.3.3.

2.3.5. Carcinoma immunocytochemistry

After collection, cells (from CTC isolation/ cell line spikes) were immediately fixed in 4% paraformaldehyde (PFA) for 10 minutes. After that, cells were cytospun (Cytospin™ 4, Thermo

Fisher Scientific) onto glass slides at 2000 rpm for 5 minutes at medium acceleration. Cells were then dried and stored in a desiccator at 4°C or progressed straight to staining.

Cells were incubated in blocking buffer (10% NDS/ 10% Glycine/ 5% Human FcR block/ 3% BSA/ 0.2% TX in PBS) for 15 minutes before incubating in stain buffer (10% NDS/ 3% BSA/ 0.2% TX in PBS) containing pan-cytokeratin clone CK3-6H5 conjugated to FITC (Miltenyi Biotech, Bergisch Gladbach, Germany), pan-cytokeratin clone C11 conjugated to Alexa Fluor® 488 (Cell Signalling Technology, Danvers, United States of America), pan-cytokeratin clone AE1/AE3 conjugated to Alexa Fluor® 488 (ThermoFisher Scientific), EpCAM conjugated to FITC (clone VU-1D9, ThermoFisher Scientific), CD16 conjugated to phycoerythrin (PE) (clone 3G8, BioLegend), CD45 conjugated to PE (clone HI30, BD Biosciences) and vimentin conjugated to Alexa Fluor® 647 (clone v9, Abcam) (Table 2) for 1 hour. Following this incubation cells were washed three times for 5 minutes each in 1% BSA in PBS, washed in PBS for 5 minutes, dried and mounted with Fluoromount Gold plus DAPI (ThermoFisher Scientific). Mounted slides were stored at room temperature for 12-18 hours before imaging using an automated inverted Eclipse Ti-E Nikon® fluorescence microscope (detailed in section 2.3.8). After imaging, slides were immediately submerged in PBS to remove coverslips (~15 minutes). After coverslip removal slides were washed five times for 5 minutes each in PBS before incubating in 1mg/mL BH_4 in PBS solution for 180 minutes in darkness, with the BH_4 in PBS solution being replaced after 90 minutes. Slides were then washed six times for 5 minutes each in PBS before incubating in 100Mm tris solution for 1 hour. Slides were once again washed three times for 5 minutes each in PBS before quenching endogenous peroxidase activity with 0.3% H_2O_2 for 20 minutes. Slides were incubated in blocking buffer for 15 minutes before incubating in stain buffer containing PD-L1 (clone 28.8, Abcam) for 1 hour. Slides were then washed three times with 1% BSA in PBS for 5 minutes each and then incubated in stain buffer containing anti-rabbit horseradish peroxidase (HRP) (1/200, Perkin Elmer) for 30 minutes. Slides were again washed three times with 1% BSA in PBS for 5 minutes each before incubating in TSA Plus working solution (TSA plus Cy5 kit, Perkin Elmer) for 5 minutes. Finally, slides were once again washed three times with 1% BSA in PBS for 5 minutes each, washed once in PBS for 5 minutes, dried and mounted with Fluoromount Gold plus DAPI (ThermoFisher Scientific).

2.3.6. Patient recruitment

Patients beginning or receiving treatment for NSCLC were recruited by Professor Michael Millward (Sir Charles Gairdner Hospital, WA), Dr Samantha Bowyer (Sir Charles Gairdner Hospital, WA), Dr Adnan Khattak (Fiona Stanley Hospital, WA) and Dr Afaf Abed (Fiona Stanley Hospital, WA) prior to commencing therapy. Procedures were approved by Human Research Ethics Committees at Edith Cowan University (No. 18957) and Sir Charles Gairdner Hospital (No. 2013-246). Clinical data such as disease stage, prior therapy and forthcoming therapy was collected for all patients. At least 9mL of blood was collected from each patient into EDTA tubes, with one tube being used for each enrichment method where more than one method was employed. Samples were processed within 24 hours of blood collection.

2.3.7. CTC enrichment and immunofluorescent staining

Blood samples were enriched using the Parsortix system (Angle Plc) and in some cases, also processed with the ClearCell FX system (Biolidics Ltd, Singapore). Enriched cells were processed in accordance with the immunofluorescence staining methods described for NSCLC patients in section 2.3.5. Patient samples were periodically processed in tandem with high, medium and low PD-L1–expressing cell line controls to ensure consistent results.

2.3.8. CTC identification and PD-L1 scoring

Stained slides were scanned using an automated inverted Eclipse Ti-E Nikon® fluorescence microscope to visualise immunofluorescent staining and analysis was performed using the NIS-Elements High Content Analysis software, version 4.2.01. For the first scan, slides were scanned using; a DAPI filter with excitation and emission (Ex/Em) wavelengths of 395/460 nm with an exposure time of 300ms, a FITC only filter (Ex/Em = 470/535 nm) with an exposure time of 700ms and 2x2 binning, a TRITC filter (Ex/Em = 555/607 nm) with an exposure time of 500ms and 2x2 binning, and a Cy5 filter (640/720 nm) with an exposure time of 1s and 2x2 binning. For the second scan, slides were scanned using; a DAPI filter (Ex/Em =395/460 nm) with an exposure time of 300ms and a TRITC filter (Ex/Em = 555/607 nm) with an exposure time of 100ms. Look up tables, adjusted based on control slides, were used to remove background fluorescence and adjust displayed intensity appropriately.

CTCs were identified among nucleated cell populations based on the size/morphology of cell, nuclear features and expression of cytokeratin, EpCAM and/or vimentin in the absence of

blood-lineage CD16 or CD45 expression. Detectable PD-L1 expression was scored as high or low based on positive controls.

2.3.9. Tumour PD-L1 expression assessment

Patient tumour biopsies were stained for PD-L1 at PathWest clinical laboratories using the 22C3 antibody concentrate in Dako ASL48 Autostainers. Patient tumour PD-L1 score was determined by pathologists, considering external controls. Correlation between tumour tissue, scored as per pathologist estimation, and CTC PD-L1 positivity, scored based on positive controls, was assessed by plotting into a contingency table categorising each as either positive or negative.

3. CHAPTER 3: Results

Section 3.1 includes sections from the following publication, of which I am an author, and responsible for the comparison of PD-L1 expression between tumour tissue and CTCs.

Khattak, M. A., Reid, A., Freeman, J., Pereira, M., McEvoy, A., Lo, J., Frank, M.H., Meniawy, T., Didan, A., **Spencer, I.**, Amanuel, B., Millward, M., Ziman, M., & Gray, E. (2019). PD-L1 Expression on Circulating Tumour Cells May Be Predictive of Response to Pembrolizumab in Advanced Melanoma: Results from a Pilot Study. *Oncologist*. doi:10.1634/theoncologist.2019-0557

3.1. Comparison of PD-L1 expression on CTCs assessed by flow cytometry to PD-L1 expression in matched tissue biopsies.

As described in 1.6.2, a previous study in our laboratory assessed PD-L1 expression on CTCs from a cohort of metastatic melanoma patients prior to receiving pembrolizumab treatment (Khattak et al., 2019). CTCs were detected in 25/40 patients, with 14/25 CTC positive patients having ≥1 PD-L1 positive CTC.

To compare PD-L1 expression between CTCs and matched tissue samples, a subgroup of patients was analysed. PD-L1 expression in the tumour was evaluated using the PD-L1 IHC 22C3 pharmDx. Only, 25 of the 40 patients assessed for CTCs by flow cytometry had tissue available for PD-L1 IHC. 14 cases had PD-L1 status data available for both the tumour and CTCs samples.

Six patients were PD-L1+ in both tumour and CTCs, and all of them responded to therapy (Figure 15). Three patients were PD-L1+ in tumour (≤10% TPS) but not on CTCs, of which two of them responded to treatment. Two patients were PD-L1+ on CTCs but not in tissue, with one responding and the other not responding to treatment. Finally, three patients were PD-L1- in both tissue and CTCs, and all failed to respond.
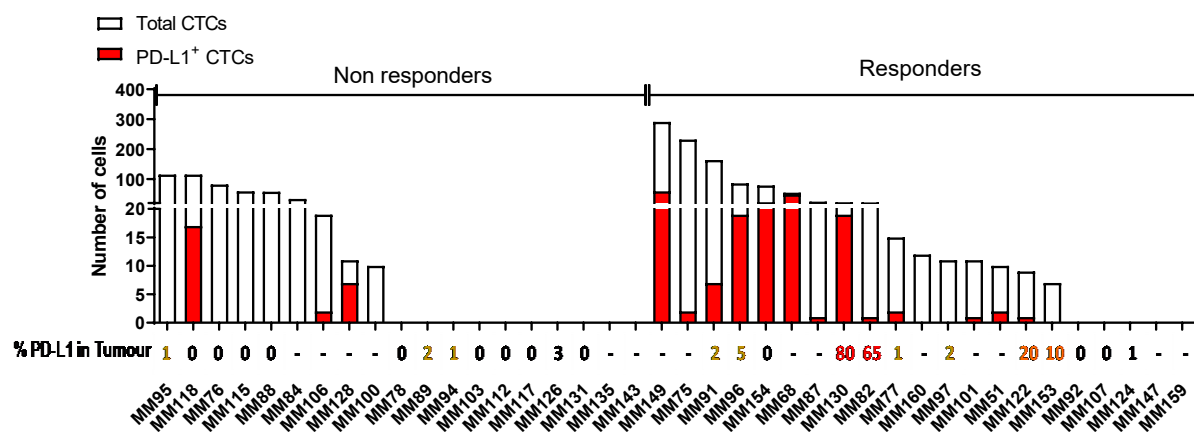
Figure 15. Proportion of total CTCs (full bars) that express PD-L1 (red bars) at baseline in patients treated with pembrolizumab monotherapy. Patients were grouped based on therapeutic objective response. Tumour Proportion Scores indicating PD-L1 expression in the tumour tissue are indicated for each patient. (-) indicates not available tissues.

Representative images of PD-L1 immunohistochemistry are shown in Figure 16. Only tumour cell PD-L1 is counted for assessment and tumour proportion score, with PD-L1 staining

depicted in brown and tumour cells being identified by pathologists. Of the three samples shown, one was concordant (MM130), and two were non-concordant with blood PD-L1 assessment.
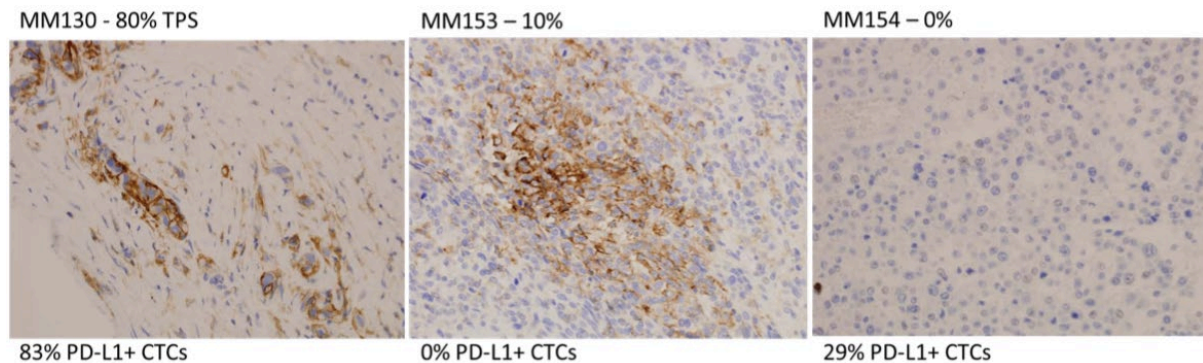
Figure 16*. Photomicrographs showing immunohistochemical staining of PD-L1 with 22C3 antibody in three of the melanoma tissues evaluated. PD-L1 staining is evidenced by the presence of the brown chromogen, whereas blue is haematoxylin counterstain. Tumour proportion score (TPS) for each sample is indicated as well as the percentage of CTCs identified as PD-L1+.*

Interestingly, PD-L1 positivity in tumour tissue (≥1% TPS) was significantly associated with response to treatment (*P=0.047*) (Figure 17B). Furthermore, there were more responders in the PD-L1+ cohort irrespective of PD-L1 assessment through tissue or CTCs (*P=0.014*), suggesting both methods could be complimentary (Figure 17C).
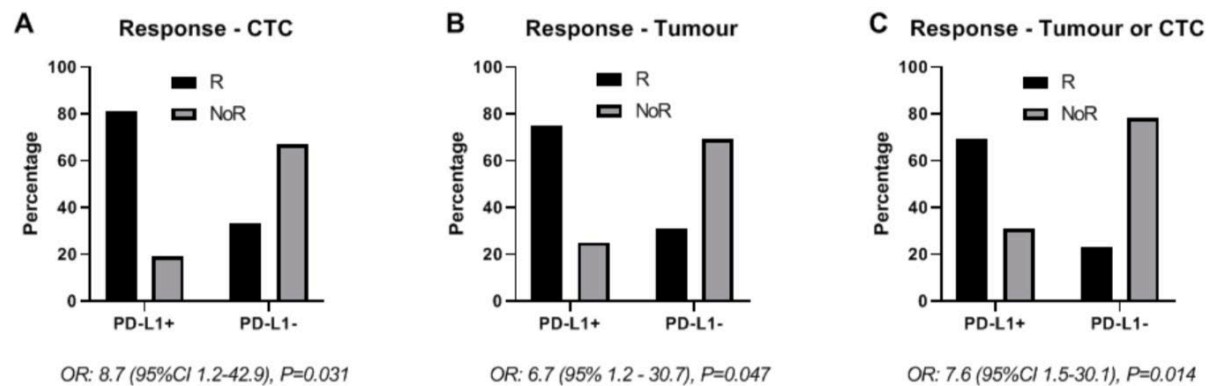
Figure 17*. Comparison of response relative to PD-L1 expression on CTCs (A), tumour tissue (B) and either CTC or tumour tissue (C). Odds ratios (OR), 95% confidence intervals and P-values of two-sided Fisher's exact tests are indicated for each graph.*

3.2. Develop an immunocytochemistry assay to evaluate PD-L1 expression in melanoma CTCs

3.2.1. Selecting a suitable anti-PD-L1 antibody

There is a range of commercially available anti-PD-L1 antibodies to detect the presence of the PD-L1 protein in tumours (Hirsch et al., 2017). Of those, 130021 (R&D systems), 22C3 (DAKO) and 28.8 (DAKO) were chosen to test for their suitability to be incorporated into an immunocytochemistry panel for identification of CTCs (Table 3). First, each antibody was tested by flow cytometry, following the protocol detailed section 2.2.2 in the Materials and Methods.

The melanoma cell line SKMEL28 was chosen to test the antibodies as it is known to constitutively express PD-L1, albeit at low levels (Johnson et al., 2016; Kim et al., 2018). Preliminary results showed that only the 28.8 antibody was effective at identifying PD-L1 expression on SKMEL28 cells with a 1.9-fold shift in median intensity relative to the isotype-matched control. Meanwhile the 130021 and 22C3 antibodies had only 1.1 and 1.2-fold shifts in median fluorescence intensity relative to their control samples respectively (Figure 18).

Table 3. *PD-L1 antibody clones assessed for suitability to include in immunocytochemistry panels.*

Clone	Company	Uses	References
130021	R&D systems	Used in previous CTC study	(Khattak et al., 2019)
22C3	DAKO	FDA approved as companion diagnostic test for pembrolizumab treatment in NSCLC patients	(Herbst et al., 2016; Reck et al., 2016)
28.8	DAKO	FDA approved as complementary diagnostic test for nivolumab treatment in NSCLC patients	(Kazandjian et al., 2016)
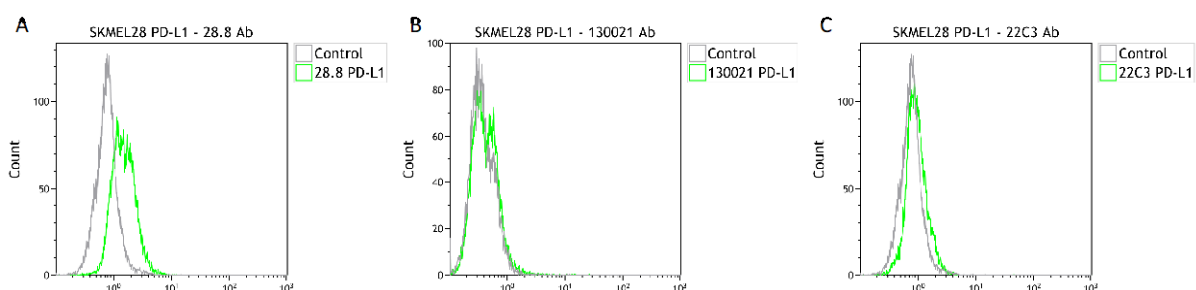
Figure 18. *Histogram plots from flow cytometric analysis of PD-L1 expression on SKMEL28 cells using the 28.8 (A), 130021 (B) and 22C3 (C) antibodies. The 130021 antibody was conjugated to FITC, while 22C3 and 28.8 antibodies were used as unconjugated primary antibodies with AF488 conjugated donkey anti-mouse and AF488 conjugated donkey anti-rabbit used as secondary antibodies*

To enhance PD-L1 expression, SKMEL28 cells were treated with different concentrations of interferon gamma (IFN-γ) over 24 hours. Cells were then analysed by flow cytometry using the 28.8 PD-L1 clone (Figure 19a). The 5.8-fold shift in median fluorescence intensity relative to the non-induced cells, demonstrated that 100 ng/ml of IFN-γ significantly induced PD-L1 expression and so this concentration was used for all subsequent IFN-γ inductions. However, neither the 130021 or 22C3 antibodies were able to clearly detect the increased PD-L1 expression on SKMEL28 cells treated with 100 ng/ml of IFN-γ. The 130021 and 22C3 antibodies had 1.2 and 1.7-fold shifts in median fluorescence intensity relative to the non-induced cells, still reading low median fluorescence intensities (Figure 19b & c). This confirms that both the 130021 and 22C3 PD-L1 antibodies cannot be used to effectively measure PD-L1 expression using flow cytometry.
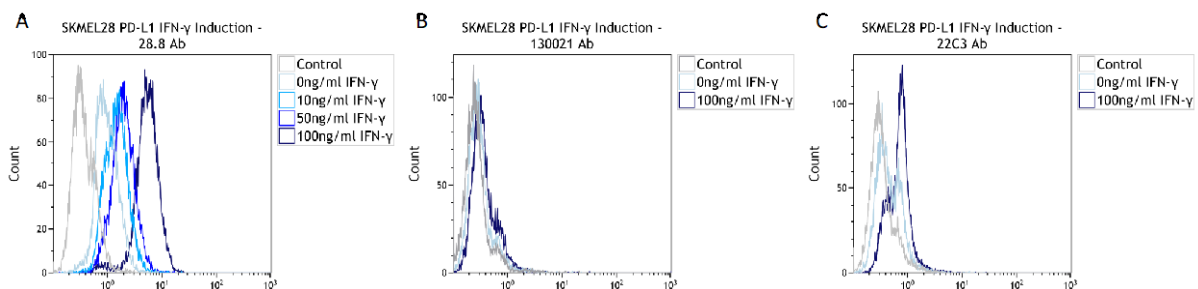
Figure 19. Histogram plots from flow cytometric analysis of PD-L1 expression on SKMEL28 cells after induction of PD-L1 expression with 100 ng/mL of IFN-γ, using the 28.8 (A), 130021 (B) and 22C3 (C) antibodies. The 130021 antibody was conjugated to FITC while 22C3 and 28.8 antibodies were used as unconjugated primary antibodies with AF488 conjugated donkey anti-mouse and AF488 conjugated donkey anti-rabbit secondary antibodies respectively. All primary antibodies were diluted 1/100, both secondary antibodies were diluted 1/500. A FITC isotype control was used as the control for the 130021 antibody. SKMEL28 cells stained with secondary antibody but no primary antibody was used as the control for 22C3 and 28.8.

As flow cytometry was only to be used to validate the antibodies and identify control cell lines and would not be used for the identification of PD-L1 on CTCs in patient samples, the three candidate PD-L1 antibodies were also tested by immunocytochemistry. SKMEL28 cells were seeded onto coverslips and allowed to grow for 48 hours before staining. Cells were then incubated in staining buffer (section 2.2.5) containing either the 28.8, 22C3 or 130021 antibody clones. The coverslips were then washed three times for 5 minutes each in 1% BSA in PBS before incubating in staining buffer containing either AF488 conjugated donkey anti-

rabbit secondary (28.8) AF488 conjugated donkey anti-mouse secondary (22C3) or no secondary antibody (130021). This was followed by washing three times for 5 minutes each in 1% BSA in PBS, washing once in PBS for 5 minutes, drying and mounting with Fluoromount Gold plus DAPI (ThermoFisher Scientific). Analysis of the immune stained cells demonstrated that only 28.8 was able to clearly detect PD-L1 expression, with the 130021 and 22C3 antibodies only producing weak, inconsistent staining (Figure 20). This conclusion was consistent with the previous flow cytometry results (Figure 18).

Next, SKMEL28 cells were seeded onto coverslips and grown for 24 hours before induction with 100 ng/mL of IFN-γ for 24 hours. Cells were then processed for PD-L1 immunocytochemistry in the same manner as described above for non-induced cells. Results demonstrated that the SKMEL28 cells incubated with IFN-γ had a significantly increased signal from the 28.8 antibody, suggesting an increase in PD-L1 expression. However, while the PD-L1 signal from these cells using the 130021 and 28.8 antibodies was higher than the signal when using the non-induced cells, it was drastically weaker than the signal from the 28.8 antibody (Figure 21). Once again, these immunocytochemistry results are consistent with the previous flow cytometry results (Figure 19). From this point forward all PD-L1 analysis used the 28.8 antibody.
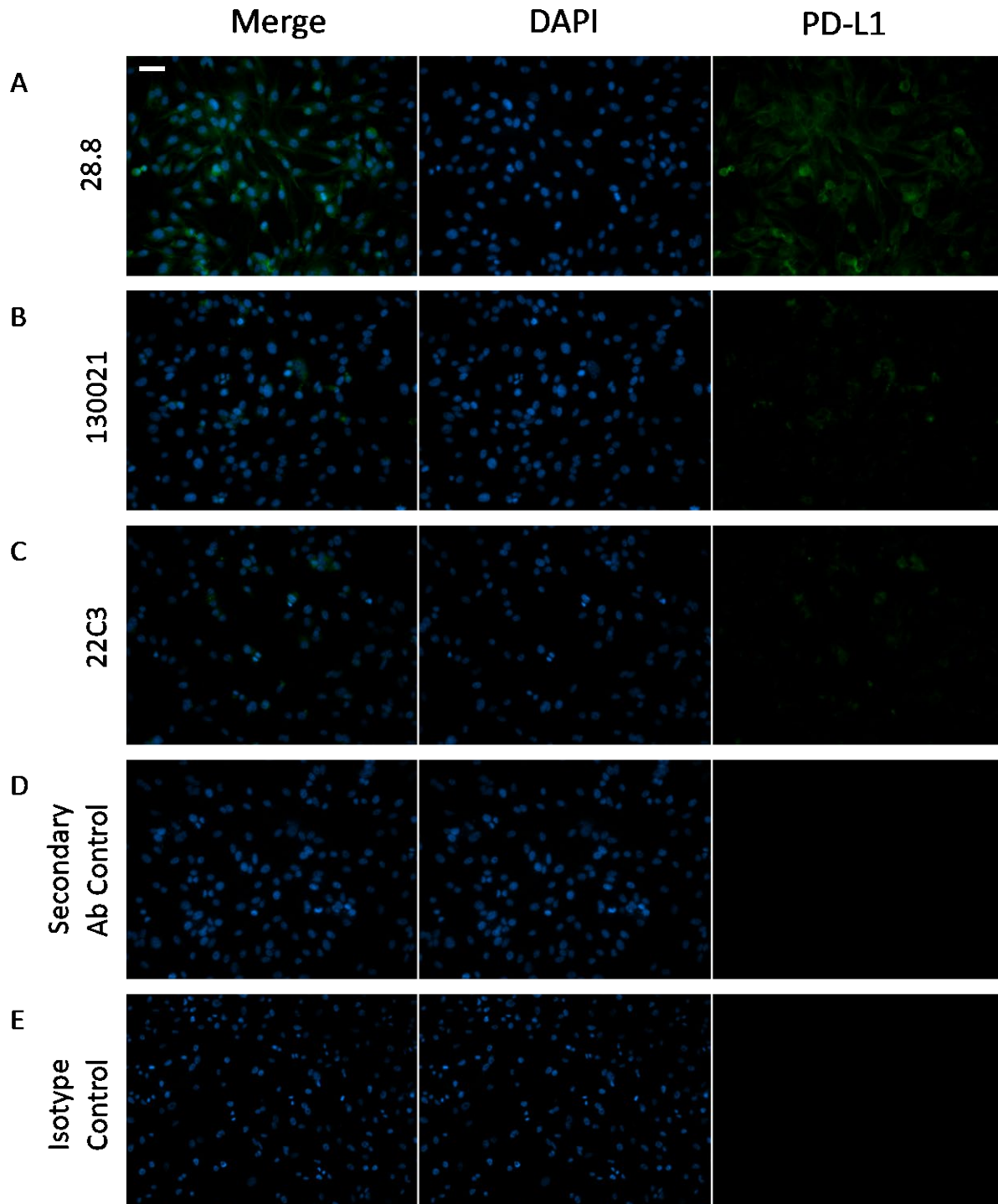
Figure 20. Photomicrographs depicting PD-L1 expression (green) on SKMEL28 cells grown on coverslips, using 28.8 (A), 130021 (B) and 22C3 (C) antibodies. Controls included a combined donkey anti-rabbit and donkey anti-mouse secondary antibodies but no primary antibody (D) and a FITC isotype control (E). The 130021 antibody was conjugated to FITC while 22C3 and 28.8 antibodies were used as unconjugated primary antibodies with AF488 conjugated donkey anti-mouse and AF488 conjugated donkey anti-rabbit secondary antibodies respectively. All primary antibodies were diluted 1/100, both secondary antibodies were diluted 1/500. Scale bar (top left) represents 50μm.
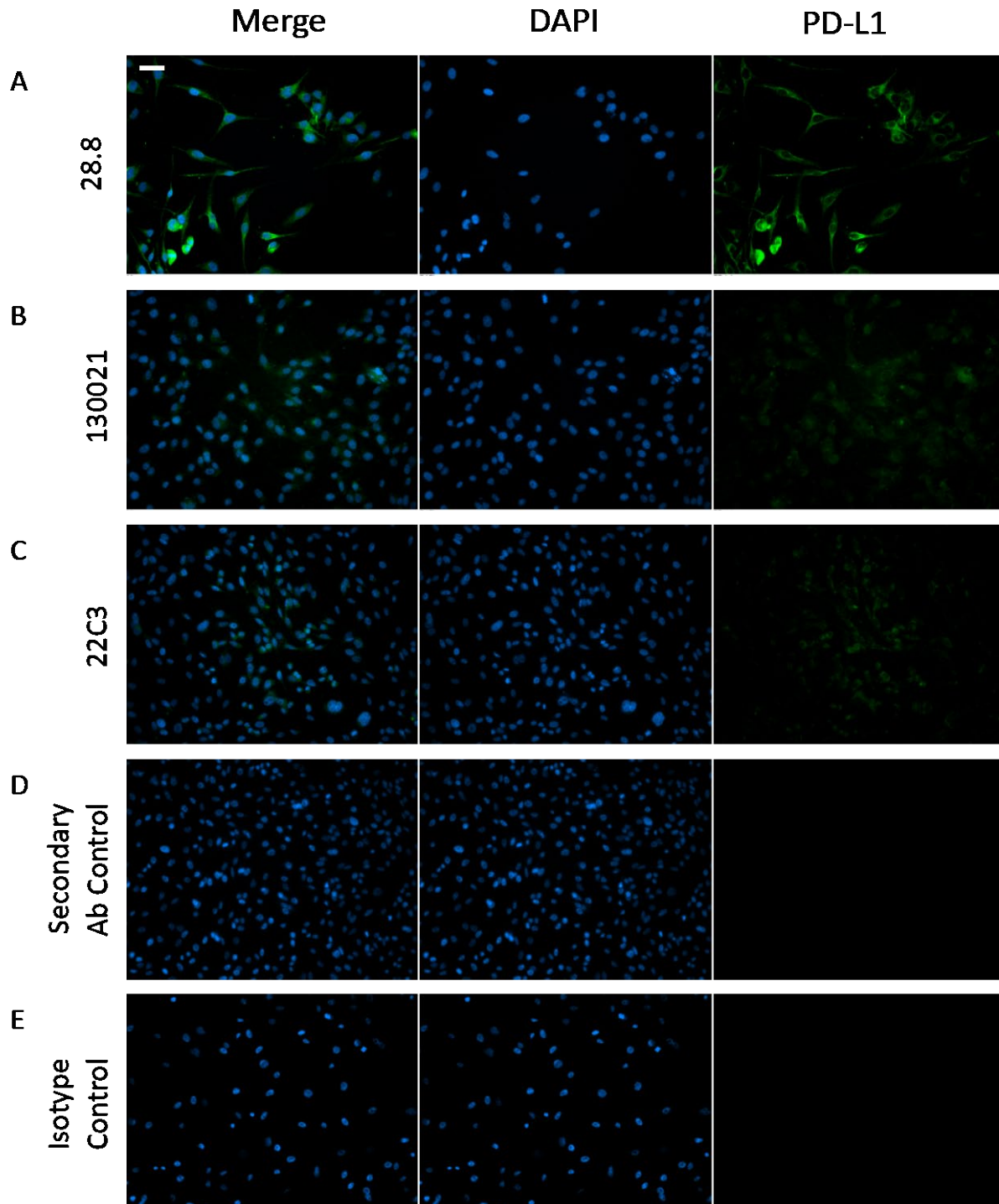
Figure 21. *Photomicrographs depicting PD-L1 expression (green) on SKMEL28 cells grown on coverslips and induced with 100 ng/mL of IFN-γ, using 28.8 (A), 130021 (B) and 22C3 (C) antibodies. A control with combined donkey anti-rabbit and donkey anti-mouse secondary antibodies but no primary antibody (D) and a FITC isotype control (E). The 130021 antibody was conjugated to FITC while 22C3 and 28.8 antibodies were used as unconjugated primary antibodies with AF488 conjugated donkey anti-mouse and AF488 conjugated donkey anti-rabbit secondary antibodies respectively. All primary antibodies were diluted 1/100, both secondary antibodies were diluted 1/500. Scale bar (top left) represents 50µm.*

3.2.2. Identifying control cell lines for PD-L1 expression to use in the melanoma immunocytochemistry panel

We aimed to identify melanoma cell lines that could serve as controls with high, low and negative PD-L1 expression. From previous results, SKMEL28 cells were found to constitutively express low levels of PD-L1 with a 1.9-fold shift in median fluorescence intensity relative to the primary control (Figure 18A), while SKMEL28 cells incubated with 100 ng/mL of IFN-γ over 24 hours had a 5.8-fold shift in median fluorescence intensity relative to the non-induced cells (Figure 19A).

Flow cytometry was used to assess several melanoma cell lines, demonstrating a range of PD-L1 expression. (Figure 22). MP41 cells expressed negligible levels of PD-L1 with a 1.25-fold shift in median fluorescence intensity relative to the primary control (Figure 22b), and therefore chosen to be used as negative control.

Overall, SKMEL28 induced with 100 ng/mL of IFN-γ for 24 hours was chosen as the high expression control, SKMEL28 without induction was chosen as the low expression control and MP41 was chosen as the negative control.
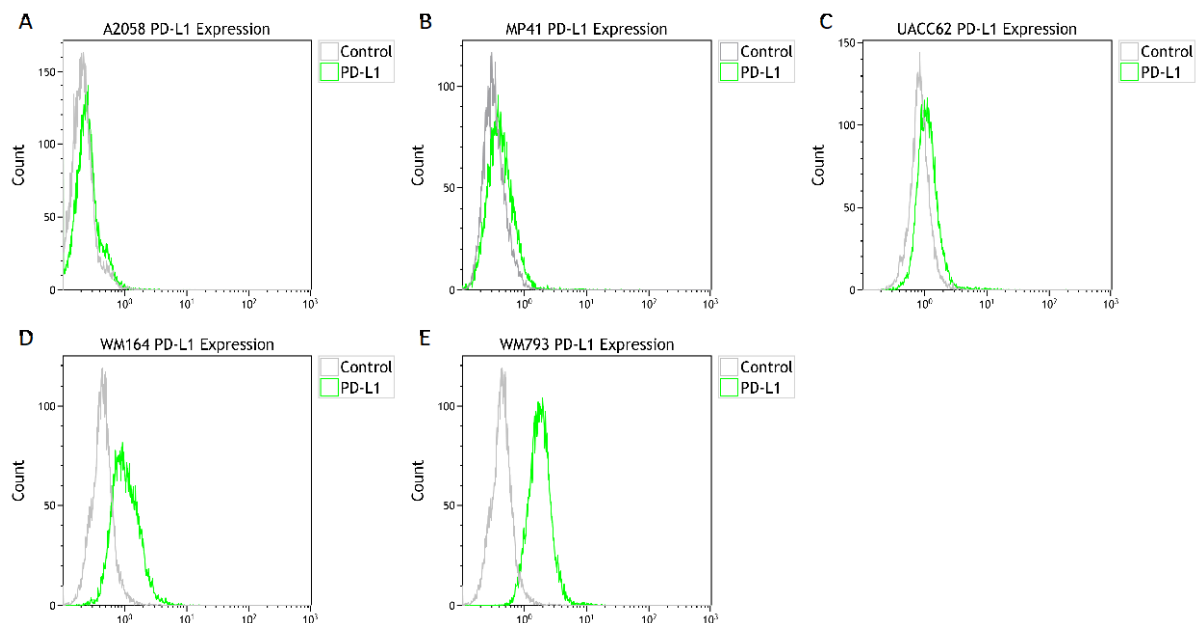
Figure 22. Histogram plots from flow cytometric analysis of PD-L1 expression on A2058 cells (A), MP41 cells (B), UACC62 cells (C), WM164 cells (D) and WM793 cells (E). The 28.8 antibody was diluted 1/100 with AF488 conjugated donkey anti-rabbit secondary antibody diluted 1/500. Cells stained with secondary antibody but no primary antibody was used as the control.

3.2.3. Developing a melanoma immunocytochemistry panel

A melanoma antibody cocktail consisting of S100, gp100 and MLANA was previously validated for detecting melanoma CTCs by Aya-Bonilla *et al*. (Aya-Bonilla et al., 2017). This melanoma marker cocktail was used to identify melanoma cells via two step staining using a donkey anti-rabbit secondary antibody bound to Alexa Fluor® 488. As this cocktail was already validated, we added the 28.8 PD-L1 antibody conjugated to Alexa Fluor® 647, to the existing immunocytochemistry protocol. SKMEL28 cells, cytospun onto a glass slide, were stained with the 28.8-Alexa Fluor® 647 antibody to ensure appropriate results. As the Alexa Fluor® 647 PD-L1 antibody is also rabbit derived, the two step staining used by Aya-Bonilla *et al*., could not be employed (Aya-Bonilla et al., 2017). To compensate, a third step was added, introducing the PD-L1 antibody only after the donkey anti-rabbit secondary and another wash step so as to avoid unintentional binding (Figure 23). However, false amplification of the PD-L1 signal was observed as the PD-L1 antibody bound to the remaining binding site on the bound donkey anti-rabbit secondary (Figure 24C).
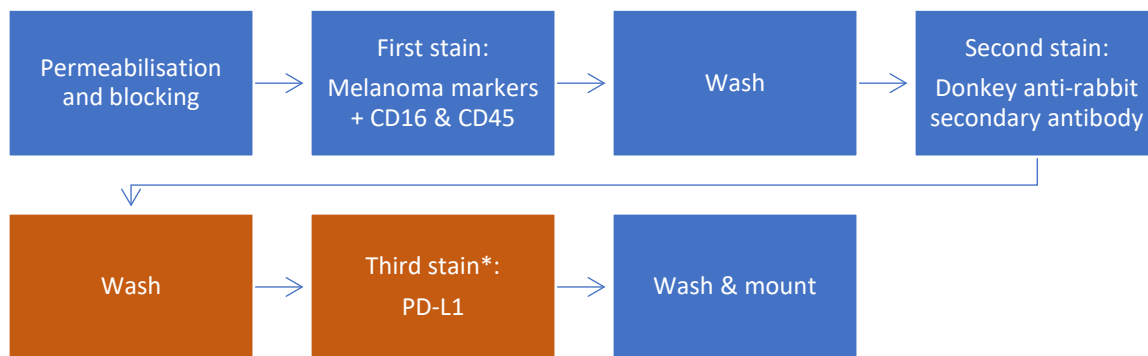
Figure 23*. Workflow of immunocytochemistry staining for melanoma samples after cytospinning onto slides. Additional steps over standard staining (Aya-Bonilla et al., 2017) are indicated in orange boxes. * indicate steps were 10% normal rabbit serum was included.*

We modified the protocol by adding 10% normal rabbit serum to the PD-L1 staining buffer used in the third stain (Step 6, Figure 23). This reduced the unspecific binding of the PD-L1 antibody to the donkey anti-rabbit conjugate, providing appropriate PD-L1 staining with similar intensity as was seen without the presence of the other antibodies, and maintaining a good staining of melanoma markers (Figure 24D).
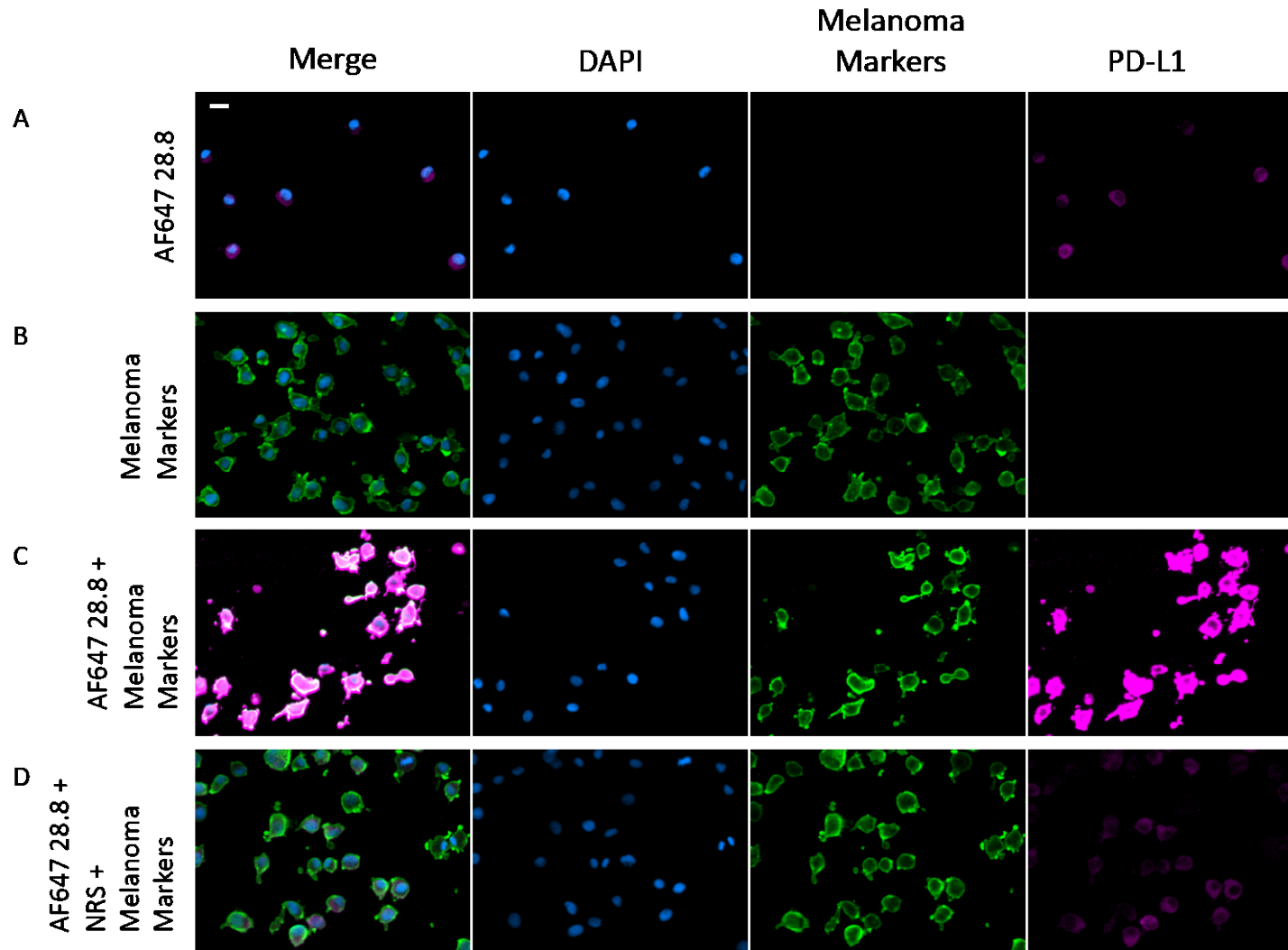
Figure 24. *Photomicrographs depicting SKMEL28 staining with the 28.8 PD-L1 antibody alone and in combination with melanoma markers. AF647 PD-L1 alone with no melanoma marker staining (A), AF488 melanoma markers alone with no AF647 PD-L1 (B), AF488 melanoma markers in combination with AF647 PD-L1 (C) and AF488 melanoma markers in combination with AF647 PD-L1 using normal rabbit serum to block non-specific binding (D). AF488 melanoma markers in green, AF647 PD-L1 in purple. The melanoma markers were used as unconjugated primary antibodies with AF488 donkey anti-rabbit secondary antibody. The melanoma marker S100 was diluted 1/500, the melanoma markers gp100 and MLANA were diluted 1/50 and the PD-L1 was diluted 1/100. Scale bar (top left) represents 20μm.*

Aya-Bonilla *et al.* used an anti-CD45 antibody to identify WBCs (Aya-Bonilla et al., 2017). However, we found that CD45 only provides staining of 60-80% of WBCs in Parsortix processed blood, as this process enriches in larger WBCs such as granulocytes which express low levels of CD45. Thus, we decided to bolster WBC identification with the addition of CD16. Both CD16 and CD45 are WBC markers, however, they can be differentially expressed on sub-populations of cells. CD45 is not expressed on natural killer cells and is only weakly expressed on granulocytes, where CD16 expression on both granulocytes and natural killer cells is higher (Cherian et al., 2010). The addition of CD16 improved the WBC staining coverage from ~80% (CD45 alone) to 100% (CD45 plus CD16) (Figure 25).

To test PD-L1 staining combined with the full immunocytochemistry panel for melanoma CTC detection, MP41, non-induced SKMEL28 and induced SKMEL28 cells were spiked into WBCs to provide control examples. Staining was carried our as described in section 2.2.5 of Materials and Methods which includes the key steps described above, addition of donkey serum with the anti-PD-L1 antibody and the use of CD45 and CD16 for WBC exclusion. The results demonstrated that the assay could effectively distinguish between high, low and negative PD-L1 expression whilst providing a good exclusion of WBCs (Figure 26). This staining protocol was then used to stain melanoma patient samples.
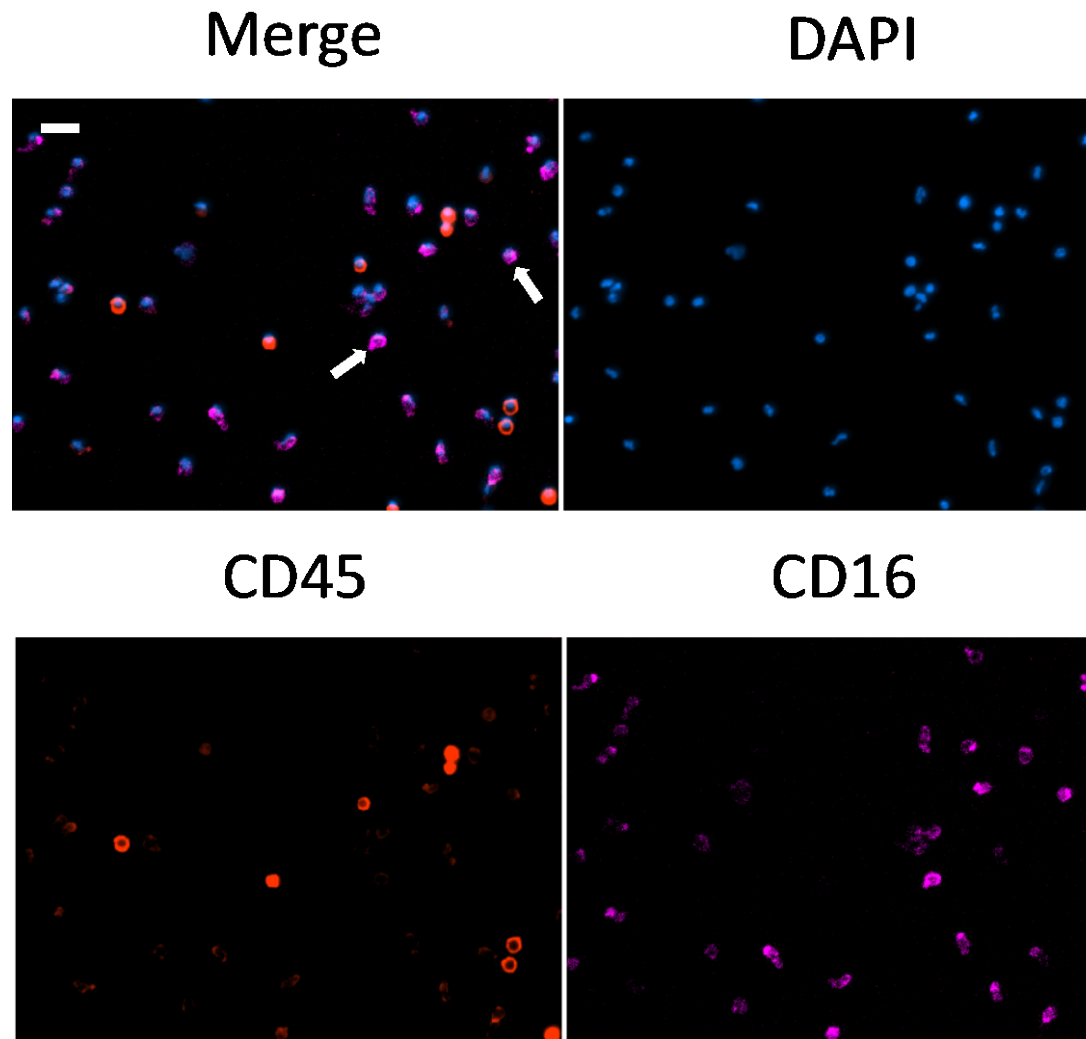
Figure 25. Photomicrograph depicting WBCs stained with CD45 conjugated to PE (red) and CD16 conjugated to Cy5 (purple). Both the CD16 and CD45 antibodies were diluted 1/50. Scale bar (top left) represents 20μm. Arrows identify cells with questionable/negative CD45 staining and strong CD16 staining.
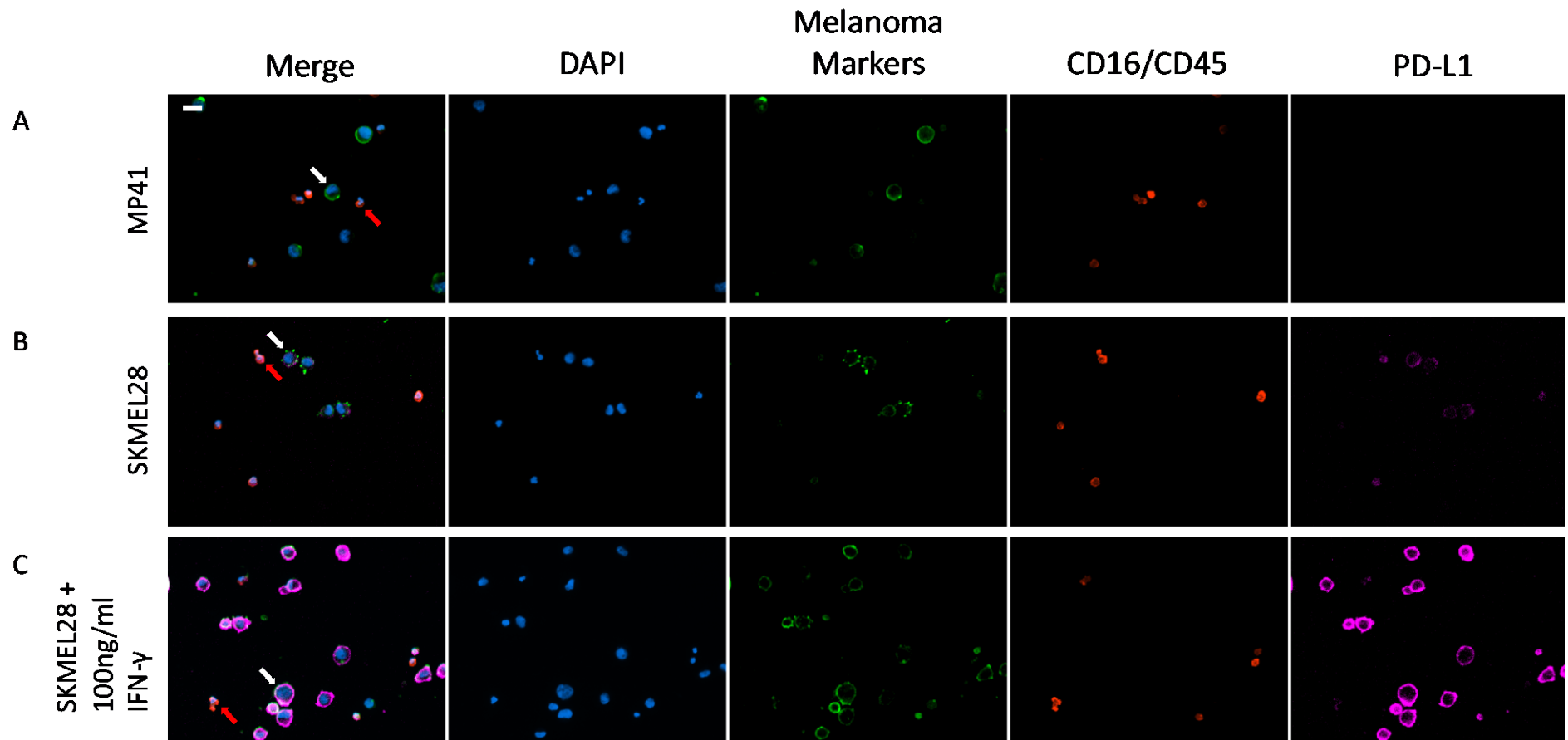
Figure 26. *Photomicrographs depicting final immunocytochemistry staining of MP41 (A), SKMEL28 (B) and SKMEL28 induced with 100 ng/mL of IFN-γ (C) spiked into WBCs. Cells were cytospun and stained with AF488 melanoma markers in green, PE CD16 & CD45 in red, AF647 PD-L1 in purple. The melanoma markers were used as unconjugated primary antibodies with AF488 donkey anti-rabbit secondary antibody. The melanoma marker S100 was diluted 1/500, the melanoma markers gp100 and MLANA were diluted 1/50, the CD16 and CD45 antibodies were diluted 1/50 and the PD-L1 was diluted 1/100. Scale bar (top left) represents 20μm. White arrows identify cancer cells, red arrows identify WBCs.*

3.2.4. Melanoma patient samples

Once the final methodology and control cell lines for assessment of the PD-L1 on melanoma cells by immunocytochemistry were developed, we proceed to analysed patient samples. As CTC analysis requires fresh blood samples recruitment and analysis were carried out from August 2019 to December 2019. Blood samples from 8 melanoma patients were collected, enriched, stained and evaluated for CTC number and PD-L1 expression. All samples were collected as baselines before commencing systemic therapy. CTCs were enriched using the Parsortix system and harvested cells were cytospun onto glass slides. The patient clinical characteristics are detailed below (Table 4).

Table 4. Clinical characteristics of melanoma patients analysed

Clinical characteristic	n
Patient median age* 77 (38-83)	8
Gender	
Male	6
Female	2
Prior therapies†	
0	3
1+	5

At the time of blood sample collection. †Prior therapies were either chemotherapy (2 patients), targeted therapy (1 patient), immunotherapy (1 patient) or all of the above (one patient).

In this pilot analysis, CTCs were detected in 3/8 (38%) of the patients, with the detected CTC number ranging from 1 to 9 (Figure 27). PD-L1 status of CTCs was evaluated in all 3 CTC positive samples, with 2/3 patients having at least one PD-L1 positive CTC (7 total). All 7 PD-L1 positive CTCs demonstrated weak PD-L1 expression (Figure 28), comparable to signal observed in non-induced SKMEL28 cells.
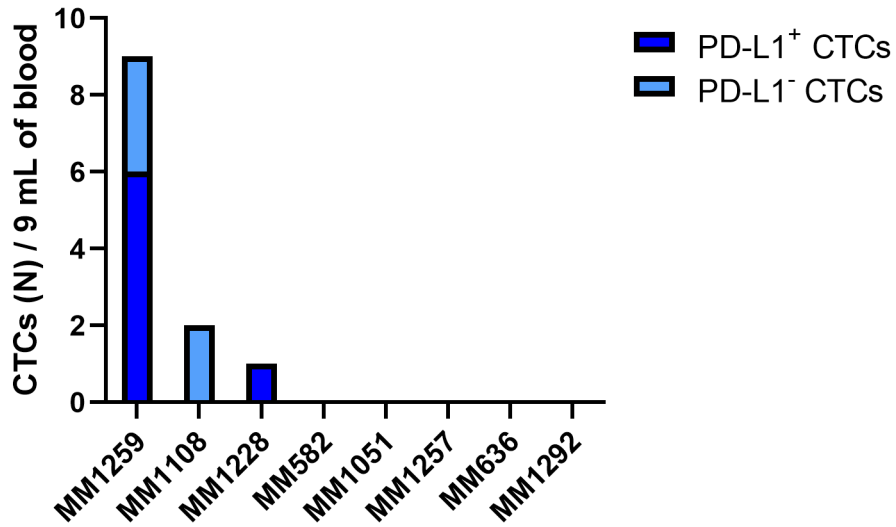
Figure 27. *Bar chart depicting the number and PD-L1 status of CTCs detected in the melanoma patient cohort.*

Of the 8 melanoma patient samples assessed, 7 samples exhibited a number of cells positive for the melanoma markers and for CD45/CD16, thus not deemed as CTCs. Some of these double positive cells also expressed PD-L1 (Figure 28).
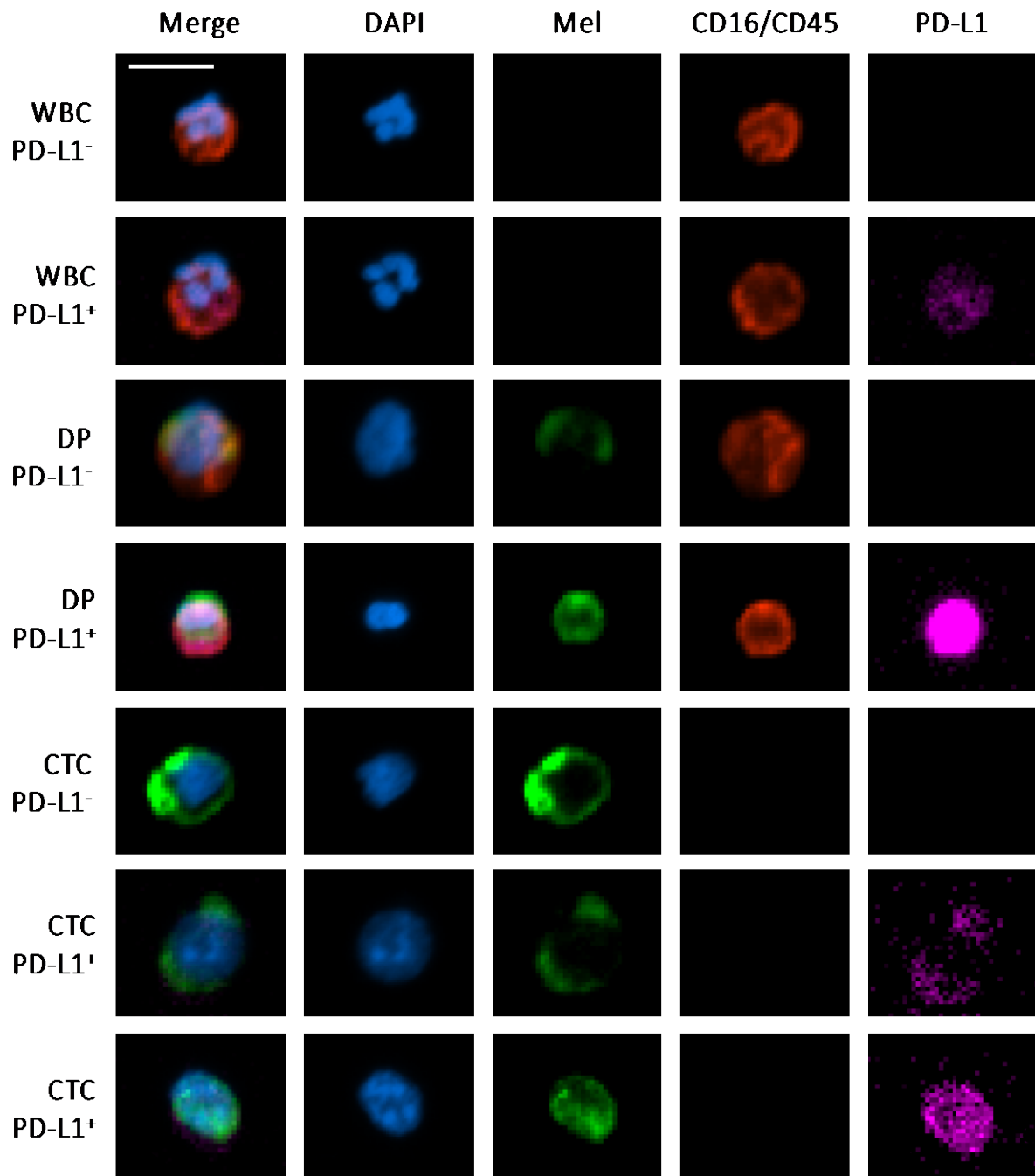
Figure 28. *Photomicrographs depicting representative cells isolated from melanoma patient blood samples. DP – Double positive for melanoma markers and CD16/CD45. AF488 melanoma markers in green, PE CD16 & CD45 in red, AF647 PD-L1 in purple. The melanoma markers were used as unconjugated primary antibodies with AF488 donkey anti-rabbit secondary antibody. The melanoma marker S100 was diluted 1/500, the melanoma markers gp100 and MLANA were diluted 1/50, the CD16 and CD45 antibodies were diluted 1/50 and the PD-L1 was diluted 1/100. Scale bar (top left) represents 10µm.*

3.3. Develop an immunocytochemistry assay to evaluate PD-L1 expression in carcinoma CTCs

3.3.1. Identifying control cell lines for PD-L1 expression to use in the carcinoma immunocytochemistry panel

We next aimed to identify carcinoma cell lines that could serve as controls with high, low and negative PD-L1 expression. Flow cytometry was used to assess three available carcinoma cell lines for PD-L1 expression (Figure 29). MDA-MB231 and MCF7 are breast cancer cell lines, while LNCaP is a prostate cancer cell line. Lung cancer cell lines were not used to develop the staining panel as none were readily available, meanwhile; both breast and prostate cancer are epithelial carcinomas, making them appropriate for use as controls in the development of this protocol. The results demonstrated that MDA-MB-231 cells constitutively expressed high levels of PD-L1 with a 9.2-fold shift in median fluorescence intensity relative to the primary control (Figure 29B). MCF7 and LNCAP cells had no apparent shift in median fluorescence intensity relative to the primary control.

MCF7 cells incubated with 100 ng/mL of IFN-γ for 24 hours had a 3.7-fold shift in median fluorescence intensity relative to the primary control (Figure 29A). Based on these flow cytometry results, MDA-MB-231 was chosen as the high expression control, MCF7 induced with 100 ng/mL of IFN-γ for 24 hours was chosen as the low expression control and MCF7 without IFN-γ induction was chosen as the negative control.
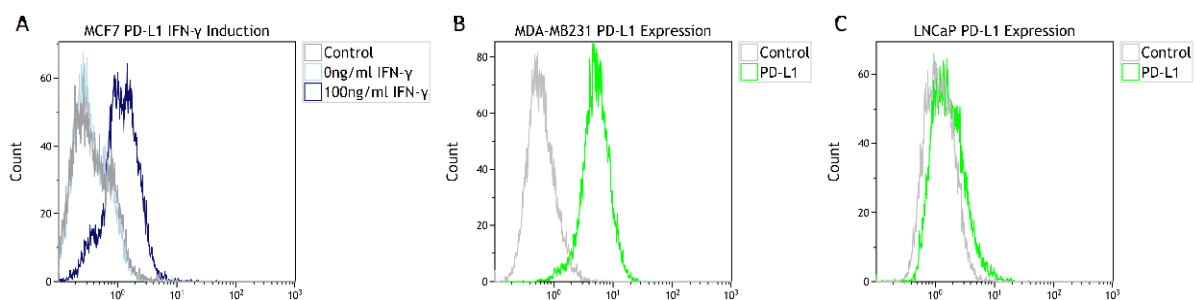
Figure 29. *Histogram plots from flow cytometric analysis of PD-L1 expression on MCF7 cells (A), MDA-MB-231 cells (B) and LNCaP cells (C) using the 28.8 antibody diluted 1/100 with AF488 conjugated donkey anti-rabbit secondary antibody diluted 1/500. Cells stained with donkey anti-rabbit secondary antibody, but no primary antibody, were used as controls. MCF7 cells were analysed with and without induction of PD-L1 expression by incubation with IFN-γ for 24 hours.*

3.3.2. Developing a carcinoma immunocytochemistry panel

We sought to devise an immunocytochemistry staining panel for detection of PD-L1 expression on carcinoma CTCs. Initially we tested a panel using three mixed pan-cytokeratins and anti-EpCAM antibodies conjugated to FITC to detect CTCs, anti-CD16 and anti-CD45 conjugated to AF647 to exclude WBCs and the 28.8 antibody conjugated to AF555 for PD-L1 assessment. This antibody combination was initially unsuccessful for carcinoma staining as the 28.8-AF555 antibody demonstrated weak staining on cells known to have high PD-L1 expression, such as MDA-MB-231 cells. This weak signal required longer exposure time (1s) to detect, causing signal from the AF647 conjugated CD16 & CD45 to bleed-through into the PD-L1 detection channel, providing false positive staining (Figure 30, note the exact colour pattern of WBC in the PD-L1 and CD16/CD45 channels). Bleed-through fluorescence from the CD16/CD45-AF647 into the PD-L1 channel was evident as not all WBCs will express PD-L1 (Ilié et al., 2017), yet every cell positive for CD16/CD45 was also positive for PD-L1. Increasing the concentration of the PD-L1 did not sufficiently increase the strength of the fluorescent signal to resolve this issue (data not shown).
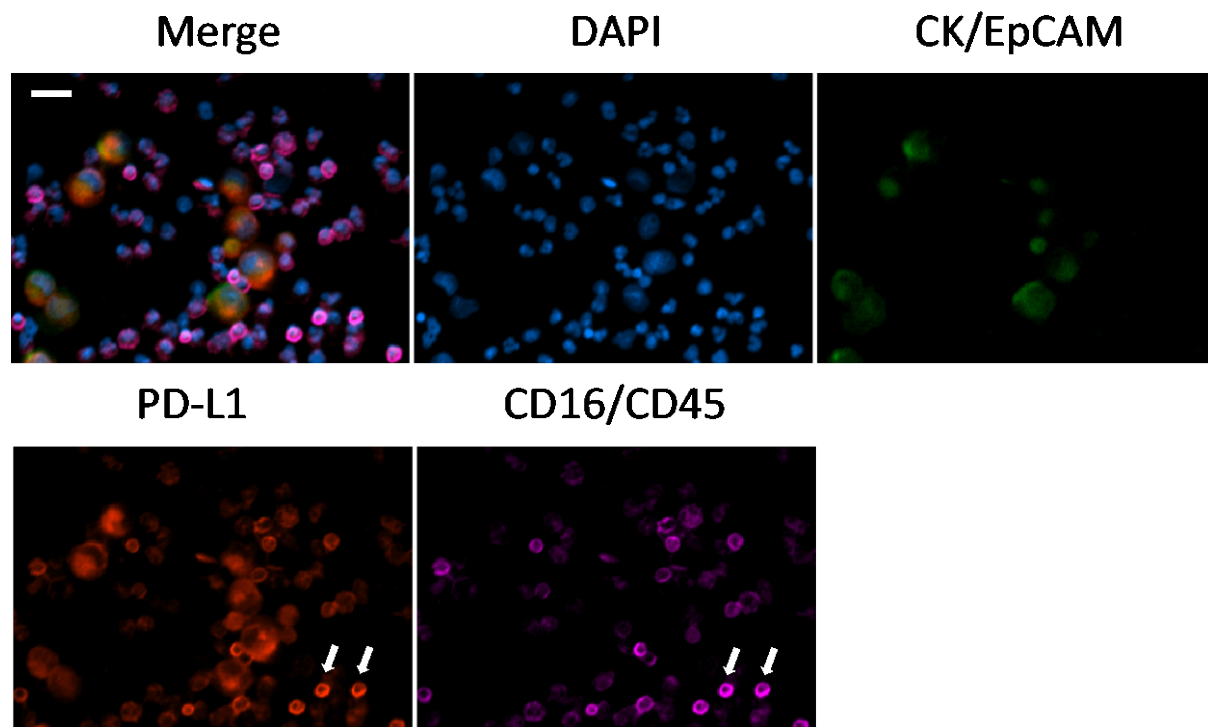
***Figure 30**. Photomicrograph depicting MDA-MB-231 cells spiked into WBCs and stained with FITC/AF488 conjugated mixed pan-cytokeratins & EpCAM (green), AF555 PD-L1 (red) and AF647 CD16 and AF647 CD45 (purple). The pan cytokeratin CK3-6H5 was diluted 1/50, the pan cytokeratin C11 was*

To increase the signal for PD-L1 detection, we next introduced a tyramide signal amplification (TSA) step. TSA is an enzyme-mediated detection technique utilising the catalytic activity of HRP to generate a high-density labelled target protein thereby providing an intensified staining signal (Litt and Bobrow, 2002). Validation of the TSA staining protocol was performed on MDA-MB-231 cells seeded onto coverslips and allowed to grow for 48 hours before staining. Cells were fixed in 4% PFA for 10 minutes and incubated in blocking buffer for 15 minutes before incubating in stain buffer containing unconjugated 28.8 PD-L1 antibody diluted 1/100 for 1 hour. Cells were then washed three times for 5 minutes each in 1% BSA-PBS before incubating in stain buffer containing anti-rabbit horseradish peroxidase diluted 1/100 for 30 minutes. Slides were again washed three times with 1% BSA-PBS for 5 minutes each before incubating in TSA Plus working solution containing a Cy3 fluorophore for 5 minutes. Following this incubation cells were washed three times for 5 minutes each in 1% BSA-PBS, washed in PBS for 5 minutes, dried and mounted with Fluoromount Gold plus DAPI. The initial concentrations of 1/100 for both the HRP and PD-L1 produced oversaturated photomicrographs (not shown). This was rectified by adjusting the concentrations of the PD-L1 and HRP to 1/400 and 1/200 respectively. The scanning settings were also adjusted to scan at 100ms without binning to provide optimal signal with minimal background (Figure 31).
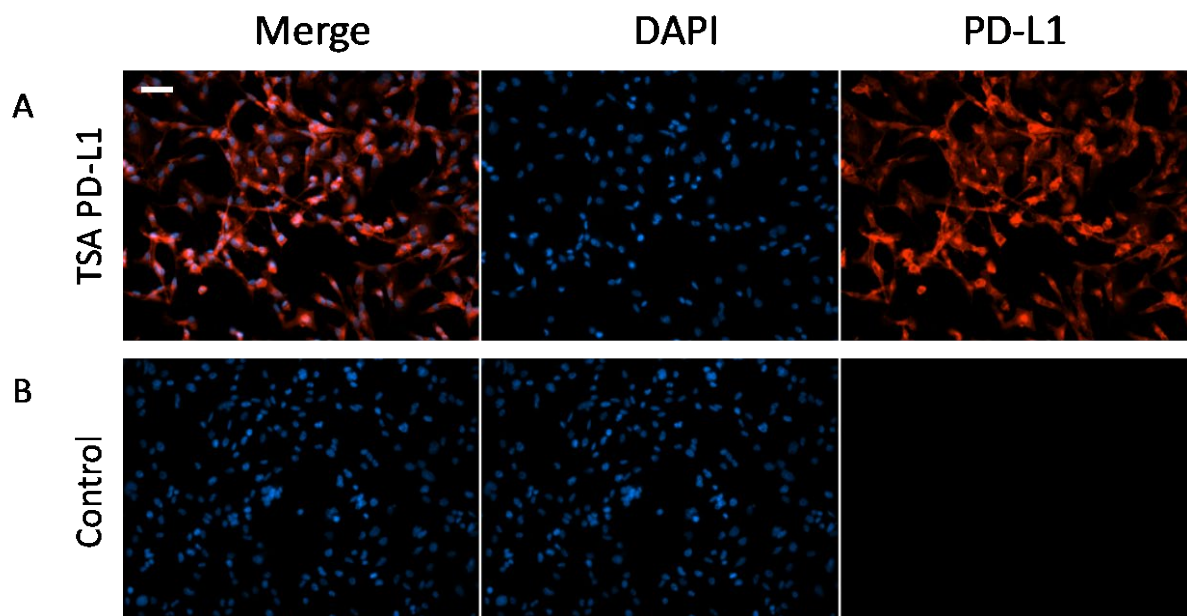
To validate the PD-L1 TSA staining in combination with the full NSCLC CTC staining protocol, MDA-MB-231 cells were spiked into WBCs and cytospun onto a glass slide. The cells were processed and stained as per the carcinoma staining detailed in section 2.3.5 of the Materials and Methods, with a few exceptions. For this test the CD16 and CD45 antibodies were conjugated to AF647 and the PD-L1 TSA was performed in tandem with the other antibody incubations.

Although the TSA successfully increased the intensity of the PD-L1, it also resulted in the TSA fluorescent signal bleeding-through into the FITC channel. The bleed into the FITC channel from the TSA affected the appearance and reliability of the cytokeratin/EpCAM staining which is critical for accurate CTC identification (Figure 32).

To overcome this problem, we separated the identification of carcinoma cells from PD-L1 detection by performing two rounds of staining on the same cells/slide. We utilised borohydride to quench the fluorescent signal after the first round of immunostaining. The borohydride quenching protocol was adapted from (Adams et al., 2016)*.* who performed the same protocol with the only difference being a reduced incubation time in borohydride of 90 minutes, compared to the 180 minutes used in our protocol. Their protocol demonstrated that a 90 minute incubation in borohydride reduced fluorescence by 99% (Adams et al., 2016). The inclusion of borohydride quenching in our new protocol would allow identification of CTCs and exclusion of WBCs in the first immunostaining, while the second stain would allow characterising PD-L1 expression.

As PD-L1 was removed from the first stain rounds in this protocol, a channel became available for assessment for additional makers. Thus, vimentin could be added to potentially identify further CTCs. Vimentin is a structural protein expressed in mesenchymal cells and can be expressed on certain cancer cells, including CTCs (Lindsay et al., 2016). Vimentin expression in epithelial cancer cells has been demonstrated to be involved in epithelial to mesenchymal transition of these cells and so can be used as a marker to detect more mesenchymal cancer cells that may have lost epithelial markers (Liu et al., 2015). Identification of these cells that

have undergone epithelial to mesenchymal transition (EMT) is of particular value as they have higher migration and invasion potential when compared to epithelial CTCs. The effects of EMT also demonstrate increased resistance to chemotherapy and immune escape capabilities beyond their epithelial counterparts (Satelli et al., 2017).

The resulting final protocol is detailed in section 2.3.5 of the Materials and Methods. Final validation of this method was performed by spiking the control cell lines (MCF7 – negative for PD-L1 expression, MCF7 induced with IFN-γ – low PD-L1 expression & MDA-MB-231 – high PD-L1 expression) into WBCs and performing the entire staining protocol (Figure 33). As expected, MCF7 cells demonstrated strong CK/EpCAM staining and no detectable vimentin staining while MDA-MB-231 cells demonstrated weak CK/EpCAM staining and strong vimentin staining. PD-L1 was expressed at low levels in the IFN-γ induced MCF7 cells and strongly expressed in the MDA-MB-231 cells. All MCF7 cells, both IFN-γ induced and not, and all MDA-MB-231 cells were negative for the WBC markers CD16 and CD45.
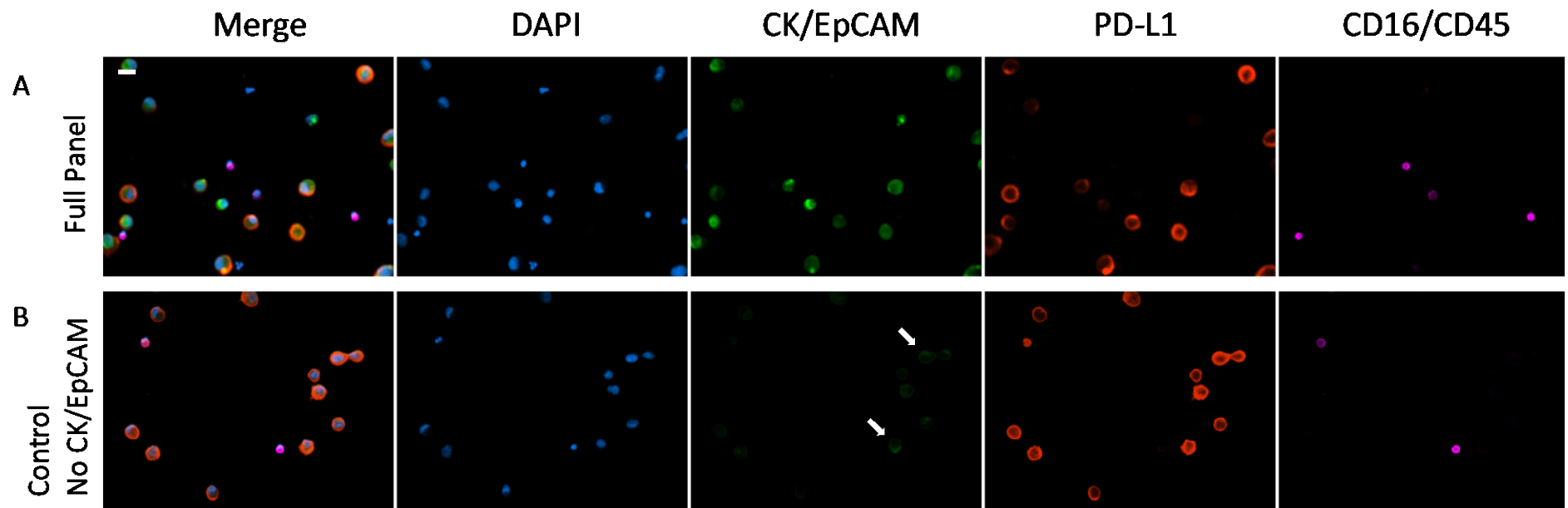
Figure 32. Photomicrograph depicting MDA-MB-231 cells spiked into WBCs and stained with Cy3 TSA PD-L1 (red), AF647 CD16 and AF647 CD45 (purple) plus FITC/AF488 conjugated mixed pan-cytokeratins & EpCAM (green) (A) and a control slide with no CK/EpCAM staining (B). The pan cytokeratin CK3-6H5 was diluted 1/50, the pan cytokeratin C11 was diluted 1/100, the pan cytokeratin AE1/AE3 was diluted 1/200, the CD16 and CD45 antibodies were diluted 1/50 and the PD-L1 antibody was diluted 1/400. Scale bar (top left) represents 20μm. Arrows identify cells where PD-L1 staining is affecting CK/EpCAM staining, evidenced by the identical staining pattern despite the absence of CK/EpCAM staining.
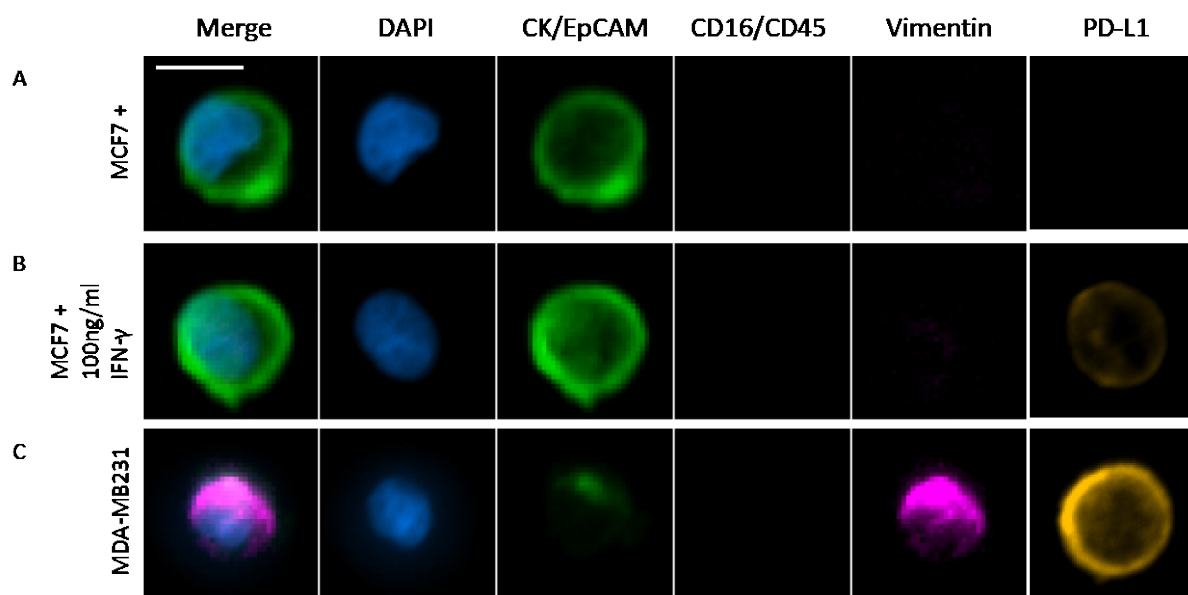
Figure 33. *Photomicrographs depicting MCF7 cells (A), IFN-γ induced MCF7 cells (B) and MDA-MB-231 cells (C) immune staining with the final carcinoma panel. Cells were stained with FITC/AF488 mixed pan-cytokeratins and EpCAM (green), PE CD16 and CD45 (red), AF647 vimentin (purple) and Cy3 TSA PD-L1 (yellow). The pan cytokeratin CK3-6H5 was diluted 1/50, the pan cytokeratin C11 was diluted 1/100, the pan cytokeratin AE1/AE3 was diluted 1/200, the CD16 and CD45 antibodies were diluted 1/50 and the vimentin antibody was diluted 1/100. The PD-L1 stain was performed after borohydride quenching and the PD-L1 antibody was diluted 1/400. Scale bar (top left) represents 10µm.*

3.3.3. NSCLC patient samples

Once the methods for the carcinoma PD-L1 immunocytochemistry panel were developed, NSCLC patient samples were analysed from May 2019 to December 2019. The patient clinical characteristics are detailed below (Table 5). Blood samples from 14 stage IV NSCLC patients were collected, enriched, stained and evaluated for CTC number and PD-L1 expression. All 14 blood samples were collected before commencing systemic therapy and processed by the Parsortix system. Seven of the 14 patients had a second sample processed by the ClearCell FX system for comparison.

Table 5. Clinical characteristics of NSCLC patients analysed.

Clinical characteristic	n
Patient median age* 67 (54-87)	14
Gender	
Male	9
Female	5
Histopathology	
Adenocarcinoma	5
Squamous-cell carcinoma	9
Stage IV subclass*	
M1a	4
M1b	4
M1c	6
Prior therapies	
0	9
1+	5
Median days between biopsy and blood	65 (30-565)

All prior therapies were chemotherapy cycles
*At the time of blood sample collection.

CTCs were detected in 5/14 (36%) of the patients analysed. In the 5 patients with detectable CTCs, the CTC number ranged from 1 to 214 (median number of CTCs = 8) (Figure 34). CTCs were detected in 4/14 total patient samples processed through the Parsortix system. For the 7 patients with samples processed through both enrichment systems; CTCs were detected in 3/7 (43%) patient samples after enrichment with the Parsortix system, while CTCs were detected in 2/7 (29%) patient samples after enrichment with the ClearCell FX system. Only one patient had CTCs detected after enrichment through both systems (L909), and it was the case with the largest number of CTCs identified. Thus, the lack of concordance between the systems might be the result of heterogeneous distribution of low number of CTCs in the collection tubes.

The identified CTCs were classified into three categories, cytokeratin/EpCAM positive - classical CTCs, vimentin positive - epithelial to mesenchymal transition (EMT) CTCs and cytokeratin/EpCAM & vimentin positive - hybrid CTCs. Classical CTCs were detected in 2/14 (14%) patients, EMT CTCs were detected in 4/14 (29%) patients and hybrid CTCs were detected in one patient. The number of classical CTCs detected ranged from 1 to 3 and the number of EMT CTCs ranged from 4 to 126 (median number of EMT CTCs 11.5). Six Hybrid CTCs were detected in the patient with the largest number of CTCs (L909) (Figure 35), with all of them detected in the sample enriched using the ClearCell FX system.
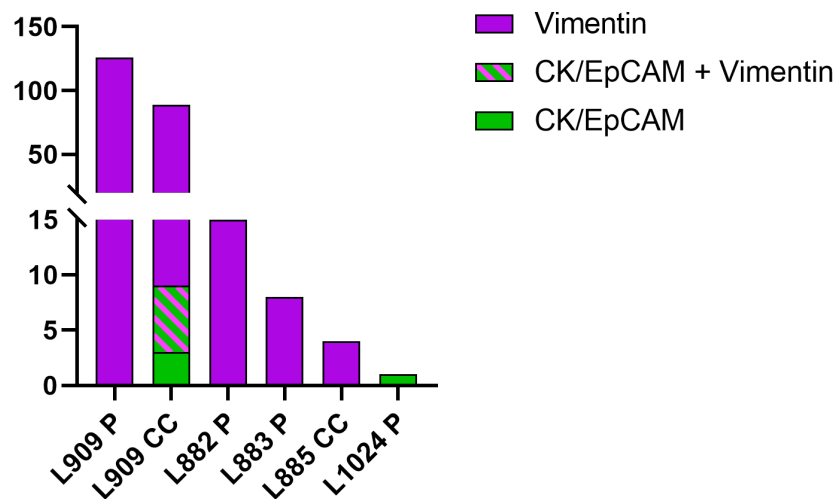
Figure 35. Stacked bar chart depicting the breakdown of CTC sub-populations detected in NSCLC patients. Samples processed through Parsortix – P, Samples processed through ClearCell FX – CC.

Significant phenotypic and morphological heterogeneity was observed in CTC detected in patient blood samples (Figure 36). CTCs were classified as DAPI$^+$, CD16/CD45$^-$, Cytoketatin/EpCAM$^+$ (classical CTCs) and/or Vimentin$^+$ (EMT CTCs) (Figure 36).

Of the 14 melanoma patient samples assessed, 10 samples exhibited a number of cells positive for CK/EpCAM and for CD45/CD16, thus not deemed as CTCs. Some of these double positive cells also expressed PD-L1 (Figure 28).
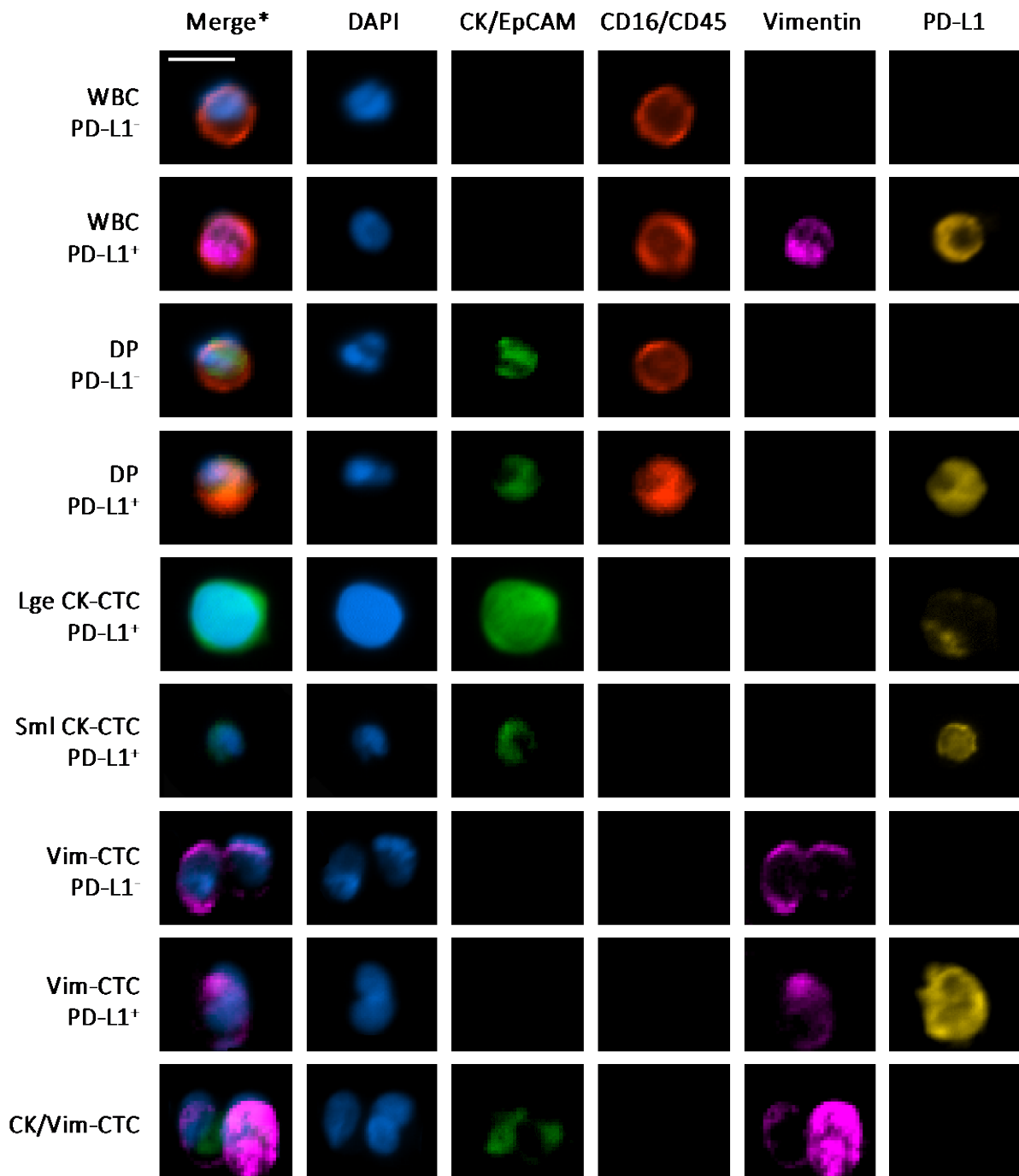
Figure 36. *Photomicrographs depicting representative cells isolated from NSCLC patient blood samples stained with FITC/AF488 mixed pan-cytokeratins and EpCAM (green), PE CD16 and CD45 (red), AF647 vimentin (purple) and Cy3 TSA PD-L1 (yellow). The pan cytokeratin CK3-6H5 was diluted 1/50, the pan cytokeratin C11 was diluted 1/100, the pan cytokeratin AE1/AE3 was diluted 1/200, the CD16 and CD45 antibodies were diluted 1/50 and the vimentin antibody was diluted 1/100. The PD-L1 stain was performed after borohydride quenching and the PD-L1 antibody was diluted 1/100. DP – double positive for cytokeratin/EpCAM and CD16/CD45. *Merge images do not include PD-L1 photomicrographs. Scale bar (top left) represents 10μm.*

PD-L1 status of CTCs was evaluated in all 5 CTC positive samples, with 4/5 patients having ≥1 PD-L1 positive CTC. The number of PD-L1 positive CTCs ranged from 1-167 (median PD-L1 positive CTCs 3.5) and the fraction of PD-L1 positive CTCs ranged from 0 to 100% of the total number of detectable CTCs (Figure 37). PD-L1 positive CTCs were further split into PD-L1 weak and PD-L1 strong CTCs based on controls, with the majority (117/175 or 67%) being strongly PD-L1 positive comparable to the signal in MDA-MB-231 cells.

All 14 patients had tumour tissue evaluated for PD-L1 status using DAKO autostainers and the 22C3 PD-L1 clone. PD-L1 status of tumour tissue ranged from 0 to 95% (median tissue PD-L1 status 75%) as determined by pathologists. Tissue PD-L1 status was compared to CTC number and PD-L1 status for the 5 CTC positive patients. No clear correlation between PD-L1 positivity in CTCs and tumour tissue was observed (Figure 37). For example, L882 had a tissue PD-L1 score of 95% while only 40% of CTCs were positive for PD-L1. On the other extreme L909 was PD-L1 negative on the tumour but 86% of CTCs evaluated were positive for PD-L1.
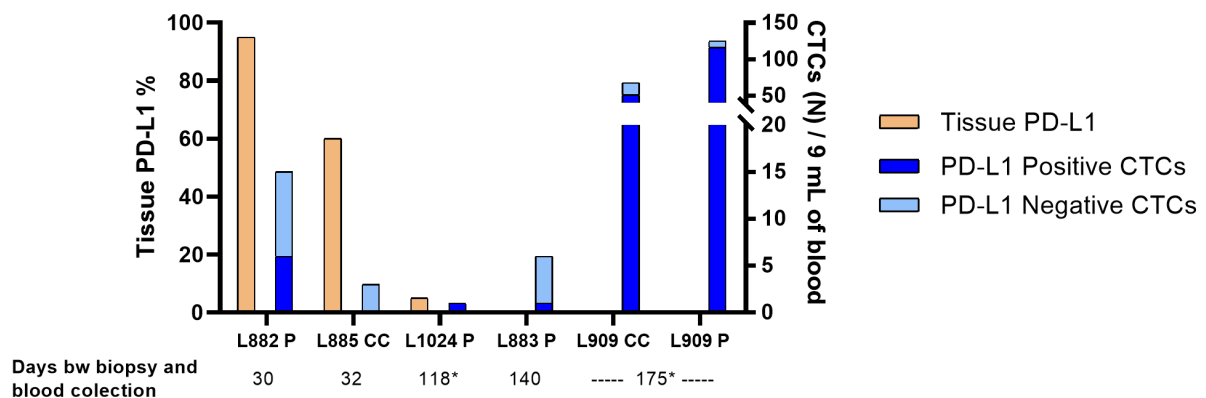
Figure 37. Bar chart comparing PD-L1 status of tissue to number and PD-L1 status of CTCs. Only including patients with detectable CTCs. Tissue PD-L1 percentage is depicted in beige, CTC number is depicted in blue split into PD-L1 positive CTCs (dark blue) and PD-L1 negative CTCs (light blue). Samples processed through Parsortix – P, Samples processed through ClearCell FX – CC. * These patients received first-line chemotherapy between tissue and blood collection.

Importantly, there were substantial differences between the dates of tissue biopsy and blood sample collection for all 14 cases, ranging from 30-365 (median delay 65 days. The delays are shown in Figure 37 with two patients receiving chemotherapy as first-line treatment. This may explain the discordances observed on PD-L1 expression.

4. CHAPTER 4: Discussion

4.1. CTC detection in melanoma patients

PD-L1 expression in tissue has been linked to anti-PD-1 therapy outcomes in melanoma patients by previous studies. However, this association has not been statistically significant or clinically applicable (Daud et al., 2016; Kaunitz et al., 2017; Topalian et al., 2012). PD-L1 expression in tissue has the potential to be a predictive marker, though, inter- and intra-tumour heterogeneity pose a problem if only a single tumour is biopsied for tissue PD-L1 analysis. Meanwhile, analysis of PD-L1 expression on CTCs has been heralded as a potential alternative as CTCs could be representative of all tumours within the body, accounting for heterogeneity. However, PD-L1 expression on CTCs has not been sufficiently studied to provide conclusive evidence of clinical utility. From our results, two clear limiting factors for evaluation of PD-L1 expression on CTCs are (i) the low number of detectable CTCs and (ii) the low CTC detection rate in melanoma patients requiring systemic treatment.

Studies assessing CTC detection in melanoma patients by immunocytochemistry have produced wildly inconsistent results, with detection rates ranging from 23 – 100% (Freeman et al., 2012; Joshi et al., 2014; Khoja et al., 2013; Po et al., 2019; Rao et al., 2011). The detection rate of 38% in this study is therefore still comparable to that of other studies. However, an interesting comparison can be made to a publication by Joshi *et al*. where they found CTCs in 100% of patient samples (Joshi et al., 2014). Like in our study, Joshi *et al*. used MLANA and S100 (albeit different antibody clones) to identify melanoma CTCs and used CD45 as a negative marker for WBCs. The significant differences between the studies are the enrichment methods, where this study used the Parsortix system and they used CD45 negative selection. Notably, we added anti-gp100 and anti-CD16 antibodies to the immunocytochemistry panel. The addition of CD16 allows exclusion of neutrophils and monocytes which stain low for CD45, improving WBC coverage. As a result, we detected double positive cells at a much higher frequency than CTCs. We found double positive cells (i.e. positive for melanoma and WBC markers) in 7/8 patient samples analysed. Joshi *et al*. and other studies mention detecting double positive cells expressing melanoma and WBC markers, but do not provide any further information, making comparison between studies difficult. The identity and role of these double positive cells are currently unknown, with them potentially being tumour-leukocyte hybrids, the result of staining artifacts or something else entirely.

To date, there have only been two other studies assessing PD-L1 expression on melanoma CTCs. In the earliest study by Khattak *et al.* we detected PD-L1 expression on 16/25 patients with the PD-L1 positive fraction of CTCs ranging from 1-89%. In this study, we found that CTC PD-L1 status was superior to other baseline clinical parameters associated with response and prognosis, including lactate dehydrogenase, disease stage and ECOG performance status (Khattak et al., 2019). However, CTCs analysed by immunocytochemistry can be recovered and used for some downstream analysis such as assessing copy number alterations to confirm that they are CTCs (Beasley et al., 2018; Carter et al., 2017). On the contrary, cells analysed by flow cytometry are lost, allowing no further analysis to be performed. It is this reasoning that led to the development of the immunocytochemistry panel for PD-L1 assessment on melanoma CTCs for our second study. For this second study, we used the Parsortix system to enrich for CTCs and developed a multimarker immunocytochemistry panel to detect them. From this cohort, we detected PD-L1 positive CTCs in 2 of the only 3 CTC positive patients. The results highlight that PD-L1 expression can be easily assessed, but the low detection rate is problematic.

As covered in section 3.2.1, the 130021 PD-L1 antibody was used in our previous study (Khattak et al., 2019), and assessed for inclusion in this second study. Interestingly, we found that 130021 could not effectively measure PD-L1 expression on cell lines when processed by either flow cytometry or immunocytochemistry. These findings contradict the results from the previous study, which demonstrated detectable PD-L1 expression on CTCs from melanoma patients (Khattak et al., 2019). This may have been caused by various factors, the most likely of which I believe to be different presentation of the PD-L1 protein between fresh CTCs and the cell lines. The epitope identified by the 130021 antibody may be more readily accessible on CTCs when compared to cell lines or to tissue, thus explaining the difference in antibody effectiveness. This could not be assessed for the second study as the antibody panel needed to be validated using cell lines before being used on patient samples.

Po *et al.* published the only other study to have assessed PD-L1 expression on melanoma CTCs, they used a multimarker immunocytochemistry panel to detect melanoma CTCs and assess PD-L1 expression. Po *et al.* detected PD-L1 expressing CTCs in 38% of CTC positive patients, a lower fraction compared to both our flow cytometry (64%) and immunocytochemistry studies (66%) (Khattak et al., 2019; Po et al., 2019). However, the

limited cohort sizes of these immunocytochemistry studies do not allow for statistically significant analysis to be performed. With only one of these three studies reporting any predictive and prognostic data, it is not yet possible to determine if PD-L1 expression on CTCs may have the potential to be either a predictive or a prognostic marker.

Only one study has compared PD-L1 expression on CTCs to that in matched tissue samples used flow cytometry for CTC analysis (Khattak et al., 2019). Patients with PD-L1 positivity on CTCs or tumour tissue had a high probability of response suggesting both methods could be complimentary, with concordant results in tissue and CTCs predicting response to treatment with 100% accuracy (Khattak et al., 2019). Intra-tumour heterogeneity could impact the correlation between CTC PD-L1 expression and tissue PD-L1 expression, due to the potential for different sections of tissue to produce significantly different results (Madore et al., 2015). This is compounded by inter-tumour heterogeneity as the PD-L1 status of different tumours within a single patient may be completely different.

4.2. CTC detection in NSCLC patients

The average CTC detection rate in patients with metastatic NSCLC was 76%, based on the 9 recent publications assessing PD-L1 expression on CTCs detailed in our review (Section 1.6.1) (Adams et al., 2017; Boffa et al., 2017; Dhar et al., 2018; Guibert et al., 2018; Ilié et al., 2017; Kallergi et al., 2018; Kulasinghe et al., 2019; Nicolazzo et al., 2016; Wang et al., 2019). In comparison, the detection rate of CTCs in our study was 36%, much lower than that seen these recent reports. However, a combination of different CTC enrichment methods, staining and classification of CTCs could explain this discordance.

Of the 9 recently published studies, 8 used label-free detection methods, with 6 different enrichment techniques being employed (Adams et al., 2017; Boffa et al., 2017; Dhar et al., 2018; Guibert et al., 2018; Ilié et al., 2017; Kallergi et al., 2018; Kulasinghe et al., 2019; Wang et al., 2019). Though this would seem to be more comparable to the data from this study, the differences in CTC detection between the two label-free detection methods utilised in this study suggest that there can be significant differences in the cell populations produced by each.

Of the 9 recent publications assessing PD-L1 on NSCLC CTCs, 6 used CD45 as a negative marker for CTCs. However, none of the publications elected to use more than one negative marker beyond CD45 (Adams et al., 2017; Boffa et al., 2017; Dhar et al., 2018; Guibert et al., 2018; Ilié et al., 2017; Kallergi et al., 2018; Kulasinghe et al., 2019; Nicolazzo et al., 2016; Wang et al., 2019). This study found that the use of CD16 significantly reduced the number of false positive CTCs detected by immunocytochemistry, by correctly identifying them as double positive cells (DAPI$^+$, CK/EpCAM$^+$ & CD16/CD45$^+$). We detected double positive cells in 10/14 (71%) NSCLC patients. As the double positive cells were generally more abundant than CTCs, these numbers more closely matching the average rate of CTC detection seen in published studies.

Use of the EMT marker vimentin to identify CTCs has the caveat of needing to exclude vimentin expressing WBCs. However, we believe that including cell features as well as a combination of negative markers (CD16/CD45) removes the background of vimentin expressing WBCs. We found that including vimentin significantly increased the detection of CTCs in our patient cohort, none of the 9 previously mentioned recent studies utilised EMT markers (Adams et al., 2017; Boffa et al., 2017; Dhar et al., 2018; Guibert et al., 2018; Ilié et al., 2017; Kallergi et al., 2018; Kulasinghe et al., 2019; Nicolazzo et al., 2016; Wang et al., 2019).

Another difference to consider between our study and the 9 recent NSCLC CTC studies is the type and number of previous treatments. In our study, 5 of the 14 patients had received chemotherapy. However, our cohort size was too small to determine what affect prior chemotherapy treatments had on CTC number or PD-L1 expression in the second line patients. Of the 9 previously mentioned studies, 4 had treatment naïve patient cohorts, 1 had only chemotherapy relapse patients and the remaining 4 did not specify if patients had prior treatments or what prior treatments they may have received. Prior treatments may have differing effects on the tumours present in a patient depending on both how recent it was and the treatment type. Any impact that a treatment has on the tumour will likely affect the type and number of CTCs present in the blood, though the degree to which treatments affect CTC production are relatively unknown.

Our study demonstrated that CTC PD-L1 expression was not concordant with tissue PD-L1 expression. Recent publications have had mixed results when assessing the correlation

between the expression of PD-L1 on CTCs and its expression in tumour tissue (Adams et al., 2017; Dhar et al., 2018; Guibert et al., 2018; Ilié et al., 2017). In addition to our study, two of the four published studies had limited size patient cohorts, thus preventing statistical analysis (Adams et al., 2017; Dhar et al., 2018). However, the two studies with large patient cohorts reported contrasting results, with Ilié *et al.* (n=106) suggesting that the two were 93% concordant while Guibert *et al.* (n=96) found no statistically significant correlation between the two (Guibert et al., 2018; Ilié et al., 2017). The contrasting results are likely due to a variety of factors that differ from study to study. Labelled and label-free CTC enrichment techniques are known to isolate different numbers and populations of CTCs from similar patient cohorts (Adams et al., 2017; Boffa et al., 2017; Dhar et al., 2018; Guibert et al., 2018; Ilié et al., 2017; Kallergi et al., 2018; Kulasinghe et al., 2019; Nicolazzo et al., 2016; Wang et al., 2019). This study even demonstrated differences in CTC detection between two label-free enrichment techniques, adding another cofounding factor for concordance between CTC and tissue PD-L1.

Another reason for the concordance, or lack thereof, between CTCs and tissue may be the delay between biopsy and blood sample collection. Our study had significant differences in the delay between tissue biopsy dates and blood sample collection, ranging from 30-365 (median delay 65 days). Of the 4 studies correlating CTC PD-L1 expression with tissue PD-L1 expression, 3 do not state the delay between sample collections (Adams et al., 2017; Dhar et al., 2018; Guibert et al., 2018). Meanwhile Ilié *et al.* collected both samples on the same day and found that they had statistically significant concordance between PD-L1 expression on the samples (Ilié et al., 2017). It is theorised that CTCs may utilise the same tumour escape mechanisms as the tumour from which they derive. However, the longer the delay between tissue and blood samples, the more opportunity the tumour has to change and express different surface markers.

Inter- and intra-tumour heterogeneity may also have a significant impact on correlation. It is believed that CTCs may arise from any tumour, yet only a single tumour is biopsied for tissue PD-L1 analysis. Intra-tumour heterogeneity could impact the correlation between CTC PD-L1 expression and that of tissue as the section of tissue being assessed can significantly affect the results (Ilie et al., 2016). This is compounded by inter-tumour heterogeneity as the PD-L1 status of different tumours within a single patient may be completely different.

Finally, the PD-L1 antibodies used to characterise the PD-L1 expression on CTCs and in tissue samples are likely to affect correlation between the two. Though efforts were made to ensure the same clone was used for both applications, this study used the 28.8 clone (DAKO) for CTCs and the 22C3 clone (DAKO) for tissue samples. The subtle differences in binding domains of each antibody is known to produce different staining patterns in tissue samples (Hirsch et al., 2017; Tsao et al., 2018). This study also observed significant differences in the pattern and intensity of ICC PD-L1 staining between three commonly used clones. The potential differences in PD-L1 characterisation from using different clones are likely to be compounded by using different clones for each application.

However, as there have been a limited number of studies, the degree to which each of the previously mentioned caveats affects correlation between PD-L1 expression on CTCs and in tissue is still unknown.

4.3.   Limitations of the study

The primary limitation of this study was realised in the low number of patients that could be analysed during the study period, compound by the number and frequency of CTCs recovered in the patients analysed. Neither the ClearCell FX or the Parsortix systems are able to successfully isolate CTCs from all melanoma and NSCLC patient blood samples processed. However, whether this issue is biological or technical remains unclear as few studies report CTC detection rates of 100% (Boffa et al., 2017; Joshi et al., 2014; Wang et al., 2019).

A secondary limitation was the varied timeframe between the tumour biopsy and blood collection for the analysis of CTC PD-L1 expression. This delay varied significantly between patient samples, with the effect of this delay on the expression of PD-L1 being relatively unknown.

The final limitation is that the length of this study, within the constraints of a master's degree, did not provide enough time to follow up disease outcome in sufficient patients to assess if PD-L1 expression on CTCs is predictive of response to anti-PD-1 therapy independently of the correlation with tumour PD-L1 status, as carried out in Khattak *et al*. This time constraint also affected the capacity to evaluate progression free survival and overall survival on these cohorts.

4.4. Future directions

Building upon the methodologies developed and preliminary evaluated in this study, future research should assess PD-L1 expression on CTCs in larger cohort of patients and evaluate if it can predict response to treatment and/or survival. In addition, the correlation between PD-L1 expression on CTCs and in tumour tissue will need to be more consistent with for several factors such as number of prior treatments, time of sample collection and anti-PD-L1 antibody utilised. Further studies will also be needed to determine if PD-L1 expression on CTCs can be a predictive or prognostic marker.

This project has provided staining protocols to act as a basis for further studies to assess PD-L1 on CTCs from melanoma and carcinoma patients. The use of borohydride quenching in the carcinoma staining panel may allow for, further improvement to these protocols through the addition of markers to improve both positive and negative CTC identification, such as N-cadherin and CD66b (Alvarez Cubero et al., 2017).

It will be important for future studies to assess PD-L1 expression on CTCs as a predictive or prognostic marker for combinations of anti-PD-1 therapy and chemotherapy. Combination of pembrolizumab with chemotherapy has been approved for the treatment of NSCLC and is seen to be highly effective (Langer et al., 2016; Paz-Ares et al., 2018; Sheela et al., 2018). However, like pembrolizumab monotherapy, this combination lacks a definitive predictor of response.

4.5. Conclusions

This project delivered multimarker immunocytochemistry assays to evaluate PD-L1 expression on CTCs from both melanoma and NSCLC patients, including the establishment of negative, low and high positive controls. Analysis of blood samples from two small cohorts show the presence of CTCs in a low proportion of patients; in 3 out of 8 melanoma and 5 out of 14 NSCLC patients. However, PD-L1 was detected in large proportion of the CTC positive cases; in 2 out of 3 melanoma and 4 out of 5 NSCLC patients. We did not observe a good correlation between PD-L1 expression on CTCs and that in matched tumour tissue samples. However, there were a variety of potential confounding factors such as small sample size, antibody clone and time differences between tumour and blood collections.

Addition of the EMT marker vimentin significantly improved detection of NSCLC CTCs, while the addition of CD16 improved exclusion of WBCs for all patient samples, overall increasing the sensitivity and specificity of the assay. The use of TSA amplification greatly improved quantification of PD-L1 expression, allowing for the reliable detection of weak expression. Finally, the introduction of multiple rounds of staining through the application of borohydride quenching provides a means to expand the immunocytochemistry panel, beyond the protocol described herein.

5. CHAPTER 5: References

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