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Macrophage polarization and colorectal cancer immune checkpoint protein expression.

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MACROPHAGE POLARIZATION AND COLORECTAL CANCER IMMUNE CHECKPOINT PROTEIN EXPRESSION

By

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ABSTRACT

MACROPHAGE POLARIZATION AND COLORECTAL CANCER IMMUNE CHECKPOINT PROTEIN EXPRESSION

Anne Macleod

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The majority of patients with colorectal cancer do not respond to treatment with immunotherapy. Immunotherapy requires the expression of cell surface immune checkpoint proteins (e.g. PDL1) to exert its effect. Most (>85%) of colorectal cancers do not express these proteins and this contributes in part to the poor prognosis and survival in patients with advanced disease. The patients in which immunotherapy *is* a potential treatment option, are a genetic subtype known as mismatch repair deficient, and have demonstrated an excellent response to anti-PD-1/PDL1 immunotherapy. A key feature of mismatch repair deficient (MMRd) cancers is their immune cell rich tumor microenvironment. Macrophages and T cells are the most abundant immune cells of the tumor microenvironment in colon cancer and T cells are known to contribute to cancer PDL1 expression via

IFNγ expression. The role macrophages play in the expression of PDL1 in cancer is not well established.

This dissertation investigated the role of macrophage polarization, and its mechanisms of inducing immune checkpoint protein expression in colorectal cancer.

These studies led to the following results:

1. Mismatch repair deficient (MMRd/microsatellite high [MSI-H]) colorectal cancers have a higher proportion of M1-like (pro-inflammatory) macrophages in their tumor microenvironment.
2. Tumors with higher proportion of M1 macrophages have increased PDL1 expression, in both mismatch repair proficient and deficient colorectal cancers.
3. The co-culture of colon cancer cell lines with M1 macrophages significantly increased cancer PDL1 expression, even in those with proficient mismatch repair systems.
4. M1 macrophages have a higher cell surface PDL1 expression compared to the anti-inflammatory M2 phenotype.
5. THP-1 derived M1 polarized macrophages increased expression of JAK/STAT pathway genes in colon cancer along with PDL1 expression, despite not producing IFNγ.

6. CXCL9 and CXCL10 gene expression was upregulated in MMRd/MSI-H tumor samples; their expression correlated strongly with M1 macrophage infiltration of the tumor micro-environment and CXCL9 and CXCL10 gene expression was significantly upregulated in our M1 polarized macrophages.
7. Increased M1 macrophage fraction and higher CXCL9 and CXCL10 gene expression were associated with improved overall CRC patient survival, including those with MMR proficient CRC.

These results suggest that M1 macrophages in the tumor microenvironment can induce PDL1 expression in colon cancer providing a potential treatment target for mismatch repair proficient CRC, which at present do not respond to immunotherapy. While IFNγ continues to play an important role in PDL1 expression, PDL1 can be induced via the JAK/STAT pathway by alternative mechanisms such as the CXCL9/CXCL10/CXCR3 axis.

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CHAPTER I: INTRODUCTION

a) Colorectal Cancer- Overview

i) Incidence and Trends

Colorectal cancer (CRC) is the third most common diagnosed cancer, and the second leading cause of cancer related mortality in the U.S annually. The American Cancer Society estimates that in 2024, 152,810 individuals will be diagnosed with CRC, and 53,010 CRC-related deaths will occur. Overall, the current lifetime risk of developing colorectal cancer is 1 in 25 (4.3%) for men, and 1 in 23 (3.9%) for women in the U.S [1-3].

Colorectal cancer continues to occur most commonly in older adults; however, the trends and patterns of disease has changed. While incidence rate overall and particularly in those >65 years of age is decreasing; the incidence of early onset (<50 years) CRC is rising. **(Figure 1a-c)**. The proportion of cases diagnosed in adults <55 years of age has risen from 11% in 1995, to 20% in 2019. CRC is now the leading cause of cancer related deaths in men <50 years of age, and second leading cause in women [1, 2]. Despite advances in screening methods, expansion of eligible age range, along with the benefits of early cancer detection and preventative polypectomy at colonoscopy, colorectal cancer remains a significant global health burden [4-7].

Figure 1: Trends in Incidence of Colorectal Cancer by Age and Stage at Diagnosis

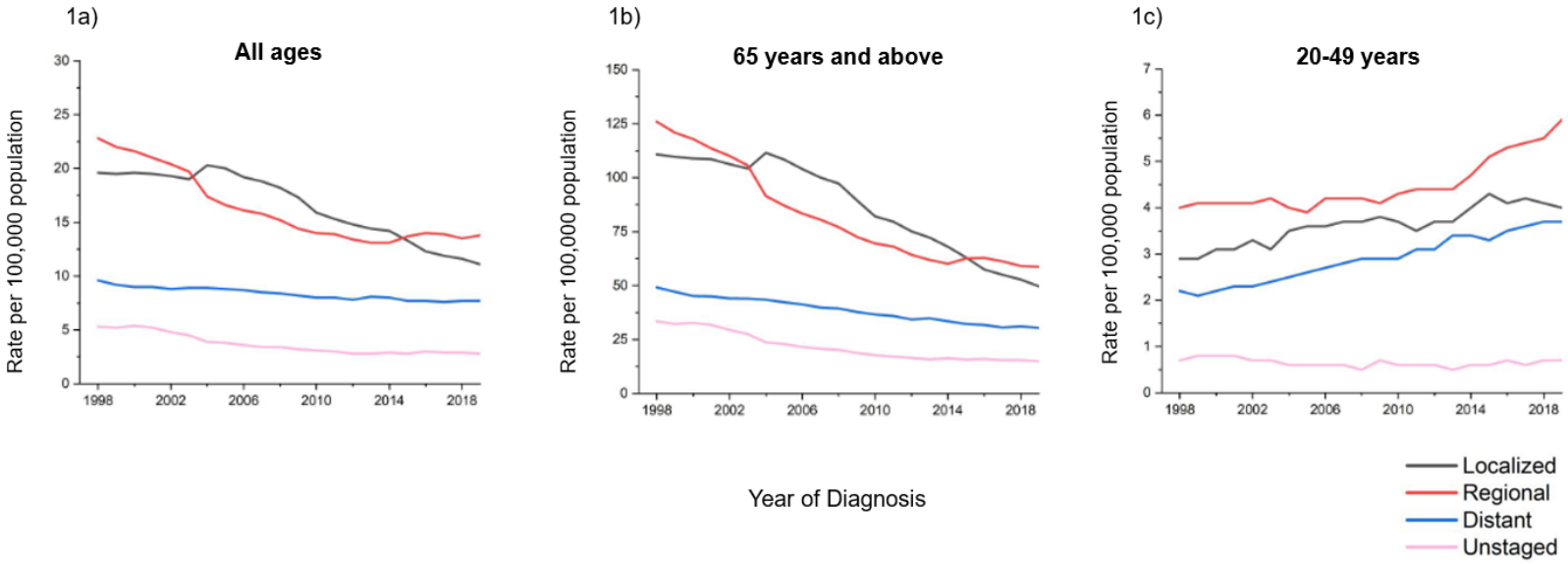


Figure 1: Colorectal Cancer trends in incidence rates per 100,000 population from 1998-2019, United States.

a) All ages, b) adults >65 years, c) adults 25-49 years. Data taken from North American Association of Central Cancer Registries, 2022. [1]

ii) Staging and Survival

Patient survival and outcomes following a diagnosis of colorectal cancer can be most accurately predicted, based on the stage of disease at diagnosis. The American Joint Committee on Cancer, Colorectal Cancer Staging System (8^{th} Ed)[8] can be found in **Table 1.** Staging is based on a combination of the T stage (extent of primary tumor) N stage (involvement of regional lymph nodes) and M stage (metastatic spread to other organs). Briefly CRC can be described as: i) *localized*, where cancer is confined to the colon/rectum (Stage I/II), ii) *regional*, involving spread of cancer to surrounding lymph nodes but not to other organs (Stage III), or iii) *distant* where cancer has spread to other organs, commonly liver and/or lung, the peritoneum or other sites [3, 8].

Table 1: The American Joint Committee on Cancer (AJCC), Colorectal Staging

T Stage	T criteria
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ, intramucosal adenocarcinoma (involvement of lamina propria no extension through the muscularis mucosae) Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into the peri-colonic tissue
T4a	Tumor penetrates to the surface of the visceral peritoneum (serosa)
T4b	Tumor invades and/or is adherent to other organs or structures

N Stage	Nodal Involvement
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	1-3 regional lymph nodes are positive (tumor in lymph nodes measuring ≥0.2mm), or any number of tumor deposits are present and identifiable lymph nodes are negative
N1a	1 regional lymph node is positive
N1b	2-3 regional lymph nodes are positive
N1c	No regional lymph nodes are positive, but there are tumor deposits in subserosa, mesentery, or non-peritonealized pericolic or perirectal tissues
N2a	4 or more regional lymph nodes are positive
N2b	7 or more regional lymph nodes are positive

M Stage	Metastatic Spread
M0	No distant metastasis
M1a	Metastasis confined to one organ or site is identified without peritoneal metastasis
M1b	Metastasis confined to two or more organs or sites without peritoneal metastasis
M1c	Metastasis to the peritoneal surface alone or with other site or organ metastases

Overall Stage		T	N	M
0	*Localized*	Tis	N0	M0
I		T1-T2	N0	M0
IIA		T3	N0	M0
IIB		T4a	N0	M0
IIC		T4b	N0	M0
IIIA	*Regional*	T1-T2	N1-N1c	M0
		T1	N2a	M0
IIIB		T3-T4a	N1-N1c	M0
		T2-T3	N2a	M0
		T1-T2	N2b	M0
IIIC		T4a	N2a	M0
		T3-T4a	N2b	M0
		T4b	N1-N2	M0
IVA	*Distant*	Any T	Any N	M1a
IVB		Any T	Any N	M1b
IVC		Any T	Any N	M1c

Table 1: *The American Joint Committee on Cancer (AJCC), Colorectal TNM Staging System 8th Edition. Edited from [3, 8]*

In 2022, the SEER registry reported, of all CRC diagnosed between 2011-2020, 21.8% of patients had distant metastases at the time of diagnosis. Cancer localized to the colon only was found in 36.1% of patients, and 35.5% were diagnosed with regional spread only [9].

The 5-year survival for all-stage CRC is 65%, however this ranges from >90% in localized disease to only 8-14% in those with distant metastatic spread at time of diagnosis. **(Figure 2).** Cancer detected early, and limited to the colon, has lower risk of recurrence and metastatic spread. Early detection and screening have resulted in good outcomes for those patients. Advanced, stage IV disease that has spread outside the colon can rarely be cured by surgery and relies on systemic treatment. Limitations and variable response rates to current treatment options for distant disease is a key factor in its poor survival, and an important unmet need in colorectal cancer management.

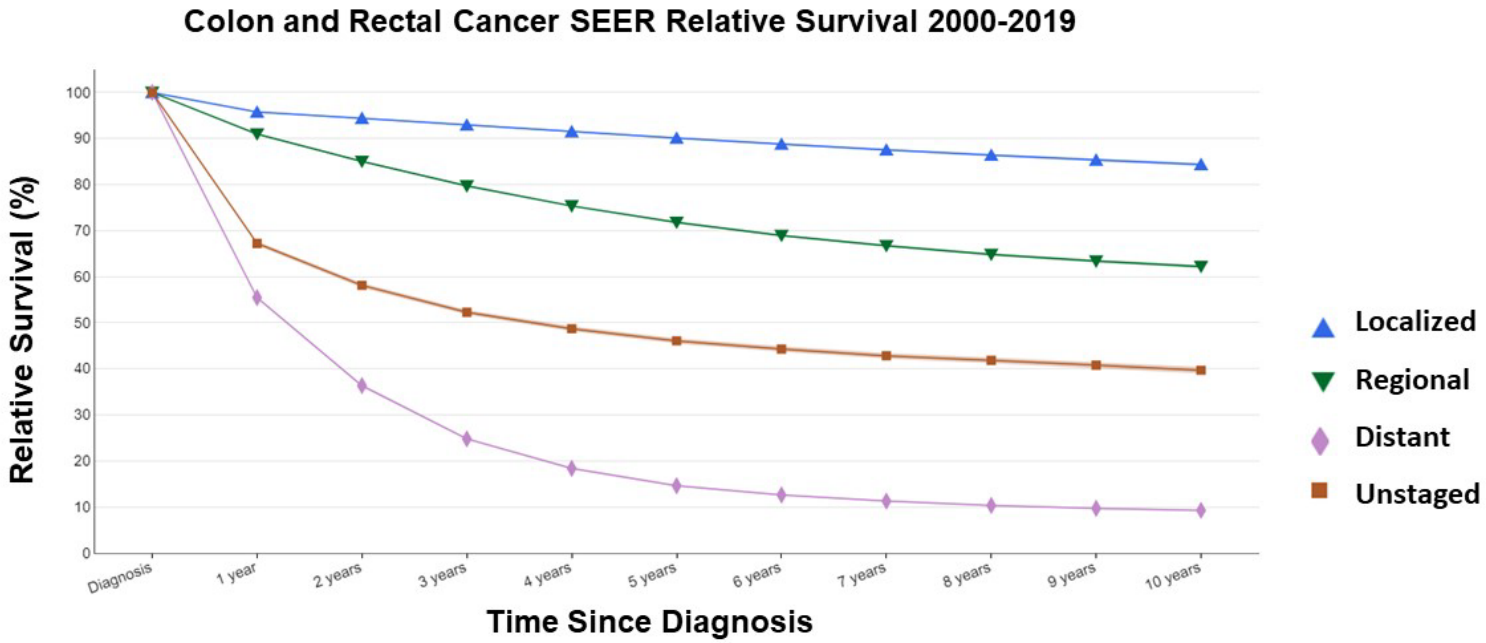


***Figure 2: Colon and Rectal Cancer SEER Relative Survival 2000-2019**:*

Survival in years from diagnosis, by stage, for colon and rectal cancer. Available from SEER explorer database [9].

iii) Treatment

The stage of cancer at diagnosis is not only an important predictor of survival, but it also helps guide the appropriate treatment approach. Surgery is the primary treatment for most curative colon and rectal cancer [3]. Local or regional cancer of the colon is usually treated with colonic resection, including removal of surrounding draining lymph nodes to complete staging. Accurate staging, as discussed, predicts the need for additional treatment based on the likelihood of recurrent disease in the future. While surgery alone is potentially curative, local, or distant recurrence may occur, and patients with Stage II/III disease and high-risk features are advised to complete adjuvant fluorouracil-based systemic chemotherapy. **(Table 2)**

Table 2: Colorectal Cancer: Indications for adjuvant chemotherapy following surgery.

Indications for adjuvant chemotherapy following surgery

• Stage II + High Risk Features*
  ▪ Microsatellite Stable/Mismatch Repair Proficient
  ▪ <12 lymphnodes in resection specimen
  ▪ Poor differentiation
  ▪ Obstruction
  ▪ Perforation
  ▪ Lymphovascular invasion
  ▪ Perineural invasion
  ▪ High level tumor budding
• Stage III

Table 2: *Clinical and pathological features of CRC indicating likely survival benefit with use of adjuvant chemotherapy follow resection with curative intent adapted from [3].*

Patients with early colorectal cancer, Tis “in situ”, or T1 cancer of an adenomatous polyp have the potential to be managed with endoscopic resection, therefore avoiding the need for extensive surgery and its associated risk. Accurate staging, polypectomy with negative margins and close follow-up surveillance are crucial to ensuring curative resection and reduced the risk of recurrence [10, 11].

The management of patients with stage IV colorectal cancer is much less well defined. It is variable, and dependent on a host of factors. These include tumor biology, particularly genetic mutations in important CRC-related genes and pathways, which will be discussed further in the latter part of this, and subsequent, chapters. Additional important considerations include the extent and location of metastatic spread; the responsiveness of the tumor to initial systemic treatment; the potential resectability of the tumor and metastases or possibility of tumor downstaging to becoming resectable [3].

Targeted molecular therapies including Bevacizumab (anti-VEGF), Cetuximab (anti-EGFR) and the anti-BRAF antibody Encorafenib can all be used alongside traditional systemic chemotherapy in select patients’ dependent on genetic mutations[12, 13]. Systemic chemotherapy agents are primarily fluorouracil-based combined with oxaliplatin and irinotecan [14, 15]. All these factors are taken into consideration, along with the general health and medical

fitness of the patient and an, often relatively individualized, treatment plan is constructed.

As described, mutational status and tumor biology are important factors in guiding appropriate treatment of colorectal cancer. Of particular importance is the MMR (mismatch repair) status of the tumor and its microsatellite stability. Mutations affecting these pathways, significantly alter the overall prognosis and treatment options in CRC, in part, due to the currently evolving role of immunotherapy in this patient cohort.

b) Pathways of Colorectal Cancer Carcinogenesis.

i) Background

Colorectal cancer has a well-studied, significant molecular and genetic heterogeneity[16]. Most cases of colorectal cancer are sporadic (70-75%). These patients have no known family history or inherited high risk cancer pre-disposition genes, and cancers develop, from adenomas, through an accumulation of genetic mutations in tumor suppressing and pro-oncogenes, discussed further, later in this chapter. Familial cancer is found in 25-30% of cases of CRC. Having a first degree relative with colorectal cancer increases the risk of developing CRC two to ten-fold, and although high risk genes are not always identified in this cohort, early screening and genetic counselling is advised on a case-by-case basis [17].

In a minority of colorectal cancers (5-10%), inherited mutations in high-risk genes are identified. The most common inherited colorectal cancer syndrome, Lynch Syndrome, accounts for 2-4% of all CRC [18, 19]. It is an autosomal dominant condition, characterized by mismatch repair (MMR) gene mutations. Other high-risk genes include: the APC gene, the cause of Familial Adenomatous Polyposis which is characterized by the formation of hundreds to thousands of polyps at a young age; BRCA gene mutations, which account for 1% of inherited colorectal cancers, and STK11 mutations resulting in hamartoma-forming Peutz-Jeghers Syndrome. **(Figure 3)**

Figure 3: Colorectal Cancer Subtypes

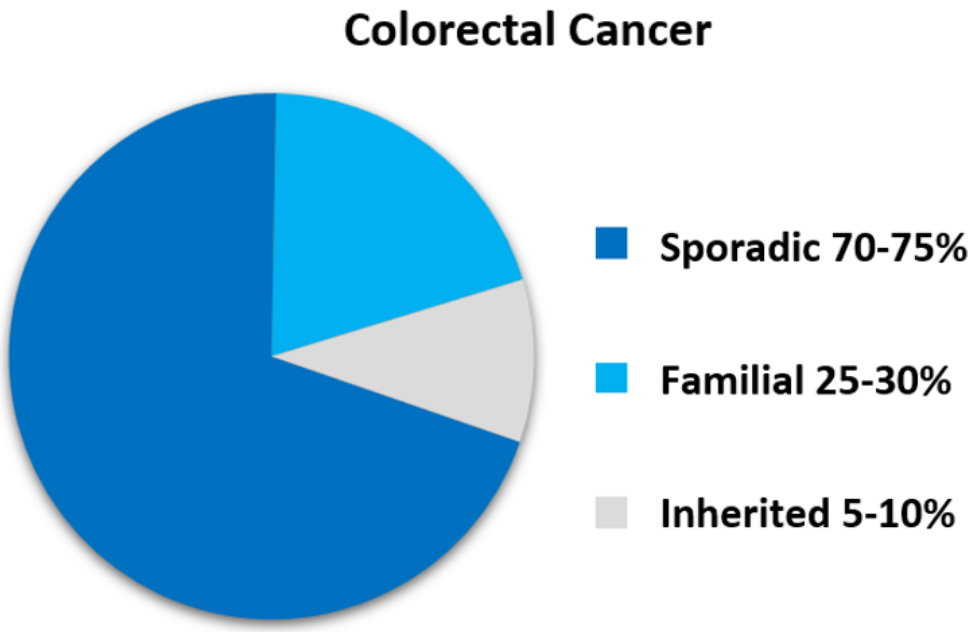


***Figure 3**: Colorectal Cancer Subtypes taken from: American Cancer Society. Colorectal Cancer Facts & Figures 2023-2025. Atlanta: American Cancer Society, Inc. 2022. [1].*

ii) Genomic Pathways of Colorectal Cancer Development.

Colorectal cancers develop due to accumulation of mutations in genes affect distinctive genomic instability pathways including the Chromosomal Instability pathway (CIN), the Microsatellite Instability pathway (MSI) and CPG Island Methylator Phenotype pathways (CIMP).

The CIN pathway is involved in ~80% of CRC and is initiated by genetic and epigenetic alterations of the colonic epithelium. It follows a progression of genetic and histological changes known as the well-established, adenoma-carcinoma sequence. Crucial to the progression from adenoma to carcinoma is the loss of normal regulation of colonic epithelial turnover. An initial loss of function of tumor suppressor gene APC is followed by activation of KRAS oncogene, and subsequent mutations and loss of p53, PIK3CA and loss of heterozygosity of ch18q [20-22]. These genetic changes lead to the characteristic stepwise histological changes from normal colonic epithelium to adenoma polyp formation due to APC inactivation and downstream activation of the Wnt-signaling pathway. Progression from adenoma to in situ and then invasive carcinoma requires activation of pro-oncogenes e.g. KRAS, SMAD. In sporadic CRC via the CIN pathway this process develops over 10-15 years. These cancers are characterized by alterations in chromosomal structure and number (aneuploidy) and loss of heterozygosity [23-25]

Microsatellite and chromosomal instability have been found in adenomas, suggesting genomic instability exists in adenomas prior to APC gene mutation and progression to malignancy [20, 26].

Cancers that develop via the microsatellite instability pathway account for 10-15% of all CRC, these cancers are often diploid and characterized by frame-shift mutations and base-pair substitutions [22, 27]. These cancers also develop from adenomas, but with higher carcinogenic potential, over a shorter time period, and via alternative gene mutations. They most commonly develop secondary to mutations in the genes of the mismatch repair system which can either be inherited, as in Lynch Syndrome sporadic.

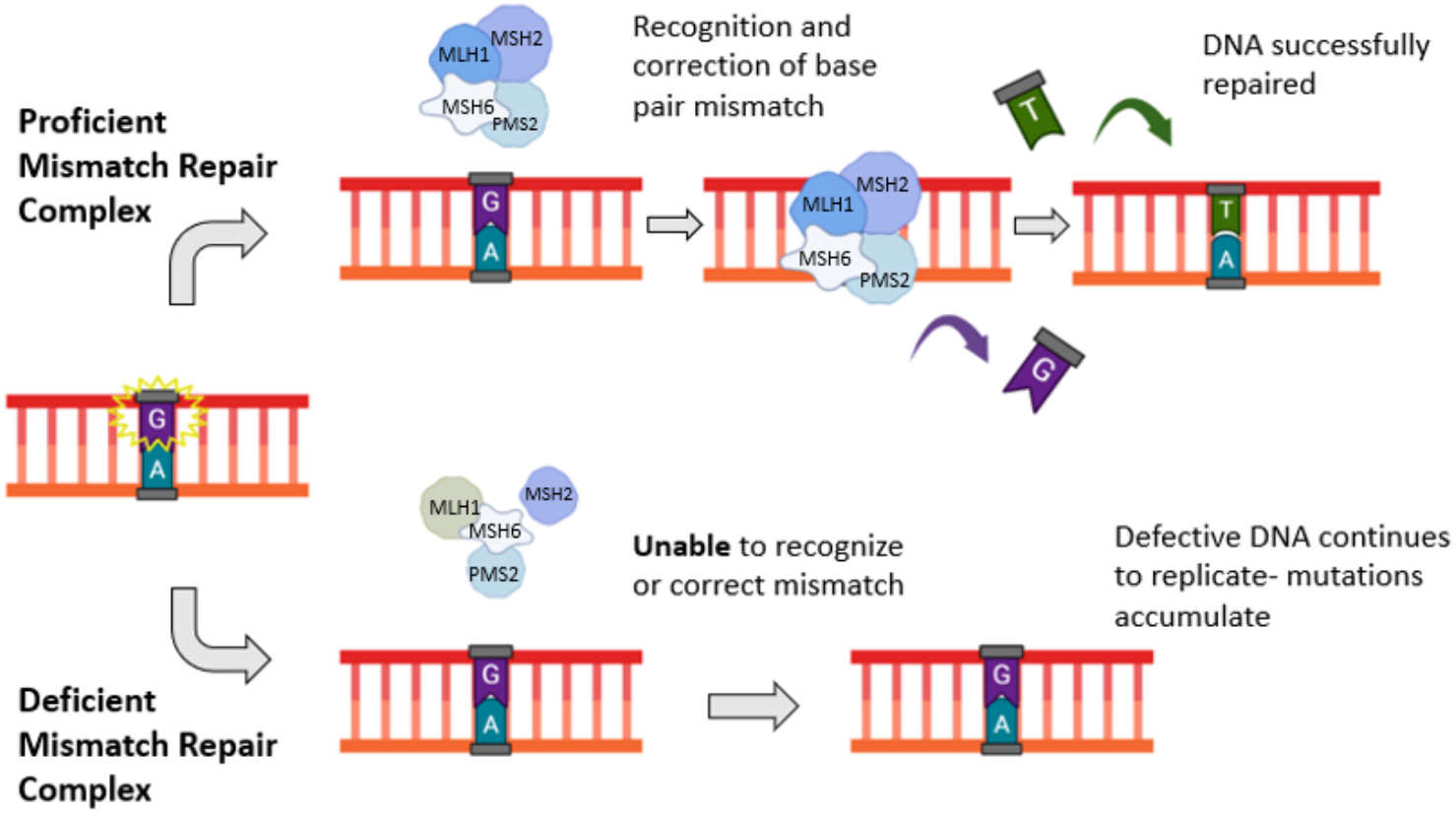
iii) The Mismatch Repair System

The mismatch repair system (MMR) is a family of proteins, encoded by mismatch repair genes. These proteins can identify and repair, incorrect base-base pairings, or small insertions and deletions, that form during DNA replication. Loss of function of any of the MMR genes results in the continued replication of defective DNA and accumulation of subsequent mutations. **(Figure 4).**

The mutations that accumulate due to deficiency in the MMR system are most commonly frame shift mutations or base pair substitutions in the microsatellites of the genome. Microsatellites, or short tandem repeats, are segments of repetitive, typically non-coding DNA scattered throughout the genome [28]. The loss of a proficient MMR system to identify and correct mutations, results in microsatellite regions that are either longer or shorter than the parent cell, referred to as Microsatellite Instability (MSI). [22, 24, 29, 30]

Cancers that develop in a deficient MMR system (MMRd) display significant Microsatellite-Instability (MSI-High) whereas an MMR proficient (MMRp) cancer, will maintain microsatellite stability (MSS). While these cancers are initiated due to mutations in MMR genes and a subsequent deficient MMR system, tumors that continue to develop are often characterized by uncorrected mutations in several oncogenes including BRAF and TGFBR2 [31].

Figure 4: The Mismatch Repair System



iv) Colorectal Cancer and the Mismatch Repair System

The majority of colorectal cancers are mismatch repair proficient/Microsatellite Stable (MMRp/MSS). These make up 80-85% of cancers and develop most commonly via genetic mutations in the APC, KRAS, and p53 genes as described above. MMRd/MSI-H colorectal cancers account for 10-15% of CRC and develop via the microsatellite instability pathway from either sporadic or inherited mutations in MMR genes. The most common affected genes are MLH1, PMS2, MSH2, MSH6.

Sporadic mutations in MMR genes account for 75% of MMRd/MSI-H CRC [32]. Loss of MLH1 function via methylation of the promotor region and subsequent silencing of the gene is found in 80-90% of sporadic MMRd/MSI-H CRC. This is often accompanied by V600E mutation of the BRAF gene, which codes for a protein kinase involved in the MAPK pathway and serves an important prognostic factor and treatment target in CRC.

Lynch syndrome, or *Hereditary non-polyposis colon cancer (HNPCC)* secondary to inherited germline mutations in MMR genes make up 2-4% of colorectal cancer and 25% of MMRd/MSI-H. MLH1 promotor hyper-methylation is extremely rare in Lynch Syndrome, and instead, most cases are due to point mutations. Mutations in MLH1 and MSH2 are the most common and are found in 32% and 38% of Lynch Syndrome patients, respectively [17, 29].

MMRd/MSI-H colorectal cancers exhibit distinct clinical and pathological features. They are commonly diagnosed at an earlier stage (20% of Stage I/II vs only 4-5% of Stage IV) [32]. They are often larger, located in the proximal colon and are frequently poorly differentiated. These tumors are characterized by significant immune cell infiltration within the tumor microenvironment, particularly tumor infiltrating lymphocytes. MMRd/MSI-H colorectal cancers have a have a better prognosis compared to MMRp/MSS CRC, if found at early stages, and are less likely to recur. MMRd/MSI-H CRC, however, have a much poorer response to standard fluorouracil- and oxaliplatin- based chemotherapy regimens. Therefore, prior to the recent developments and advances in the use of immunotherapy, the prognosis and survival in patients with MMRd/MSI-H CRC if recurrent or metastatic, has historically been much worse [12, 13].

A crucial, clinically important, difference in the evolution of treatment options, overall management, and prognosis for patients with MMRd/MSI-H vs MMRp/MSS colorectal cancer is the expression of immune checkpoint proteins and their subsequent responsiveness to immunotherapy. MMRd/MSI-H colorectal cancers have high immune checkpoint protein expression and have been found to respond to immune checkpoint inhibitor immunotherapy, while MMRp/MSS CRC, do not. The following chapters will discuss immune checkpoint protein expression, immunotherapy, and its evolving role in the treatment of colorectal cancer.

CHAPTER II: IMMUNOTHERAPY AND IMMUNE CHECKPOINT PROTEIN EXPRESSION

a) Overview

As described above, a crucial feature which alters the management and prognosis for patients with MMRd/MSI-H vs MMRp/MSS colorectal cancer is the expression of immune checkpoint proteins and their responsiveness to immunotherapy. Immunotherapy via immune checkpoint inhibitors (ICI) targets immune checkpoint proteins on the cancer cell surface.

Immunotherapy has changed the treatment paradigm and prognosis in many cancers over the past several decades. Immune checkpoint inhibitors (ICI) act through the targeting of immune checkpoint proteins such as PDL1, CTLA-4, TIM3 and LAG-3, which are expressed on cancer cells [33, 34]. Expression of immune checkpoint proteins enable cancers to evade recognition by the immune system and allow cancer growth and progression. However, the development of immunotherapy to target these proteins has resulted in this tumor promoting mechanism, now providing a potential therapeutic target. In many cancers, they also serve as a prognostic indicator of responsiveness to immunotherapy [34-36].

Immune checkpoint inhibitors function via the use of antibodies to inhibit the immune system's regulatory checks. This allows the immune system to restore their anti-tumor activity, providing a potential cancer treatment option with

significantly more tolerable side effects, and less toxicity than current systemic chemotherapies [33, 37].

b) The Development of Immunotherapy use in Cancer.

The earliest success in the use of immune checkpoint inhibition immunotherapy was in the treatment of metastatic melanoma. Metastatic melanoma was previously a disease with extremely poor prognosis and limited effective treatment options. Prior to the advent and increasing use of immunotherapy, median survival with chemotherapy was only 8-10 months, with an average 5-year survival <10% [38].

Successful in-vivo models in 1994, identified that activation of effective immune response to tumor cells could be potentiated by checkpoint inhibition [39]. In the mid-2000s, several trials confirmed the efficacy and safety of an anti-CTLA-4 antibody, ipilimumab, and went on to report improved response and overall survival in patients with metastatic melanoma. This led to its approval by the Food and Drug Administration (FDA) in 2011 [36, 40-43]. Durability of its effect was demonstrated, and a follow up study reported a doubling of 5-year survival of patients with metastatic melanoma from 8.8% to 18.2%, due to the addition of anti-CTLA-4 antibody to standard chemotherapy [44]. Subsequent pooled analysis of survival across several trials showed a 3-year overall survival in 254 patients of 22% [45].

The approval of ipilimumab was shortly followed by the first FDA-approved anti-PD-1 antibody, nivolumab in 2014. Early trials of anti-PD-1 antibodies in metastatic melanoma evaluated its efficacy and safety as both a monotherapy, and in combination with anti-CTLA-4 antibody [37, 46, 47]. The Checkmate 067 trial reported prolonged progression free survival both with anti-PD-1 monotherapy and in combination with anti-CTLA-4 vs anti-CTLA-4 alone [47]. Patients that had disease progression with anti-CTLA-4 therapy in the Checkmate 037 trial, had a greater objective response rate with anti-PD-1 antibody vs standard chemotherapy. This was also accompanied by fewer side effects [37].

The success of immune checkpoint inhibitors in melanoma has led to it now having replaced cytotoxic chemotherapy as first line treatment in metastatic disease[48, 49]. This has resulted in further studies, validation, approval and now widespread adoption in the management several cancers including; classical Hodgkin's lymphoma [50, 51] renal cell carcinoma [52], non-small cell lung cancer[53, 54] and urothelial cancer [55, 56]. Its successful role and use in colorectal cancer however, is limited [57], and will be discussed further in Chapter III.

c) Immune Checkpoint Proteins and their Inhibitors.

i) PD-1/PDL1

Programmed Death Ligand-1 (PD-1) is a transmembrane, cell surface protein, that functions as an inhibitory receptor[58]. It is expressed on activated T cells, macrophages, and B cells. Programmed Death Ligand-1 (PDL1) the complementary ligand for PD-1 is expressed by antigen presenting and immune suppressor cells. The role of the PD-1/PDL1 relationship under physiological conditions is to allow the host immune system to recognize its own cells, initiate negative feedback therefore disabling activated T cells from attacking its own healthy immune system [59, 60]. Cancer cells have developed the ability to adopt this mechanism, by expressing PDL1 they are able to evade immunosurveillance [61-63]. **Figure 5** depicts this relationship.

Immune checkpoint inhibition via the blocking of PD-1/PDL1 binding, disrupts the cancer's ability to evade immune cell detection. It re-enables the recognition of cancer expressing antigens by the T cells. This reactivates the exhausted T cells and re-initiates the hosts anti-tumor response, limiting its proliferation and growth [64, 65].

Figure 5a: PD-1/PDL1 Axis

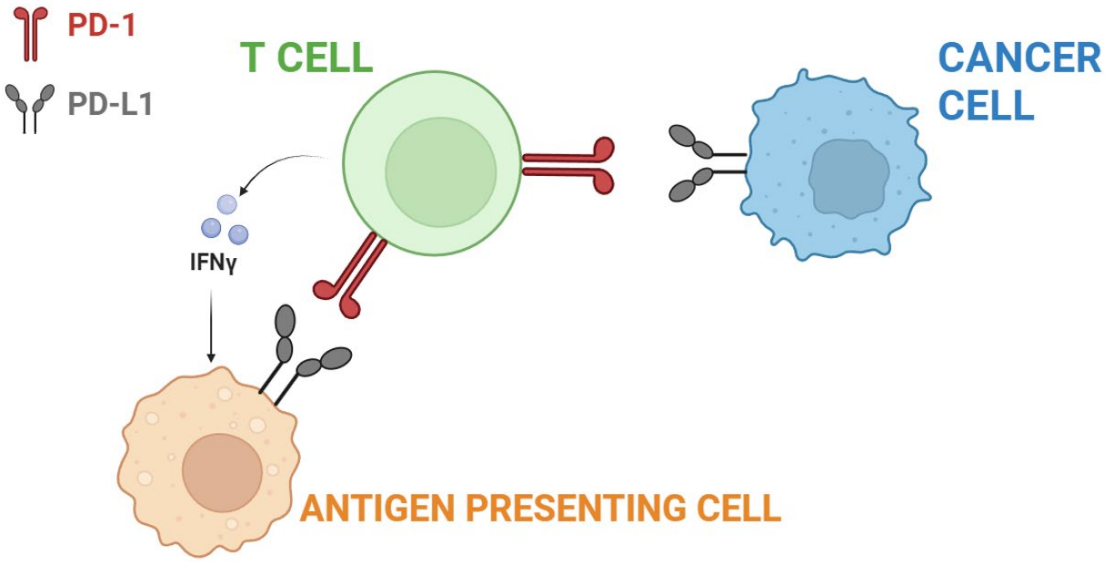


Figure 5b: PD-1--PDL1 Axis- Immunotherapy target.

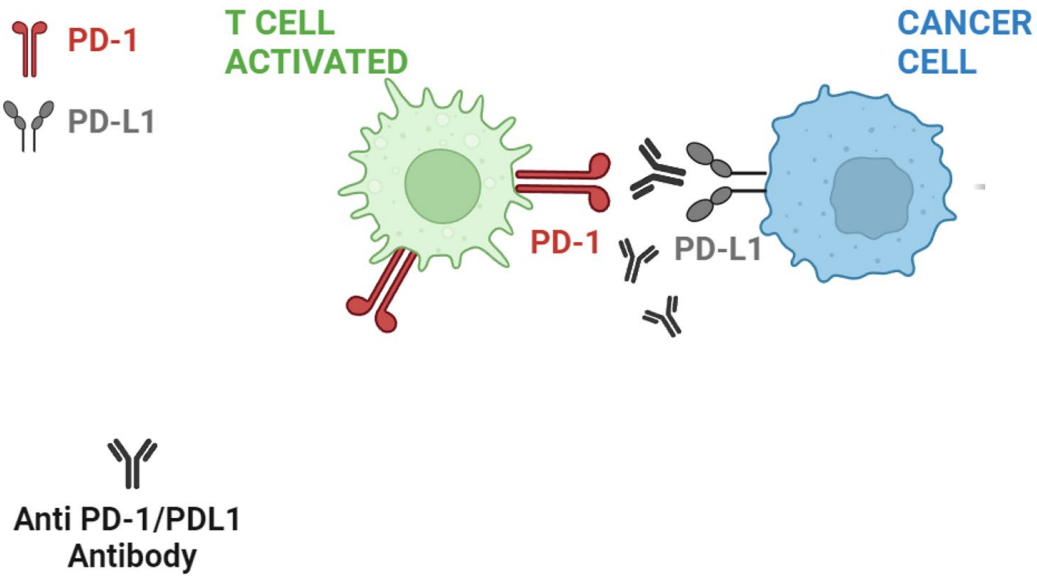


Figure 5: *Blocking of the PD-1/PDL1 axis enables recognition of cancer cell antigens by the host T cell, re-activating the T cell and immune response to cancer.*

ii) CTLA-4

Cytotoxic T Lymphocyte Associated protein (CTLA-4) blockade was the first ICI shown to exhibit anti-tumor effects, reported in in-vitro studies in 1996 [39] and the first FDA approved ICI as discussed above. CTLA-4 is part of the CD28 immunoglobulin family, is expressed mainly by Treg cells and is mediator of immunosuppression via competitive binding to CD80 and CD86, expressed during antigen presentation.[59, 66] It inhibits the activation of T cells, and similarly to PDL1, can be expressed by cancer cells to induce suppression of the immune response and enable cancer progression. It differs to the PD-1/PDL1 axis response in that it in the physiological setting, it primarily functions in *early* immune responses and in the lymphoid tissues. PD-1/PDL1 inactivation of T cells occurs *later* in the immune response and more commonly occurs in the peripheral tissues[61, 67].

iii) LAG-3

Lymphocyte activation gene (LAG-3) another T cell expressing immune checkpoint molecule, has a similar role and function to PD-1 and CTLA-4 described above [68]. High LAG-3 expression, and LAG-3, PD-1 co-expression on cancers is generally regarded as a marker for aggressive disease and has been associated with unfavorable clinical outcomes[68, 69]. While it is considered a next generation immunotherapy target, and the target of several

ongoing immunotherapy trials, with promising results [70] the mechanism of its action is less well understood [71] . Several ligands have been identified and suggested to play a role in LAG-3 mediated T cell inhibition. These include MHC class II, Galactein-3 (Gal-3) and fibrinogen like protein (FGL1). The number of different ligands identified; are likely related to the diverse role LAG-3 plays across different disease settings including in cancer, viral infections and auto-immune. While ongoing studies examining these mechanisms continue, LAG-3 continues as a promising and successful immunotherapeutic target. [68, 71-73]

CHAPTER III: COLORECTAL CANCER AND IMMUNOTHERAPY

The role of immunotherapy in colorectal cancer, while still limited, has changed significantly over the past few years. Its use has been significantly less pronounced and widespread in comparison to the success seen in other cancers, such as metastatic melanoma, and renal cell carcinoma. However, its role has significantly evolved, and continues to with many ongoing trials into novel treatment strategies, to optimize and expand its beneficial effects and overcome resistance.

a) **Previously Treated Metastatic CRC**

The first trials of immunotherapy in colorectal cancer, were in the metastatic setting. Five single-arm trials (phase 1b KEYNOTE 012 and 028, and phase II KEYNOTE 016, 164, and 158) investigated the anti-PD-1 agent pembrolizumab across different tumor types, including 149 patients with previously-treated advanced CRC, 59 of whom presented MMRp/MSS tumors [32, 74].

In 2015, the phase II KEYNOTE-016 trial evaluated the benefit of pembrolizumab (anti-PD-1 ICI) in patients with previously treated metastatic cancer including both mismatch repair proficient and deficient tumors of various cancer origins. Of the 28 colorectal cancer cases evaluated following 20 weeks of treatment, 10 (35.7%) were MMRd and of those 40% (4/10) had a partial response to anti-PD-1 immunotherapy. The overall response (ORR) in patients with MMRp CRC was 0% (0/18) and progressive disease was found in 61% (11/18) of MMRp patients[75]. The follow up KEYNOTE-164 trial of pembrolizumab in MMRd/MSI-H CRC with single vs multiple prior treatments evaluated 124 patients separated depending on number of previous therapies completed. The anti-PD-1 pembrolizumab was confirmed to be effective in this patient cohort with improved median survival and tolerable side effects [76].

The benefit of anti-PD-1 immunotherapy in MMRd/MSI-H colorectal cancer was further confirmed in the phase 2 CheckMate-142 trial. Initial results found nivolumab (anti-PD-1) monotherapy and nivolumab + ipilimumab (anti-CTLA-4) showed a durable response in patients with previously treated metastatic or recurrent MMRd/MSI-H CRC. Nivolumab monotherapy resulted in a response rate of 31%, and patients treated with monotherapy were later reported to have a 12-month survival of 74%. [77]. Combination therapy of nivolumab + ipilimumab was evaluated in 119 patients and at median follow up of 13.4 months overall response rate was 55%. Progression free survival at 9 and 12 months was 76% and 71% respectively and 80% of patients had disease control for >12 weeks[77, 78] .

b) **First Line Treatment in Metastatic CRC**

The tolerability, and positive clinical response of anti-PD-1/anti-CTLA combination therapy in *previously treated* metastatic MMRd/MSI-H CRC from Checkmate-142 trial, led to the further trial of combination immunotherapy as first line treatment in *previously untreated* MMRd/MSI-H CRC [79]. Forty-five patients were treated first line with combination anti-PD-1/anti-CTLA therapy with a median follow up of 29.0 months. An overall response rate of 69% and complete response of 13% was reported. Progression free and overall survival rates at 24 months were 74% and 79% respectively [79].

Prior to the introduction of immunotherapy, metastatic colorectal cancers were treated with systemic chemotherapy agents, primarily fluorouracil-based chemotherapy combined with oxaliplatin or irinotecan, and targeted monoclonal antibody molecular therapies. These include, anti-EFGR agents, e.g. Cetuximab for KRAS wild-type tumors or anti-VEGF antibody e.g. Bevacizumab. Anti-BRAF antibodies, e.g. Encorafenib has shown benefit and efficacy in patients with BRAF V600 mutation. Treatment was largely the same irrespective of MMR/MSI status. While MMRd/MSI-H CRC is commonly diagnosed at earlier stage, and resectable, primary disease has a more favorable prognosis than MMRp/MSS CRC. In the event of metastatic or recurrent disease, response to standard systemic chemotherapy is poorer in MMRd/MSI-H CRC.

The KEYNOTE-177 trial further confirmed the durable anti-tumor effect of anti-PD-1 immunotherapy, by comparing pembrolizumab (anti-PD-1) to systemic chemotherapy (5-Fluorouracil-based +/- anti-EGFR/VEGF) for the first line treatment of metastatic MMRd/MSI-H CRC. At interim analysis, at median follow up of 32.4 months, pembrolizumab had a superior progression free survival (16.5 vs 8.2 months) compared to the standard of care chemotherapy group. Overall response, and sustained response at 24 months were also higher (43.8% vs 33.1% and 83% vs 35% respectively). Initial randomization was 1:1; however, patients were permitted to cross into the immunotherapy arm if disease progression was found and 56 (36%) of the systemic chemotherapy arm crossed over to receive anti-PD-1/PDL1 therapy [80, 81].

c) **Primary Resectable CRC**

The success of immunotherapy in metastatic disease has subsequently led to trials of its use as upfront, first line treatment of primary, resectable CRC.

While biologically and genetically similar, due to location and operative considerations, the management of primary rectal cancer differs to that of colon cancer. Pre-operative chemoradiation has been the mainstay of treatment in most cases of rectal cancer since pre-operative radiation was shown to reduce local recurrence, and cancer related survival in several trials in the early 1990s [82-85] . The addition of chemotherapy, and various treatment combinations and

regimens have evolved over the subsequent 30 years. In 25-30% of rectal cancers a complete clinical and pathological response to pre-operative therapy is observed. The evolution of the 'watch and wait approach' with complete destruction of the cancer with chemotherapy and radiation and observation alone, without surgical resection, has been an important shift in the treatment paradigm of rectal cancer. Successful response to chemoradiation, with close surveillance, can allow for the avoidance of rectal resection and the morbidity associated with major pelvic surgery, including bowel, urinary and sexual dysfunction; infertility and the risk of requiring a permanent ostomy [86].

The responsiveness of anti-PD-1 immunotherapy in metastatic MMRd/MSI-H CRC led to the hypothesis that blockade of PD-1/PDL1 could be effective as first line treatment of primary CRC. In 2022 Cercek et al. reported a trial of anti-PD-1 monoclonal antibody in 12 patients with Stage II/III rectal adenocarcinoma. The trial design was for patients with MMRd/MSI-H cancer to complete 6 months of immunotherapy and commence standard pre-operative chemoradiation +/- surgery for residual disease. A 100% complete clinical, pathological, and radiological response rate was noted at the end of 6 months of immunotherapy; and at 6 months of follow up from treatment completion, 0/12 patients had required any additional treatment [87]. Several similar trials are ongoing [88] or have gone on to show similar results, either with patients not requiring surgery [89] or with complete, or significant pathological response at surgery or follow up imaging [90, 91] .

In the past decade, immunotherapy in colorectal cancer has progressed from a ‘last line’ treatment option for progressive/metastatic disease, to being considered the first line, and potentially sole treatment for MMRd/MSI-H CRC. This offers the potential option of avoidance of surgery and organ preservation, which is of particular importance in the management of rectal cancer .

While these advances have significantly changed the treatment and prognosis of MMRd/MSI-H CRC, and ongoing trials continue to evolve, it is crucial to note, that this benefit is only seen in this subset of colorectal cancer only. As described above, MMRd/MSI-H colorectal cancers make up only a minority (10-15%), while the majority of colorectal cancers (MMRp/MSS) remain immunotherapy resistant, and there are many current trials studying mechanisms and pathways in which its benefit can be expanded. The lack of beneficial effects on most MMRp/MMS CRC is likely due, in part, to the “immune-cold” nature of their tumor micro-environment.

CHAPTER IV: MACROPHAGES, THE TUMOR MICROENVIRONMENT AND PDL1 EXPRESSION

a) **The Tumor Microenvironment in Colorectal Cancer**

Colorectal cancers are complex heterogenous tumors with dense tumor microenvironments (TME). These microenvironments are composed of variable infiltration of immune stromal cells, endothelial cells along with cancer cells and surrounding blood vessels and extracellular matrix [92, 93]. The cells of the TME exist surrounded by, and are part of, continuous signaling pathways of both tumor promoting and inhibiting effects. Cells of the TME communicate both via cell-to-cell interaction; and via mediators, such as soluble chemokines, cytokines, and growth factors [94]. Macrophages comprise a significant portion of the immune cells of the TME and exist across the spectrum of pro- “M1” and anti- “M2” inflammatory phenotypes. These contribute both tumor promoting and inhibiting effects to this dynamic environment. In the TME of CRC most tumor associated macrophages exhibit an “M2” anti-inflammatory phenotype

Differences in the composition of the TME, as described in *Chapter I.b) iv.* is a hallmark of MMRd/MSI-H CRC. These cancers are often termed “immune hot” due to their dense immune cell infiltration and pro-inflammatory environment, secondary to the high mutation burden of the tumor. In contrast, is the “immune cold” environment of MMRp/MSS CRC which contain significantly fewer immune cells [95]. In addition to increased density of immune cells, there are studies to suggest that the immune cell phenotypes of the TME differ in MMRd/MSI-H CRC, including an increased infiltration of more M1-polarized macrophages. [95-97].

b) **Tumor Associated Macrophages**

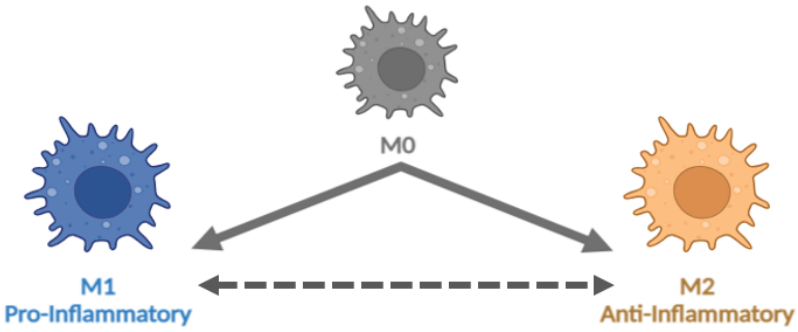
Macrophages are crucial immune cells which are characterized by their plasticity, i.e. their ability polarize between pro- and anti-inflammatory phenotypes and by their functional diversity.[98] The previously often described dichotomous polarization of ‘M1’ vs ‘M2’ macrophages, is now better appreciated as extreme ends of an activation scale. Macrophage subtypes M1, M2a, M2b, M2c and M2d have been described, each with their own activating agents, cytokine/chemokine profiles and functional properties [99, 100].

Macrophages at each end of the polarized M1-M2 spectrum have different activating factors, cytokine production and functional roles **(Figure 6).** In the newer sub-division of M2 macrophages the classic “M2-tumor associated macrophage” is closest to the “M2a” subtype. In all in-vitro work of this study

going forward, our “M2” macrophages have been analyzed extensively and closely resemble the M2a subset [101].

Classically activated macrophages demonstrate a pro-inflammatory phenotype promoting inflammation, apoptosis, extracellular matrix destruction and antibody cell mediated cytotoxicity to kill tumor cells. These are termed ‘M1’ macrophages. Alternative activation produces ‘M2’ macrophages, which exhibit pro-tumorigenic functions; promoting tumor cell metastases, inhibiting T cell mediated anti-tumor responses and lead to cancer progression [98, 102, 103]

Figure 6: Macrophage Polarization



"Pro-inflammatory"
- Anti + Pro-Tumorogenic effects
  - CD80
  - HLA-DR
  - IL-6, IL-8, IL-27, TNFα, IL-1β, IFNγ

"Anti-inflammatory"
- Pro-Tumorogenic
  - CD206, CD163
  - Arg1, VEGF
  - TGFβ, IL-10

***Figure 6**: M1 and M2 polarized macrophages exist on a dynamic continuum between these two extremes and have differing functional roles, cell surface markers and cytokine production.*

c) **Macrophages and Cancer PDL1 Expression-**

As described in Chapter IIc), T cells play a crucial and well-established role in regulating the expression of immune checkpoint proteins, their ligands, and responses to antibody blockade with immunotherapy. Cancer cells' ability to express PDL1, is largely driven by both i) extrinsic activation from T cell production of IFNγ, and ii) intrinsic constitutive expression which is driven by genetic alterations of the cancer cells [62, 104].

T cells, upon recognition of tumor antigens, release interferons, which in turn induce PDL1 expression in cancer along with other cells of the TME. This enables inhibition of the anti-tumor immune response by the binding of PD-1/PDL1 described previously. IFNγ induced cancer PDL1 expression is largely mediated by Janus Kinase and signal transducers and activators of transcription (JAK/STAT) pathway [62, 105].

While T cell induced PDL1 expression via activation of the JAK/STAT pathway is the primary role in inducing cancer cell PDL1 expression. The role of the other immune cells of the TME in inducing PDL1 expression is unclear. Macrophages are the second most abundant immune cell of the TME, and while they, as antigen presenting cells, can themselves express PDL1, how they contribute to the cancer immune checkpoint protein expression is less clear.

Zong et.al demonstrated a mechanism in hepatocellular carcinoma (HCC), another immune-rich cancer, where M1 macrophage infiltration correlated to higher overall tumor PDL1 expression. They identified M1 macrophage induced IL-1β led to increased HCC PDL1 expression through activation of IRF1 [106]. A further study reported macrophage induced PDL1 expression through cytokine IL6 and TNFα production via the NFκB/STAT3 pathway[107].

CHAPTER V: OBJECTIVE, HYPOTHESIS & SPECIFIC AIMS

a) Key Objective

To investigate the relationship between macrophage polarization and the immune checkpoint protein expression in colorectal cancer (CRC).

b) Hypothesis

Tumor Associated Macrophages of the TME differ in MMR deficient CRC and have a more M1-like phenotype than in MMR proficient CRC. Macrophage pro-inflammatory cytokines (e.g. IL-6, IFNγ, IL-1β) increase PDL1 expression in CRC via the JAK/STAT pathway.

c) Specific Aims

Aim 1:

To compare macrophage phenotypes in MMRd/MSI-H vs MMRp/MSS CRC, and their correlation to PDL1 Expression

Aim 2:

To study the effect on cancer PDL1 expression following co-culture with macrophages of both pro and anti-inflammatory phenotypes.

Aim 3:

a. To identify a mechanism, by which M1 macrophages may increase cancer PDL1 expression.
b. To examine the role of macrophage phenotype and its cytokines in the tumor microenvironment on patient survival.

CHAPTER VI: METHODS & MATERIALS

a) CELL CULTURE AND TREATMENT

The human monocytic leukemia cell line THP-1 (TIB-202), and colon cancer cell lines HT29 (HTB-38), SW480 (CRL-228), Hct116 (CCL-247), RKO (CRL-2577) were purchased from the American Type Culture Collection (ATCC, Manassas, USA) and authenticated using short tandem repeat analysis. Cells were incubated in RPMI-1640 medium (ATCC, Manassas, USA) supplemented with 10% fetal bovine serum (FBS) (ATCC, Manassas, USA), 1% L-glutamine, 10,000 units/mL penicillin, 10 mg/mL streptomycin, 25 µg/mL amphotericin B, and maintained at 37°C with 5% CO2.

For macrophage differentiation, THP-1 cells were seeded at 2×10^5 cells/mL into 24-well cell culture plates and treated for 72 hours with 100ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, Burlington MA, USA) and differentiated into M0 macrophages as per previous protocol. [101, 108]

For M1 polarization, M0 macrophages were then treated with lipopolysaccharide (LPS) 10, 50 or 100ng/mL (Sigma Aldrich) or LPS + interferon-γ (IFNγ) 20 or 50 ng/mL (Sigma Aldrich). Phosphate Buffered Saline (PBS) (Sigma Aldrich) 10ul treatment was used as negative control. Method further described in Chapter 7.2)

“M2-like” polarized macrophages were created from differentiated “M0” macrophages following 2 treatments of IL-4 and IL-13, as per previous protocol [101, 108]

b) CO-CULTURE MODEL

The co-culture model used in this work utilizes 24-well Corning®, Transwell® cell culture plates with 6.5mm diameter/0.4μm pore transwell basket inserts. THP-1 monocyte derived macrophages are differentiated and polarized in the upper chamber using 200ul $1.0x10^6$ cells. Cancer cells are plated in the corresponding well at a $0.2x10^6$/ml concentration; a total volume of 1ml is used. Basket and well are combined with media changes.

For experiments which were repeated with medium-treated cells, a similar protocol was followed. Cancer medium was used to treat macrophages grown and differentiated in the well of 24 well plates. 'Cancer medium' treatment utilized 800ul cancer medium combined with 200ul fresh RPMI+FBS+AA medium as described above. In experiments in which cancer cells were treated with macrophage medium, 1000ul medium from M1, M2 or M0 derived macrophages was combined with cancer cell lines in 24 well plate cell culture plates for times as specified by the experimental design.

Throughout results section, 'single' culture refers to single cell type cultured alone (either cancer cell or macrophage) not in co-culture.

c) QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from macrophages and cancer cell lines, using the RNeasy purification kit (Qiagen, Germantown, MD, USA) and quantified with spectrophotometry (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA).

Reverse transcription was performed with 20 ng total RNA, using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. TaqMan PCR was performed with TaqMan gene expression assays *(**CCL18:** Hs00268113_m**, CCL22**: Hs01574247_m1, **CCL3:** Hs00234142_m1, **CCL4:** Hs99999148_m1. **CD274:**Hs00204257_m1, **CD80**: Hs01045161_m1, **CD86** Hs01567026_m1 **CXCL10:**Hs00171042_m1 **CXCL9**:Hs00171065_m1, **FGL1:**Hs00189514_m1, **HLA-DRA:** Hs00219578_m1, **IFNG**: Hs00989291_m1, **IL-10**: Hs00961622_m1, **IL17A:** Hs00174383_m1, **IL1b**: Hs01555410_m1**, IL-27**: Hs00377366_m1, **IL-6:** Hs00174131_m1, **IL-8:** Hs00174103_m1, **JAK1**: Hs01026983_m1, **JAK2:** Hs01078136_m1**, LAG-3**: Hs00958444_g1, **MRC1/CD206**: Hs00267207_m1, **NFKB1**: Hs00765730_m1, **PDCD1**: Hs01550088_m1, **PPARG:** Hs01115513_m1 **RNA18S5:** Hs03928990_g1, **STAT1:** Hs01013996_m1, **STAT3:**Hs00374280_m1, **TGFB:** Hs00998133_m1, **TLR4:** Hs00152939_m1, **TNF-a** :Hs00174128_m1*) (Applied Biosystems) and Fast Advanced Master Mix (Applied Biosystems) using StepOne Real-Time PCR systems (Applied Biosystems).

Sample sizes represent individual wells of cells harvested; technical duplicated were performed for each. Results for each target gene were normalized using 18s as the housekeeping gene and are given as mean ΔCT values.

Statistical Analysis

All experiments were performed with experimental duplicates of at least n= 6, with 2 technical duplicates for each data point. Descriptive statistics for ∆CT and fold change (FC) are reported and compared between treatment groups. Mean ∆CT, FC and either 95% confidence intervals or standard deviation are presented for all data points. All PCR data were analyzed using a one-way analysis of variance (ANOVA) with a post hoc Benjamini–Hochberg correction to control the false discovery rate at 5% for normally distributed data; for non-normally distributed data, a Kruksall-Wallis analysis was performed. Data was graphed using GraphPad Prism version 10.2.0 or IBM SPSS Statistics. $P<0.05$ was used for significance.

d) FLOW CYTOMETRY

Macrophages were washed with ice-cold phosphate buffered saline (PBS, Sigma Aldrich) + 5% fetal bovine serum (FBS, ATCC) and incubated on ice for 45 mins to enable detachment. Cells were gently scraped from bottom of a 24-well plate and resuspended in 5% FBS in PBS to a concentration of $0.1x10^6$/ml. Cells were stained with either anti-CD80 (PE), anti-CD86 (FITC), anti-PDL1 (PE), anti-CD206 (PE-Cy5) or appropriate negative isotype control (BD Biosciences). Cells were stained for 25 mins in the dark at 4°C, washed with PBS and fixed with 300ul 1% paraformaldehyde.

Cancer cells were harvested for staining using Trypsin-EDTA Solution (0.25% Trypsin/ 0.53Mm, EDTA, ATCC). Following removal of media, 250ul of trypsin was added to each well and incubated at 37°C, 5% CO2 for 7 mins. Trypsin was then neutralized with equal volumes RPMI and 10% FBS media. Following centrifugation, cells were the resuspended in 5% FBS/PBS and stained as per the above protocol for macrophages.

Cells were analyzed using a FACS Calibur® flow cytometer with a minimum of 10,000 gated events acquired per sample. Data were analyzed using Cell Quest software (Becton Dickinson).

Statistical Analysis:

All experiments were performed with experimental duplicates of at least n= 6. Descriptive statistics for percent (%) positive and median fluorescence intensity (MFI) are reported as mean and standard deviation (SD). Flow results were compared between cell types and treatment groups (single vs co-culture) and analyzed using a one-way analysis of variance (ANOVA) with a post hoc Benjamini–Hochberg correction to control the false discovery rate at 5%. Statistical analysis was performed, and data graphed using GraphPad Prism version 10.2.0 or IBM SPSS Statistics. $P<0.05$ was used for significance.

e) ELISA (ENZYME LINKED IMMUNOSORBENT ASSAY)

Cell supernatant was collected at the time of cell harvest and stored until further use at -80°C. ELISA kits for Human IL6 (Catalog # 88-7066), and TNF alpha (Catalog # 88-7346) were purchased from Invitrogen, Thermofisher Scientific™ and ELISA performed as per manufacturer's instructions. Sample sizes represent individual wells of cells harvested. All experiments were performed with technical duplicates, read at 450nm using Spectra Max 384plus® spectrophotometer and analyzed using SoftMax Pro® software, Molecular Devices, CA. Results were discarded if the co-efficient variant (CV) was >20%.

f) PATIENT SAMPLES (University of Louisville)

Peripheral Blood Mononuclear Cell (PBMC) Isolation

After informed consent, blood was obtained pre-operatively from colorectal cancer patients using venipuncture. This protocol was approved by the University of Louisville Institutional Review Board (IRB: 97.0361). PBMCs were isolated from whole blood using 3% Dextran solution and Ficoll-Hypaque (1.05g/ml). Total RNA was then extracted from PBMCs using the RNeasy purification kit (Qiagen®, Germany), and quantified with spectrophotometry (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA). Conversion to DNA was performed using 20 ng total RNA, and a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol.

TaqMan qRT-PCR was used to measure gene expression, using TaqMan gene expression assays, Fast Advanced Master Mix and StepOne Real-Time PCR systems (Applied Biosystems). Technical duplicates were performed and results for each target gene were normalized using 18s as the housekeeping gene. Values are reported as mean ΔCT, and the data compared between MMRd and MMRp colorectal cancer patients using the Mann-Whitney U test ($p<0.05$ used for significance).

Tumor RNA Extraction

After informed consent (as described in the preceding paragraph), tumor samples from previously untreated, colorectal cancer patients were collected. Samples were stored in RNAlater® (Invitrogen, U.S.) at -80°C until RNA extraction was performed. Total RNA was extracted using mRNeasy Mini Kits (Qiagen®, Germany), RNA was quantified and assessed for purity with spectrophotometry (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA) prior to undergoing RNA sequencing

g) ADDITIONAL RNA-SEQUENCING DATASETS

European-Genome-Phenome Archive (EGA)

An additional 69 CRC patient tissue samples (MMRp n= 47, MMRd n= 22) were acquired from the European Genome-Phenome Archive (EGA)[109]. EGA data is held by the European Bioinformatics Institute and the Centre for Genomic Regulation under accession number EGAD00001000215. These were sequenced as paired-ends at 75bp and obtained in Fastq format. Corresponding clinical data including MMR status was acquired.

The Cancer Genome Atlas (TCGA)

There were 598 samples obtained from The Cancer Genome Atlas (TCGA)[110] in the form of raw gene counts. This included 590 MMRp and 97 MMRd CRC tissue samples. Corresponding clinical data including age, BMI, sex, cancer stage, location, survival and MMR status was acquired using cBioPortal. [111-113]. Access was approved via National Cancer for Biotechnology Information (NCBI) (dbGAP accession number phs000178.v11.p8).

h) SUMMARY OF RNA SEQUENCING & ANALYSES PERFORMED

Tumor samples obtained at the University of Louisville were sequenced as paired-ends at 150bp by Novogene (Durham, NC). Quality control analysis using FastQC indicated that the sequences were of good quality with no trimming necessary. Twenty tumor samples met criteria for further analysis. The University

of Louisville and EGA samples were aligned to the hg38 reference genome using the STAR v2.6 aligner with an average alignment rate of 97% across samples [114]. Raw read counts were generated with HTSeq [115] using the same Gencode V36 annotations used by TCGA [116].

Differential Expression and Functional Annotation Analysis

The raw read counts were normalized using relative log expression (RLE) prior to differential expression analysis with DESeq2 [117]. Differential expression was used to identify significant genes in MMRd/MSI-H vs MMRp/MSS CRC. This was followed by functional annotation of differentially expressed genes (logFC >1 or < -1) with gProfiler2 [118]. Functional annotation analysis was used to identify enriched pathways in KEGG, REACTOME, and Gene Ontology (GO). Differentially expressed genes were also inputted to Ingenuity Software, (Qiagen) and using Ingenuity Pathway Analysis, a gene network was created.

Immune Fraction Analysis

Normalized gene counts, Counts per Million (CPM) were input to the deconvolution program CIBERSORTx, Stanford CA [119]. Immune cell fractions for bulk sequenced samples were predicted using LM22 immune signatures while controlling for batch effects and running 1000 permutations. Immune

infiltration scores were also calculated using FPKM values with the xCell enrichment analysis tool [120].

Survival Analysis

Survival Analysis was performed using the 598 patient samples from TCGA in which sufficient survival information was available. Reads normalized using relative log expression were used. The surv_cutpoint function from the R survminor package was used to identify a boundary for high and low gene expression where the greatest survival differences were seen, and overall survival curves analyzed using Kaplan-Meier curves and compared using log rank test.

Correlation Analysis

Correlation analysis was performed using RLE normalized gene counts from n=598 TCGA patients and M1 immune cell fractions based on the CIBERSORTx tool. The Spearman coefficient was calculated using R software.

CHAPTER VII: RESULTS

a). MACROPHAGES, THE TUMOR MICRO-ENVIRONMENT AND IMMUNE CHECKPOINT PROTEIN EXPRESSION- AN RNASEQ ANALYSIS.

i) Introduction:

The optimism surrounding the success of immunotherapy in MMRp/MSI-H colorectal cancer, whilst encouraging, must be considered in conjunction with the minimal response, and continued poor outcomes for patients with MMRp/MSS CRC. These cancers make up the majority of CRC (>80%) and the treatment of advanced stage disease remains a significant unmet need associated with poor patient outcomes. To capitalize on the success of MMRd/MSI-H CRC with immunotherapy and to potentially alter MMRp/MSS CRC to respond to this treatment, we aim to explore mechanisms in which the immune cells, particularly macrophages of the TME contribute to the responses seen with immune checkpoint inhibitor immunotherapy.

The profound immune cell infiltration of the TME and the significant tumor mutation burden of MMRd/MSI-H CRC is well established. Immune cells of the TME contribute to the overall immune checkpoint protein expression, directly by their own immune checkpoint protein expression, and indirectly via cell-to-cell interaction, altering surrounding cancer cells' expression. This is most notably due to the role of T cells in the TME. Tumor associated macrophages (TAMs), also contribute to the cancer's overall immune cell density, however their role in expression of targets for immunotherapy (e.g. PDL1) and contribution to tumor immunotherapy response is unclear.

Macrophages exist across a dynamic spectrum between pro (M1)- and anti (M2)- inflammatory phenotypes. Given the significant pro-inflammatory, immune rich environment of the MMRd/MSI-H TME in colon cancer, it is considered that macrophages may exist in a more M1-like state, which may also contribute to these cancers' PDL1 expression and response to immunotherapy.

To begin exploring this relationship further, and address the mechanism of TAMs contribution to immunotherapy response, the aims of this study were to use RNA-sequenced CRC tumor data to:

i) Predict the immune cell fractions in the tumor microenvironment of MMRd/MSI-H vs MMRp/MMRp CRC

ii) Perform differential gene expression analysis of MMRd/MSI-H vs MMRp/MMRp CRC, and identify genes involved in immune checkpoint protein expression; and

iii) Correlate the immune cell fractions of macrophages and immune checkpoint protein gene expression.

ii) Results

Immune Cell Fraction Prediction

Immune cell fractions were calculated using RNA sequence data from tumors of 687 patients (MMRd/MSI-H n=97 (14.1%), MMRp/MSS n= 590 (85.9%). These included colorectal cancer patient genomic data from i) The Cancer Genome Atlas (TCGA) n= 598, ii) European-Phenome-Genome Atlas (EGA) n=69 and iii) University of Louisville patients (n=20).

Immune cell fractions for all CRC using the LM22 signature, CIBERSORTx were calculated and summarized in **Figure 7**. As expected, macrophages and T cells were the most predominant immune cell type at 31% and 43%, respectively.

Figure 7: Predicted Immune Cell Fractions in 687 Colorectal Tumors.

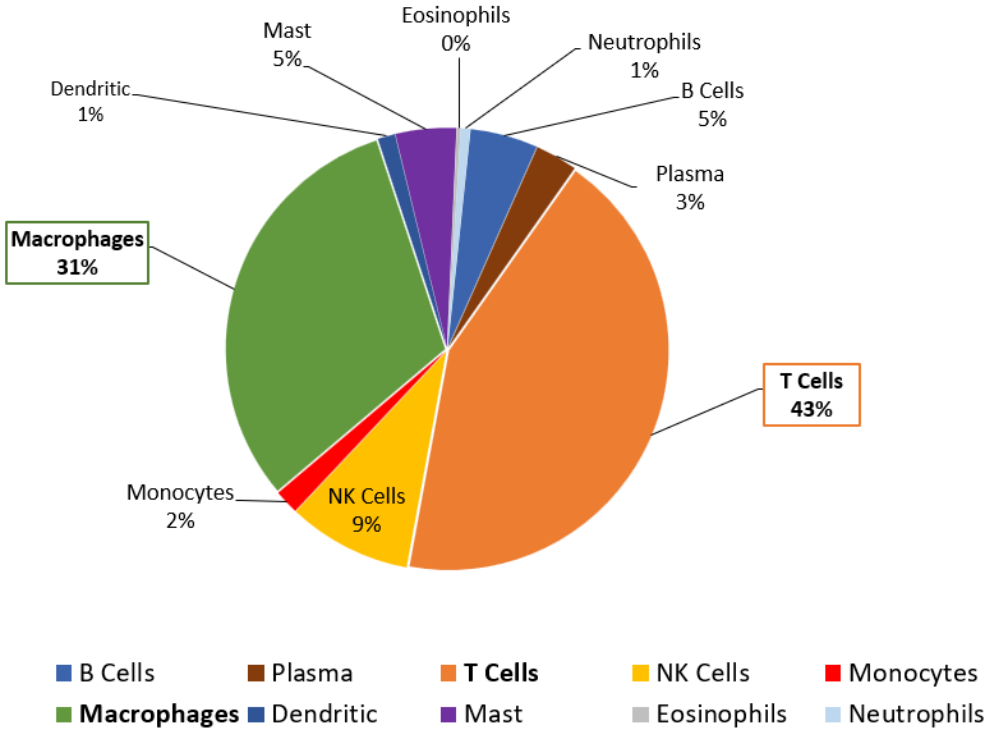


Figure 7: *Immune cell subtype composition of the tumor micro-environment of 687 colorectal cancers. Fraction prediction calculated using CIBERSORTx, deconvolution program with LM22 signature [121]*

Immune cell subtypes (LM22) by individual cell type, compared between MMRd/MSI-H (n=97) and MMRp/MSS CRC's (n=590) are shown in **Table 3.** Several cell subtypes were found to have higher fractions in MMRp/MSS CRC including naïve B cells, plasma cells, while CD8+ T cells were higher in MMRd/MSI-H CRC (7.26 vs 4.45 %).

Macrophage subtypes comparing MMRd/MSI-H and MMRp/MSS are show in **Figure 8.** MMRp/MSI-H CRC's were found to have a higher fraction of pro-inflammatory M1 macrophages (5.9 vs 3.2%) and a lower fraction of non-polarized M0 macrophages (15.2 vs 17.3%). There was no difference in M2 Macrophages between MMRd and MMRp cancers.

Table 3: Predicted CRC Immune Cell Subtype: Mismatch Repair deficient vs Mismatch Repair proficient.

	MMRd/MSI-H N= 97		MMRp/MSS N= 590		
	Mean (%)	SD	Mean (%)	SD	P value
B cells naive	4.324	3.891	5.222	3.549	0.023
B cells memory	0.314	1.696	0.124	0.693	0.055
Plasma cells	2.217	2.429	4.025	4.192	<.001
T cells CD8	7.259	6.186	4.556	3.409	<.001
T cells CD4 naive	0.000	0.000	0.043	0.498	0.4
T cells CD4 memory resting	28.007	8.082	29.216	6.933	0.121
T cells CD4 memory activated	1.646	1.991	2.525	2.957	0.005
T cells follicular helper	1.889	1.995	0.915	1.349	<.001
T cells regulatory (Tregs)	4.699	3.174	5.444	3.426	0.046
T cells gamma delta	0.033	0.324	0.008	0.112	0.155
NK cells resting	8.688	3.058	8.385	2.693	0.314
NK cells activated	0.991	1.546	0.197	0.723	<.001
Monocytes	1.801	1.794	1.806	1.248	0.976
Macrophages M0	15.154	7.055	17.275	8.560	0.021
Macrophages M1	5.843	3.267	3.757	2.486	<.001
Macrophages M2	10.249	4.625	9.904	3.943	0.437
Dendritic cells resting	0.261	0.471	0.401	0.703	0.059
Dendritic cells activated	0.971	1.794	0.930	1.272	0.782
Mast cells resting	0.836	1.576	1.230	2.065	0.073
Mast cells activated	3.534	3.780	3.358	2.882	0.594
Eosinophils	0.337	0.871	0.241	0.630	0.188
Neutrophils	0.946	1.689	0.440	1.027	<.001

Table 3: *Comparison of immune cell fractions in MMRp/MSS vs MMRd/MSI-H colorectal cancer, using LM22 signature, CIBERSORTx [122]. P values calculated by one-way Analysis of Variance (ANOVA*

Figure 8: Macrophage Subtypes of the Tumor Microenvironment: MMRp vs MMRd

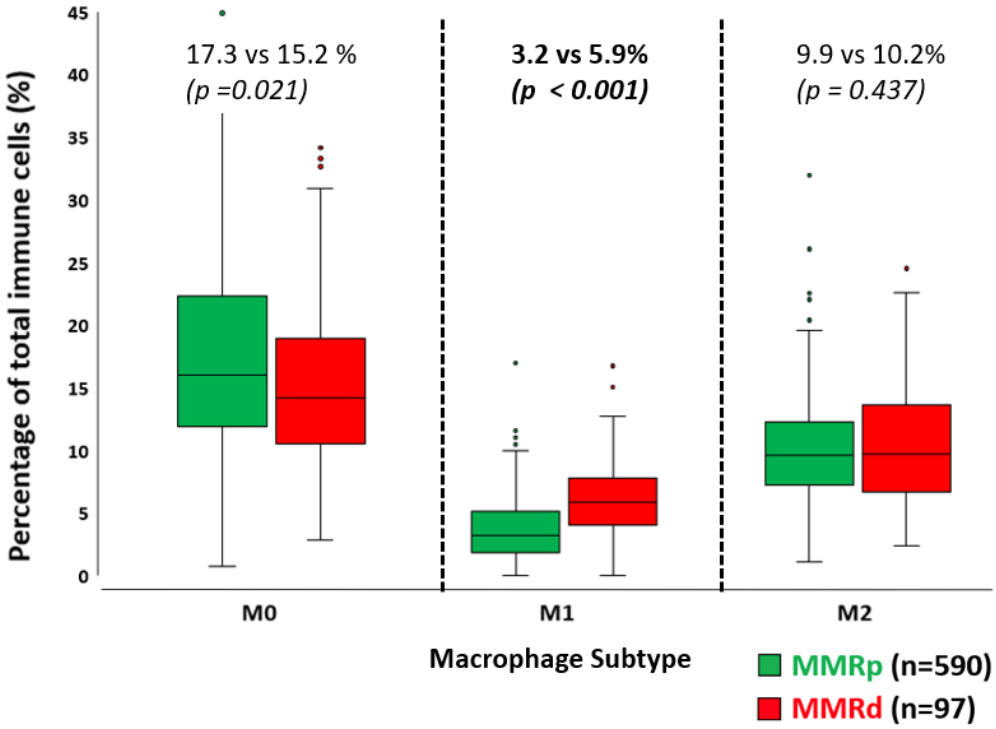


Figure 8. *Box and whisker plot comparing macrophage subtypes between MMRd/MSI-H and MMRp/MSS CRC. Results based on CIBERSORTx prediction.*

A second method of immune cell prediction was used to confirm these findings. Using the xCELL enrichment analysis tool [120], immune cell infiltration scores were calculated. M1 macrophage enrichment scores were found to be higher in MMRd/MSI-H vs MMRp/MSS CRC's [0.069 ± 0.02 vs 0.053 ± 0.018, $p<0.001$]. To further validate these techniques, a correlation analysis was performed comparing the M1 fraction prediction by CIBERSORTx and the M1 immune infiltration score; a moderate correlation was found between the M1 fraction and infiltration score using these two methods (Spearman Coefficient 0.48, $p<0.0001$).

Differential Gene Expression and Immune Checkpoint Proteins

Differential expression analysis was performed using the same patient cohort as above. Gene expression was compared between MMRd/MSI-H and MMRp/MSS cancers and genes were considered differentially expressed if the log_2FC was < -1.0 or >1.0. Analysis identified 762 genes to be differentially expressed, 317 were upregulated and 445 were downregulated.

The immune checkpoint proteins **PDL1** *($Log_2 FC = 1.5$; $p(adj)log_{10} = 28.5$),* **PD-1** *($Log_2 FC = 1.27$; $p(adj)log_{10} = 17.5$)* and **LAG-3** *($Log_2 FC = 1.66$; $p(adj)log_{10} = 35.1$)* and its ligand **FGL1** *($Log_2 FC = 2.14$; $p(adj)log_{10} = 4.62$)* were all significantly upregulated in MMRd/MSI-H vs MMRp/MSS CRC.

Correlation Analysis

Given the above findings, we performed a correlation analysis to evaluate whether there was an association between the tumors with the highest M1 infiltration and their gene expression of immune checkpoint proteins. **Figure 9** shows some of these results. The Spearman co-efficient was calculated using CIBERSORTx M1 fraction and the Relative Log Expression (RLE) normalized gene counts for the genes of interest above.

The M1 macrophage fraction was correlated with gene expression of PDL1 (R = 0.554), LAG-3 (R=0.50) and PD-1 (R= 0.418); p(adj) <0.001 for all. There was no correlation with M1 macrophage infiltration and FGL1 gene expression

Figure 9: Macrophage and Immune Checkpoint Protein Correlation

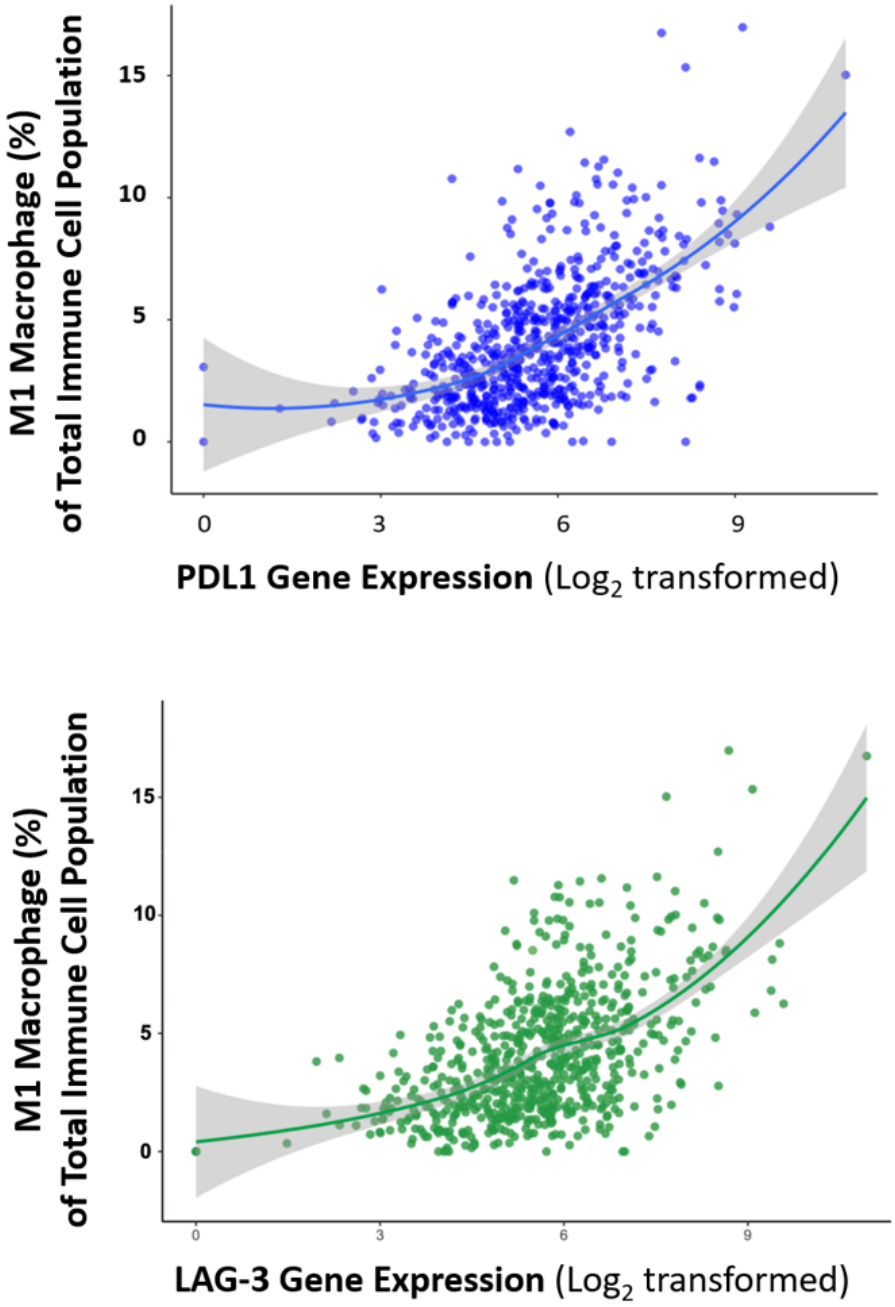


Figure 9: *Spearman Correlation between M1 fraction of total immune cells and log_2 transformed gene expression of LAG-3 (R=0.55 p<0.001) and PDL1 (R=0.54 p<0.001).*

iii) Discussion

Analysis of RNA sequenced data from over 600 CRC tumors revealed a distribution of immune cells in the tumor microenvironment as expected. Tumors were mainly composed of macrophages and T Cells, with M2, anti-inflammatory macrophages being the predominant TAM phenotype. Using two different RNAseq data deconvolution platforms, we identified small, but potentially relevant differences in the M1 macrophage infiltration in CRC's depending on MMR status.

MMRd/MSI-H CRC's were found to have a higher proportion of M1 macrophages compared to MMRp/MSS CRC's, and this was not accompanied by differences in M2 macrophages. The TME of MMRd/MSI-H cancers are known to be immune dense, and a higher M1 macrophage infiltration contributes to their overall pro-inflammatory environment.

Secondly, as anticipated, and clinically reported, this analysis confirmed that MMRd/MSI-H cancers have increased expression of PD-1 and its ligand PDL1, along with LAG-3 and one of its ligands FGL1. Increased gene expression of immune checkpoint proteins PD-1, PDL1 and LAG-3 also correlated with tumors containing the highest M1 macrophage infiltration.

While there are acknowledged limitations to the use of deconvolution programs and prediction of immune cell subtypes, these results support existing literature, that suggest that one of the many mechanisms in which MMRd/MSI-H

CRC's express higher PDL1 *may* be through the differences in their macrophage phenotype.

These results report a correlation only, between M1 macrophages, and PDL1 expression, both of which are associated with MMRd/MSI CRC's; however, these results warrant further study into the role and mechanism by which a pro-inflammatory macrophage phenotype can alter the tumor immune checkpoint protein expression. The next step of this work is to address how macrophage polarization may alter cancer cell immune checkpoint protein expression in an in-vitro cell-line model.

b). MONOCYTE TO M1 MACROPHAGE POLARIZATION PROTOCOL

i) Introduction

Colorectal cancers (CRC's) are composed of dynamic complex tumor microenvironments (TME) with infiltration of immune, structural and cancer cells. The cells of the TME and mediators of this environment contribute to all aspects of cancer behavior, including proliferation, differentiation, response to therapy and metastases.[123, 124]. It is therefore imperative that in vitro studies into colon cancer cells, also consider, as able, the effects of the surrounding cells and the complex signaling environment in which they grow [125].

The model of our overall work is built on exploring the relationship and signaling mechanisms between macrophages of the TME and cancer cells. Macrophages are crucial immune cells within the tumor microenvironment and as described, exist on a continuous spectrum between pro-inflammatory (M1-like) and anti-inflammatory (M2-like) macrophages [100]. We have previously studied the relationship of M2-like macrophages and CRC, and work with an established M2a-like macrophage cell line model [101, 108].

To study, in vitro, the role macrophage phenotypes play in the immune checkpoint protein expression of CRC, the aim of this work was to design a reproducible protocol of the differentiation and polarization of the human THP-1 monocyte cell line [126] into an M1-like macrophage that could sustain an M1 like phenotype for co-culture with cancer cell lines.

ii) Experimental Variables

Figure 10 provides an overview of the steps of a previously established THP-1 to M2 polarization protocol and the unknown variables to be determined. The treatment choice, treatment dose, timing of treatment and duration of rest which were required to be determined to polarize the M0 differentiated macrophages into M1-like macrophages.

To determine the optimal protocol, polarization from M0 using lipopolysaccharide +/- IFNγ using 5 different dose combinations, with 2 treatment times and 6 rest time variables were considered. The variable experimental conditions used to determine the optimal polarization protocol are summarized in **Table 4**. M1 polarization was confirmed based on gene and protein expression of pro- and anti- inflammatory cytokines and macrophage markers, summarized in **Table 5**

Figure 10: THP-1 to Macrophage Differentiation and Polarization Protocol

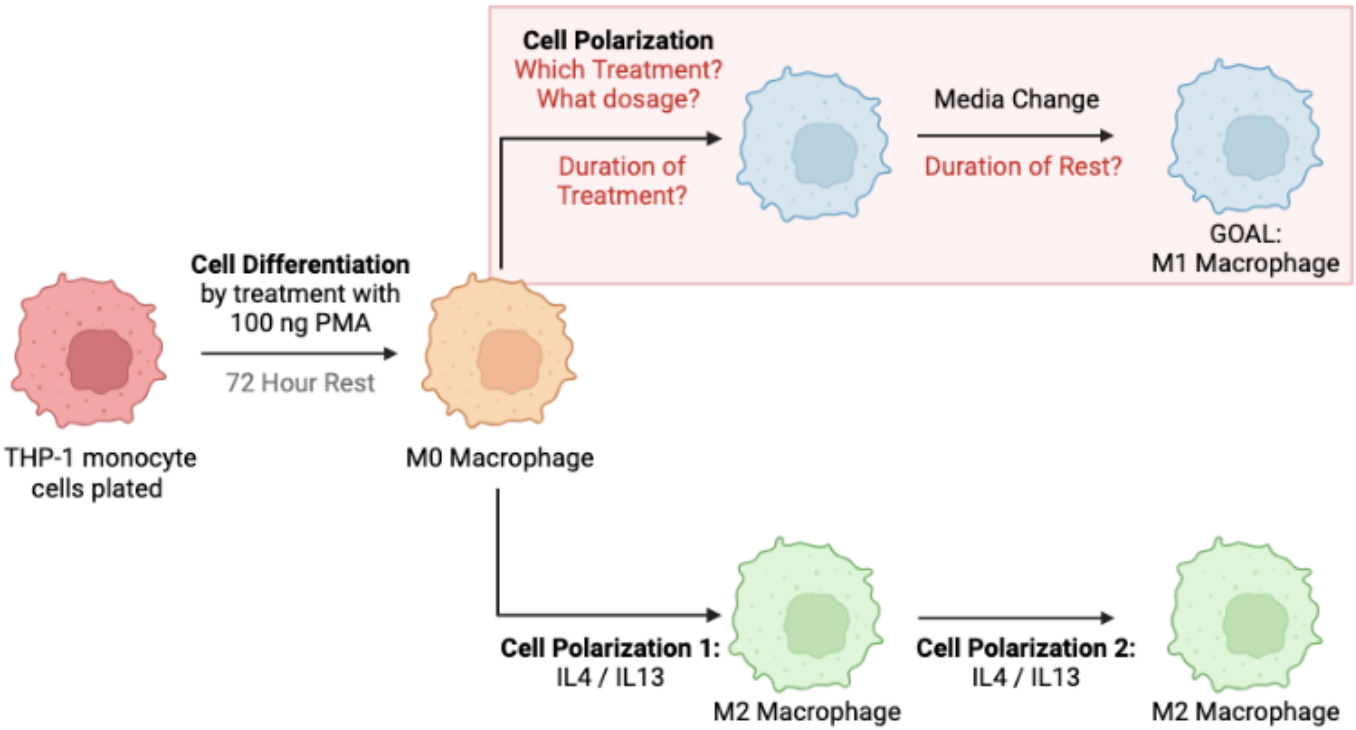


Table 4. Experimental Variables for M1 Polarization Protocol

Treatment	Treatment Dose	Treatment Time	Rest Time
LPS alone	10ng/ml 50ng/ml 100ng/ml	1hr 2hr	3hr 6hr 9hr 12hr 18hr 24hr
LPS 100ng/ml + IFNγ	(IFNγ) 20ng/ml 50ng/ml		

Table 4: *The treatment choice, dose, combination and duration, as well as rest period for M1 polarization of THP-1 monocytes following differentiation to M0 macrophages. LPS: Lipopolysaccharide, IFNγ: Interferon-γ*

Table 5. Macrophage Markers and Cytokines used to Confirm Polarization.

	Gene Expression		Protein Expression	Cell Surface Markers
M1-Macrophage (Pro-inflammatory)	CD80 CD86 HLA-DR	TNFα IL-1β IL-6	IL6 TNFα	CD80 CD86
M2 Macrophage (Anti-inflammatory)	CD206 IL10 TGFβ		IL-10	CD206

Table 5*: Pro and anti-inflammatory macrophage markers and cytokines used to determine successful M1 and M2 macrophage polarization as measured by gene expression (qRT-PCR), protein expression (ELISA) and cell surface markers (Flow Cytometry).*

iii) Results

M1-like macrophages were characterized, and polarization confirmed via the measurement of gene and protein expression of macrophage markers and cytokines using qRT-PCR, ELISA and flow cytometry. The optimal treatment protocol based on gene and protein expression as well as cell viability was found to be 1 hour of 100ng/ml LPS, followed by a rest period in complete (RPMI + 10% FBS + 1% AA) media for 18 hours. Representative results are described and discussed below.

The protocol allowed for the use of M1 macrophages to be used in a co-culture model (described in chapter VIIc) with cancer cells lines. It also enabled the use of M1 macrophage media to study the effects of treatment with media alone vs cell co-culture combination

Gene Expression

Changes in gene expression following LPS treatment + rest period are shown as mean fold change (FC) and standard deviation (SD) in **Table 6** Upregulation was determined by comparing to the gene expression of untreated M0 macrophages (using ΔCT values and significance calculated using Kruskall Wallis test). CD80 and pro-inflammatory cytokines TNFα, IL1β, and IL6 were significantly upregulated at all time points. Downregulation of M2 marker CD206 was noted at 18 and 24 hours, and there were no changes in TGF-β compared to untreated control. ($p<0.05$).

Table 6: Inflammatory Cytokine and Macrophage Marker Gene Expression

Cytokine/ Macrophage Marker	Rest Period following 1-hour LPS treatment.					
	3-hours	6-hours	9-hours	12-hours	18-hours	24-hours
Pro-inflammatory (M1 markers)						
CD80	**43.1 ± 5.1**	**115.1± 18.9**	**267.2 ± 64.9**	**258.3 ± 18.2**	**88.5 ± 9.4**	**69.7 ±16.7**
TNFα	**103.4 ± 37.1**	**36.9 ± 6.9**	**27.8 ± 6.5**	**14.5 ± 3.2**	**4.88 ± 0.6**	**9.2 ± 0.6**
IL1β	**78.9 ± 8.9**	**207.5 ± 41.5**	**115.1 ± 19.9**	**79.0 ± 30.4**	**7.5 ± 1.7**	**8.0 ± 1.5**
IL6	**1663.2 ± 484.2**	**3479.2 ± 564.2**	**1651.1 ± 252.5**	**1624.7 ± 212.3**	**111.1 ± 29.8**	**83.5 ± 14.6**
Anti-inflammatory (M2 markers)						
CD206	-1.5 ± 0.3	-2.01 ± 0.9	1.0 ± 0.2	-2.2 ± 1.1	**-3.6 ± 1.1**	**-9.2 ± 8.7**
TGF-β	-1.3 ± 0.6	1.15 ± 0.1	1.2 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	1.1 ± 0.2

Table 6: *Mean FC ± SD gene expression following 1-hour 100ng/ml LPS treatment and rest. ΔCT results were compared with Kruskall Wallis test and* ***Grey shaded & boldface=p<0.05. N=12 for all time points*****.**

Comparison of the significantly upregulated genes described above and results from Table , to 2-hour 100ng/ml LPS treatment is found in **Table 7.** Fold changes and SD are expressed, and the 1-hour vs 2-hour treatment was compared for each gene and rest period time point.

The M1 marker CD80 showed similar upregulation in gene expression following both treatment times for the earlier time points (3- and 6-hours rest); however, the difference in fold change was significantly higher following 2 hours of treatment after 12-, 18- and 24-hours rest (258.3 vs 408.2; 88.53 vs 105.6; and 69.7 vs 101.7, $p<0.05$) respectively. Interestingly, as will be discussed, this difference did not translate to a difference in CD80 cell surface marker on subsequent protein analysis.

The pro-inflammatory cytokines TNFα and IL6 were both significantly upregulated. TNFα had its highest upregulation at 3 hours rest, following 2 hours LPS (FC = 225.88), however for ongoing upregulation at subsequent time points there was either no difference between treatment times, or the 1-hour treatment had a greater effect.

Similarly, IL6 had the most significant increase in gene expression with 2 hours of LPS 100g/ml followed by 3- and 6- hour of rest (FC 31,552.0 and 19,116.0 respectively). Fold-change was significantly higher in the 2-hour vs 1-hour LPS treatment group, and this pattern continued for the remaining rest periods studied (9-, 12-, 18-, 24- hour

Table 7: Gene Expression Fold-Change following 1 vs 2-hour LPS treatment + Rest Period.

Gene		Rest Period Following LPS Treatment											
		3 hours		6 hours		9 hours		12 hours		18 hours		24 hours	
	LPS	Mean FC	SD	Mean FC	SD	Mean FC	SD	Mean FC	SD	Mean FC	SD	Mean FC	SD
CD80	1 h	43.14	5.04	115.07	18.93	267.18	64.89	258.26	18.20	88.53	9.39	69.67	16.68
	2 h	45.39	14.54	112.09	6.85	176.00	31.04	408.24	90.44	105.65	9.70	101.69	7.37
P value			*0.752*		*0.750*		***0.025***		***0.009***		***0.017***		***0.004***
TNF	1 h	103.38	37.09	36.87	6.90	27.80	6.53	14.60	3.21	4.88	0.55	9.17	0.61
	2 h	225.88	16.07	35.69	3.46	15.11	2.75	6.35	1.66	3.94	0.60	3.24	0.70
P value			***0.000***		*0.750*		***0.006***		***0.004***		***0.020***		***0.000***
IL1β	1 h	78.39	8.96	207.46	41.49	115.13	19.93	79.03	30.43	7.48	1.75	8.04	1.46
	2 h	73.08	11.17	261.05	23.93	64.72	12.91	16.68	5.32	3.75	0.81	13.19	2.52
P value			*0.431*		*0.055*		***0.005***		***0.004***		***0.004***		***0.004***
IL6	1 h	1663.25	484.18	3479.22	564.24	1650.87	252.53	1623.69	212.31	111.06	29.77	83.42	14.55
	2 h	31552.0	10108.8	19166.0	3854.44	3449.34	880.39	2992.28	1035.21	38.76	7.96	149.26	55.90
P value			***0.000***		***0.000***		***0.004***		***0.020***		***0.002***		***0.026***
MRC1	1 h	-1.51	0.32	0.50	0.16	1.00	0.16	-2.23	1.10	-3.62	1.11	-9.21	8.64
	2 h	1.83	0.22	-1.88	0.36	-2.70	0.51	-3.46	0.80	-1.94	2.45	-7.60	2.53
P value			***<0.0001***		***0.000***		***0.000***		*0.064*		*0.100*		*0.353*
TGFβ	1 h	-1.30	0.59	1.11	0.11	1.26	0.14	1.04	0.20	1.36	0.09	1.41	0.16
	2 h	-1.05	0.13	-1.44	0.13	1.01	0.15	1.27	0.25	1.74	0.20	1.48	0.29
P value			*0.386*		***<0.0001***		***0.025***		*0.096*		***0.004***		*0.353*

Table 7: *Gene expression of macrophage markers and cytokines expressed as Mean FC and SD. N=12 for all time points. P value represents statistical comparison between 1h vs 2h LPS for each gene and time point.*

Protein Expression

Macrophage protein expression was measured in cell supernatant via Enzyme Linked Immunosorbent Assay (ELISA). TNFα and IL6, two hallmark cytokines of M1 macrophages were measured for the same experimental variables as above.

ELISA results are shown in **Figure 11.** This demonstrated an increased protein expression of both TNFα and IL6 following treatment of differentiated M0 macrophages with 100ng/ml LPS for 1- or 2- hours compared to a negative control (untreated M0s). Protein expression was significantly increased for both 1- and 2- hours of LPS treatment and following 6-, 9-, 12-, 18- and 24- hours of rest for IL6. TNF was additionally significantly increased at 3- hours of rest. For all rest times following LPS treatment there was no difference between protein levels following 1 or 2 hours of treatment for either IL6 or TNFα. These results suggest no benefit of 2 hours treatment over 1-hour LPS 100ng/ml treatment, despite the differences observed in gene expression as discussed above.

Figure 11: TNFα and IL6 Protein Expression Following 1h vs 2h LPS Treatment

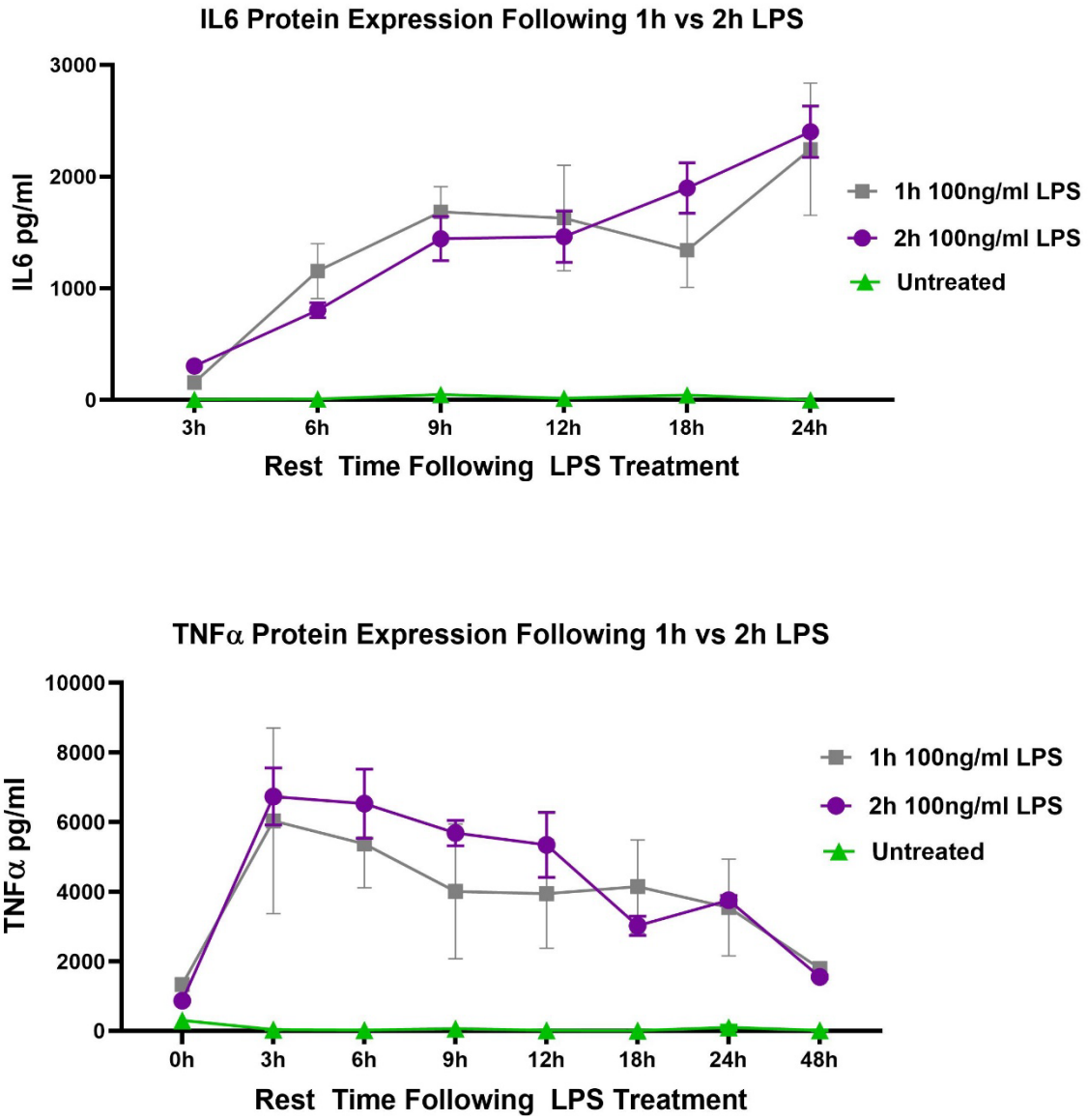


Figure 11*: IL6 and TNFα protein expression following 1 vs 2 hours of 100ng/ml LPS + rest. Mean concentration ± SD presented. All treated times points were significantly increased from untreated other than 3h, IL6 with no difference in 1 vs 2 hours LPS. (p<0.05, Two-way ANOVA for each time with Bonferroni correction). N=6 for all time points and treatments. LPS: Lipopolysaccharide*

.Cell Surface Protein Expression

Further confirmation of successful M1 macrophage polarization was demonstrated by the measurement of cell surface Cluster of Differentiation (CD) markers. CD80 and CD86 are both expressed on M1 macrophages, while CD206 (MRC1) is a marker of anti-inflammatory M2 macrophages.

Macrophage marker expression is shown in **Figures 12 and 13.** These are representative flow quadrant plots, and histograms confirming increased expression of CD86 (80.3%) and CD80 (55.3%) on macrophages following 1-hour 100ng/ml LPS treatment and 18-hour rest. This was a significant increase from M0 macrophages in which there was minimal expression of CD80 or CD86, and polarization was additionally confirmed by low (0.2%) expression of CD206.

Figure 12: Macrophages treated with 1h hour 100ng/ml LPS + 18-hour rest.

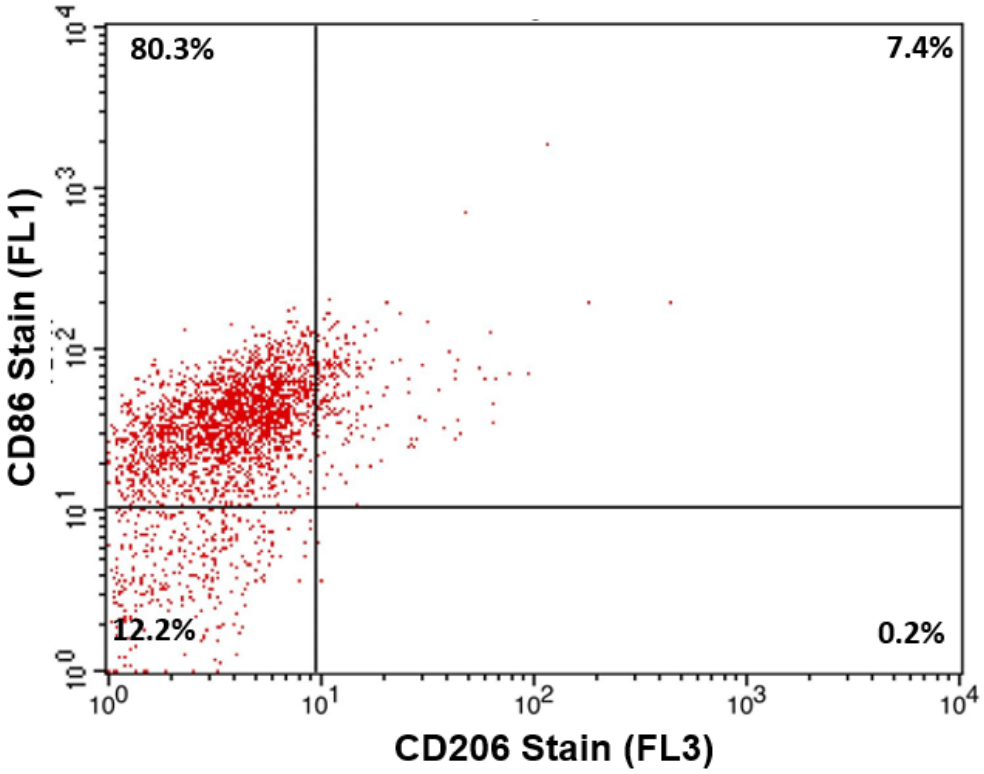
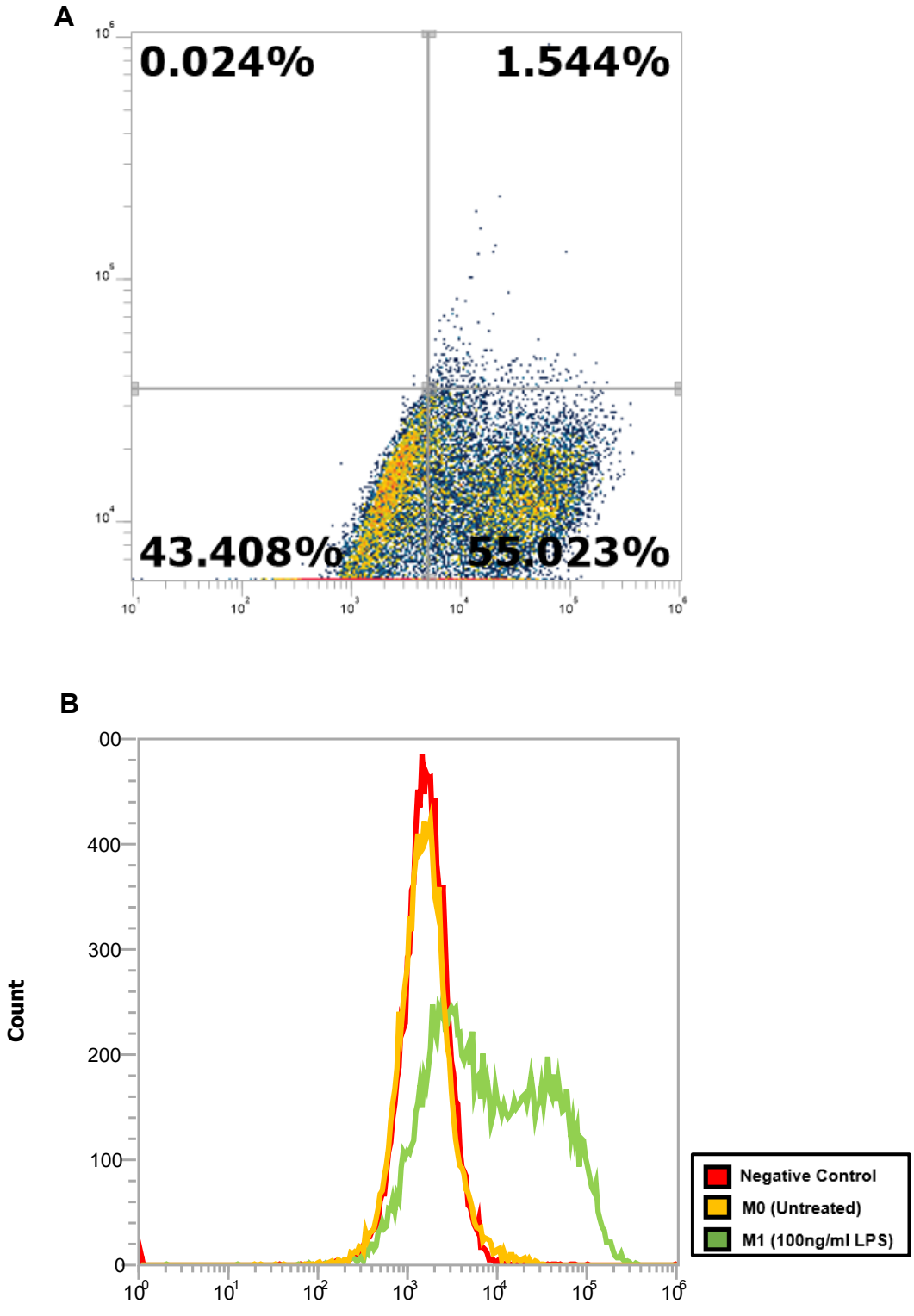


Figure 13: Macrophages CD80 Expression



Figures 12 and 13

Representative flow quadrant plots and histogram confirming the successful polarization to M1 like macrophages. Fig 12: Cell surface markers CD80 and CD206 showed minimal expression in gated cells. Fig 13.A) Flow quadrant plot demonstrating 55% of M1 polarized macrophages expressed CD86. B) Histogram demonstrating increasing in CD86 expression in M1 macrophages vs untreated M0 macrophages. Minimum 10,000 gated events were confirmed for each analysis.

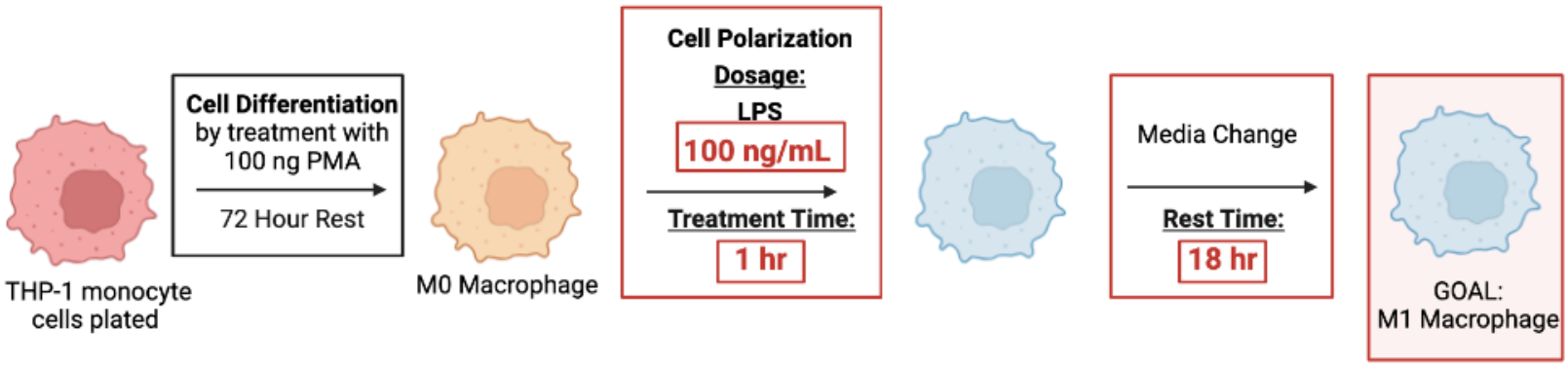
iv) Conclusion

The successful polarization of THP-1 differentiated, non-polarized M0 macrophages to a pro-inflammatory M1-like phenotype was confirmed through measurement of cell supernatant protein expression of IL6 and TNFα, upregulation of the pro-inflammatory M1 markers and pro-inflammatory cytokine gene expression, along with positive CD80 and CD86 cell surface expression.

The final treatment, dose, duration of treatment and duration of rest were based on a balance of increased gene and protein expression while maintaining cell viability, both in the immediate treatment period and to ensure that cells remained viable during co-culture with cancer cell lines. The final protocol is outlined in **Figure 14** and has allowed for the in vitro study of colon cancer cells lines with both M1-like and M2-like co-culture.

While cancer-macrophage cell culture cannot entirely replicate the complexity of the tumor microenvironment, and there are limitations in the use of cell lines compared to human macrophages[127, 128], our cell line model has the benefit of enabling the study of reproducible, standardized, consistent M1-like macrophages in an in vitro model.

Figure 14 : M1 Macrophage Polarization Protocol



c). MACROPHAGE PHENOTYPE AND COLON CANCER PDL1 EXPRESSION - A CELL LINE CO-CULTURE MODEL.

i) Introduction

As discussed previously, macrophages make up a significant part of the tumor micro-environment as Tumor Associated Macrophages (TAMs). These exist on continuum between extreme ends of M1 (pro-inflammatory) and M2 (anti-inflammatory) states. Whilst the majority of TAMs in the TME of CRC are M2-like; pro-tumorigenic and anti-inflammatory, we have shown in our immune cell prediction analysis (Chapter 7.1), that MMRd/MSI-H CRC's contain a proportionally higher fraction of macrophages of an M1-like phenotype compared to MMRp/MSS CRC. There was no difference in their fraction of M2-like macrophages. A moderate correlation between tumors of higher M1-like macrophage proportion and higher PDL1 expression was also shown.

To further examine the effect of macrophage subtypes on cancer PDL1 expression, to confirm the findings from our RNAseq analysis and to identify pathways in which macrophage phenotype may alter PDL1 expression; the aims of this work were to:

i) Measure the PDL1 expression of colon cancer cell lines.

ii) Study the changes in cancer PDL1 expression following co-culture with THP-1 monocyte derived M1-like vs M2-like macrophages.

iii) Compare the effect of macrophage co-culture on cancer PDL1 expression in MMRd/MSI-H vs MMRp/MSS CRC.

Colon Cancer Cell Lines & Culture Model

To compare the different effects macrophage co-culture may have depending on baseline PDL1 expression and MMR status, we used 4 different colon cancer cell lines. HT29 and SW480 are derived from MMRp/MSS tumors which are traditionally “immune-cold” and PDL1 negative. The Hct116 and RKO cell lines which originate from MMRd/MSI-H colon cancers. **Table 8** summarizes the clinical and pathological features of these cell lines [129-134]. The HT29 cell line originates from a more advanced MMRp/MSS cancer than SW480, and also contains the BRAF V600E mutation- a poor prognostic indicator in colorectal cancer; however, molecular therapies targeting this mutation are available. Hct116 contains a single nucleotide mutation in the MLH1 gene- [NM_000249.4(MLH):c.755C>A (p.Ser252Ter)] a recognized mutation in Lynch Syndrome. The replacement of C>A at codon 252 results in the substitution of a serine in place of a stop codon [135]. RKO, is derived from a cancer with MSI due to promotor region hypermethylation, commonly associated with sporadic MSI cancers as previously described.

We hypothesized that PDL1 expression in colon cancer cell lines will be increased with M1 macrophage co-culture and may decrease following M2 macrophage co-culture. **Figure 15** shows the design of the co-culture model and experiment.

Table 8: Colon Cancer Cell Lines: Clinical and Genetic Characteristics

Cell Line	Clinical-pathological Features	MMR Status + Gene Mutations	Other CRC Gene status
HT29	44y Female Caucasian Stage III	MMR proficient/MSS	BRAF- V600E KRAS- WT TP53- mut
SW480	51y Male Caucasian Stage II	MMR proficient/MSS	BRAF- WT KRAS- mut TP53- mut
Hct116	48y Caucasian Male Stage II Lynch Syndrome	**MMR deficient/MSI-H** **MLH1**-mut: *SNV* c.755C>A (p.Ser252Ter)	BRAF- WT KRAS-mut TP53-WT
RKO	63y Male Stage III Poorly differentiated	**MMR deficient/MSI-H** **MLH1**-silenced (Promotor hypermethylation)	BRAF- V600E KRAS- WT TP53- WT

Table 8: *Clinical and genetic characteristics of the colon cancer cell lines used in the macrophage-cancer co-culture model. WT= wild type, mut = mutation, BRAF V600E, common oncogenic mutation associated with colon cancer in which targeted molecular therapy (anti-BRAF/anti-MEK antibodies) can be offered as an adjuvant therapy.*

Figure 15: Macrophage-Cancer Co-Culture Model

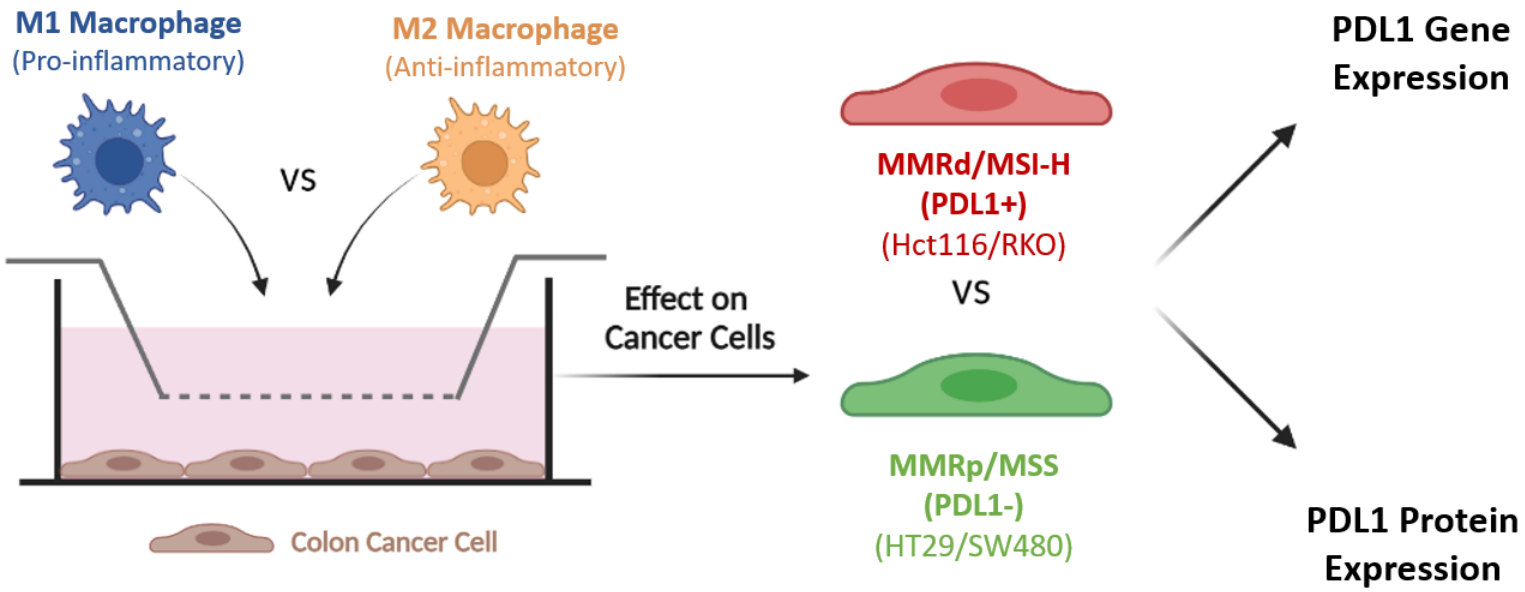


***Figure 15**: Macrophage-Cancer Co-Culture Model: Cancer cell lines are plated at 0.2x10^6/ml in the wells of a 24 well plate. Following differentiation and polarization as described in Chapter 7.2 M1 or M2 macrophages in upper chamber, semi-permeable transwell baskets are then combined with colon cancer for 24 hours of co-culture.*

ii) Results

Gene Expression

As expected, at baseline, the MMRd/MSI-H colon cancer cell line RKO had the highest PDL1 gene expression (mean DCT 12.8 ± 0.2). The single culture gene expression of the remaining 3 cell lines was similar **(Table 9).** The effect of co-culture with M1 and M2 macrophage is noted in Table 7.3b (mean ∆CT) and shown in **Figure 16** as fold change (FC) relative to single culture.

Interestingly, despite its significantly higher baseline gene expression, RKO cell lines had a negligible change in gene expression following co-culture with either macrophage phenotype (FC ± SD = *M1:*1.68 ± 0.45; *M2:* 1.31 ± 0.19). Hct116 cell line, the second MMRd/MSI-H cell line had a 4.5-fold increase in PDL1 expression following M1 macrophage co-culture. Both MMRp/MSS cell lines HT29 and SW480 also had an upregulation in PDL1 following M1 macrophage co-culture, FC= 5.86 ± 1.71 and 2.03 ± 0.62 respectively. Combining cancer cells with M2 macrophages for 24 hours resulted in no or biologically negligible changes in PDL1 gene expression in all 4 cancer cell lines. **(Table 9)**

Each experiment co-culture was also repeated using M1 and M2 media as treatment, rather than cells in co-culture baskets. Results (not shown) showed similar results with each of the macrophage and cancer cell line combination.

Table 9: Cancer Cell PDL1 Expression Single vs Co-Culture.

	Single			M1 Co-Culture				M2 Co-Culture			
	Δ CT	**SD**	***n***	**Δ CT**	**SD**	***n***	**p value**	**Δ CT**	**SD**	***n***	**p value**
MMRp/MSS Cell Lines											
HT29	20.95	0.8216	*32*	18.71	0.3791	*16*	<.0001	21.74	0.3166	*18*	<.0001
SW480	20.74	0.3755	*6*	20.03	0.4506	*10*	0.0389	20.76	0.3135	*10*	0.949
MMRd/MSI-H Cell Lines											
Hct116	20.07	0.904	*39*	17.95	0.8128	*51*	<0.0001	21.01	0.3359	*18*	<0.001
RKO	12.8	0.228	*10*	11.88	0.3075	*10*	0.0035	12.36	0.1916	*10*	0.1426

Table 9: *Cancer Cell PDL1 expression single vs co-culture. CT= Cyle Threshold, SD = Standard Deviation, P values calculated using ΔCT values, compared to single culture. One-way ANOVA performed with a post hoc Benjamini–Hochberg correction to control the false discovery rate at 5%. N= experimental duplicates.*

Figure 16: Cancer PDL1 Gene Expression following Macrophage Co-Culture

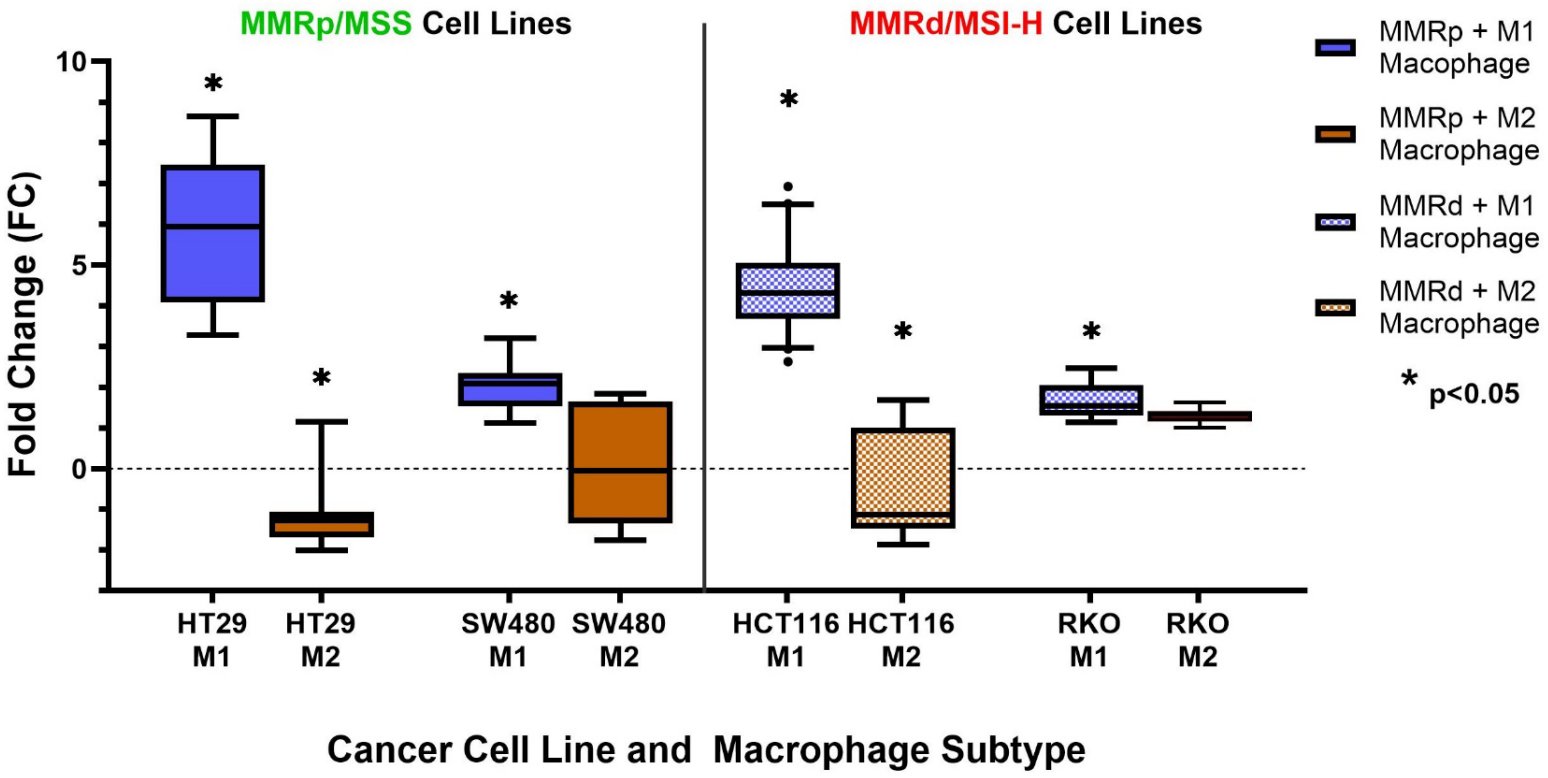


Figure 16: *Box and Whisker plot demonstrating the change in gene expression (Fold-Change) following Co-Culture of Cancer cells with M1 macrophages (blue) or M2 macrophages (orange). Solid color represents the result in MMRp/MSS CRC cell lines HT29 and SW480. Dashed color represents the MMRd/MSI-H cell lines Hct116/SW480***.**

Protein Expression

PDL1 cell surface protein was also measured. Percentage (%) of cells positive for PDL1 and median fluorescence intensity (MFI) of positive cells was compared between cell lines and macrophage phenotype co-cultures.

Cell surface protein expression was highest in RKO and Hct116 cells lines. Analysis of RKO found that 98.4 ± 0.62 % of cells expressed PDL1 at baseline. The RKO cell line maintained a >99% expression following co-culture with both M1 and M2 macrophages. Unlike gene expression, the Hct116 cell line had a significantly higher baseline protein PDL1 expression, 29.1 ± 0.62 %, compared to the two MMRp/MSS cancer cell lines HT29 (1.94 ± 1.64 %) and SW480 (7.99 ± 1.18) $p < 0.05$ for both.

Following co-culture with M1 macrophages, HT29, SW480 and Hct116 all increased their cell surface PDL1 to 26.7, 19.3 and 66.8 % respectively. Hct 116 was the only cell line to also increase expression with M2 culture, although to a lesser extent. **(Figure 17 and Table 10)**

MFI is also shown in Table 7.3c, while there could be no increase in % of PDL1 expressing RKO cells, it was the only cell line to significantly increase MFI following co-culture. Across all conditions, MFI was significantly higher in RKO compared to all other cells lines.

Figure 17: Cancer Cell PDL1 Protein Expression

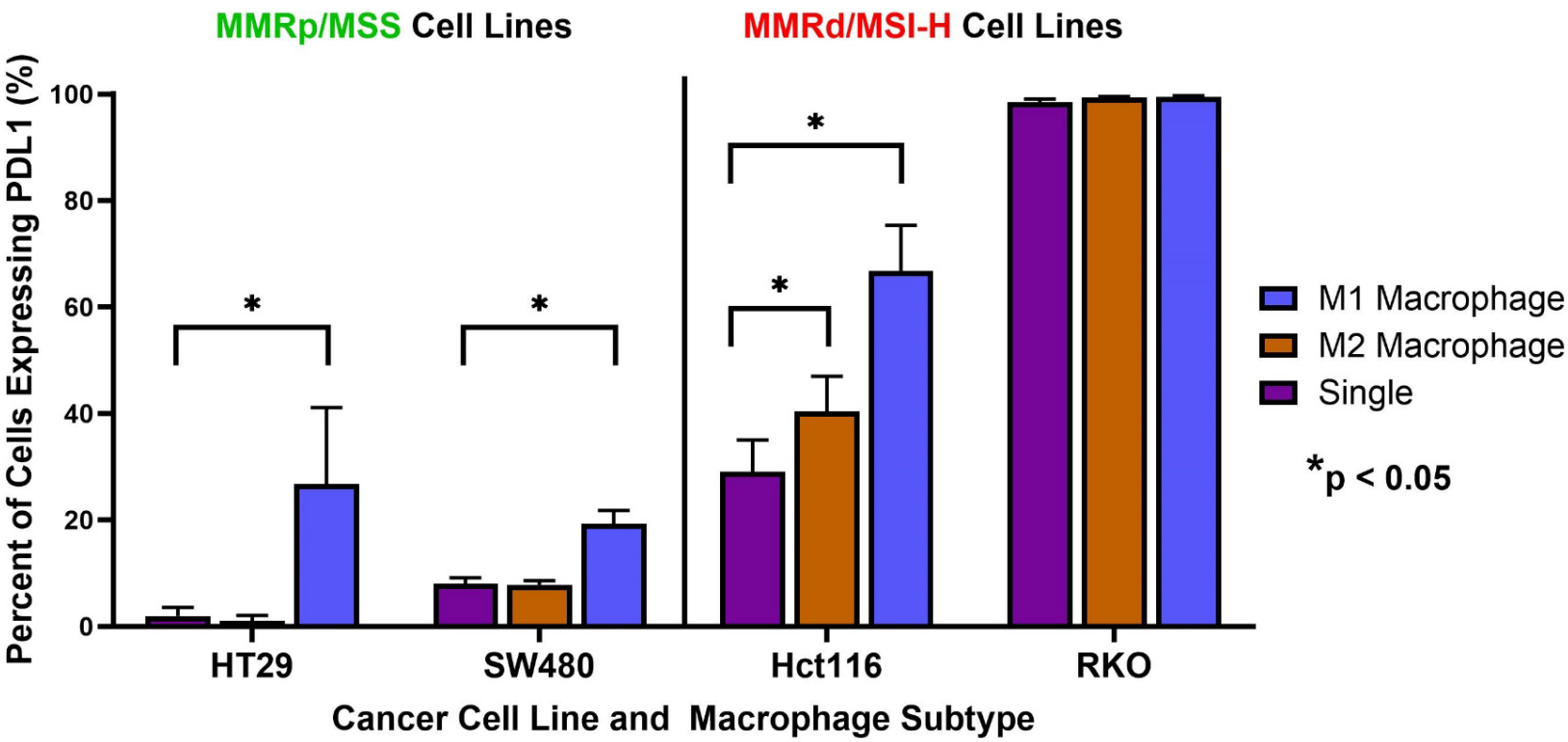


Table 10: Cancer Cell Surface PDL1 Protein Expression

Percent (%) of Cells PDL1+

	Single			*M1 Co-Culture*				*M2 Co-Culture*			
	N	**Mean %**	**SD**	**N**	**Mean %**	**SD**	**P value**	**N**	**Mean %**	**SD**	**P value**
HT29	8	1.94	*1.64*	**20**	**26.7**	***14.4***	**<0.0001**	12	1.04	*1.04*	0.7194
SW480	10	7.99	*1.18*	**10**	**19.3**	***2.49***	**0.0002**	6	7.84	*0.76*	0.9656
Hct116	17	29.1	*5.93*	**25**	**66.8**	***8.51***	**<0.0001**	**6**	**40.4**	***6.59***	**0.0001**
RKO	12	98.4	*0.62*	16	99.5	*0.15*	0.6738	8	99.3	*0.25*	0.7743

Median Fluorescence Intensity (MFI) of PDL1+ Cells

	Single			*M1 Co-Culture*				*M2 Co-Culture*			
	N	**MFI**	**SD**	**N**	**MFI**	**SD**	**P value**	**N**	**MFI**	**SD**	**P value**
HT29	8	14.31	*2.83*	20	16.0	*2.62*	0.755	12	14.97	*1.29*	0.9103
SW480	10	12.32	*0.19*	10	13.86	*0.35*	0.7912	6	12.52	*0.10*	0.9764
Hct116	17	17.83	*2.53*	25	24.99	*4.88*	0.0813	6	14.03	*0.06*	0.5375
RKO	12	177	*24.58*	**16**	**252.4**	***31.34***	**<0.0001**	**8**	**227.5**	***11.47***	**<0.0001**

Table 10. *Cancer PDL1 Protein Expression. Percent of all cells expressing PDL1 (mean %, SD) and median fluorescence intensity (MFI) of positive cells (Mean, SD). P values are compared to single culture for each cell line.* ***Boldface and grey = significant <0.05*** *One-way ANOVA performed with a post hoc Benjamini–Hochberg correction to control the false discovery rate at 5%. N= experimental duplicates*

iii) Discussion

PDL1 expression is a hallmark of MMRp/MSI colorectal cancer, and its expression allows for cancer to be targeted, and treated with anti-PD-1/PDL1 immune checkpoint inhibition. MMRp/MSS CRC do not express PDL1, and generally do not respond to immunotherapy. As described above, there are many contributing genetic, morphological, and immune related factors which cause these cancers to differ, one of these as described in Chapter 7.1 is the phenotype of the TAMS of their TME.

We previously reported an association between MMRd/MSI-H CRC, PDL1 expression and higher M1 macrophage infiltration, and we now show the potential for increasing PDL1 expression with M1 macrophages, in a co-culture model. We found, as expected, baseline cell surface protein expression was higher in MMRd/MSI-H cancer cell lines. Gene and protein expression could be upregulated in all cancer types. Interestingly, gene expression was increased to a similar extent (4-5-fold) in HT29 (MMRp/MSS) and Hct116 (MMRd/MSI-H) cell lines despite their differences in baseline cell surface PDL1 expression. This suggests that cancer cells which lack PDL1 surface expression have the capacity to be manipulated to express the protein, and therefore potentially benefit from immunotherapy.

RKO cells which had the highest PDL1 gene expression; and almost 100% of its cells expressed PDL1 surface protein, did not show any change in

gene expression in the co-culture model, however MFI did increase with M1 co-culture. This suggests that pro-inflammatory M1 mediated cytokines may cause the transportation of previously intracellular PDL1 to the cell surface. This; however' also suggests that in already highly expressing PDL1 cancer types, attempts to further increase PDL1 expression, may be of limited benefit.

This in vitro work shows the ability of M1-like macrophages and their cytokines to increase PDL1 expression of surrounding cancer cells. This supports the previous findings of patient sample RNAseq analysis showing higher PDL1 expression in CRC tumors with higher M1 macrophage infiltration.

This finding supports the studies that suggest manipulation and re-programming of TAMs, which are largely M2-like to a more M1 like phenotype is an important adjuvant to increasing responsiveness to anti-PD-1 immunotherapy in currently treatment-resistant, “immune-cold” cancers. The next steps of this work is to explore the M1 mediated cytokines and intracellular mechanisms whereby M1 macrophages can upregulate PDL1 expression.

d). MACROPHAGE PDL1 EXPRESSION AND EFFECT OF CO-CULTURE

i) Introduction

Tumor immune checkpoint protein expression enables the use of immunotherapy as a targeted treatment of many cancers. As discussed, the immune cells of the tumor microenvironment (TME) comprise a significant portion of the tumor, and immune cells themselves contribute to the tumors' immune checkpoint protein expression e.g. PDL1 and response to immunotherapy.

Tumor associated macrophages (TAMs) as one of the most abundant immune cells within the TME, mediate tumor progression and immunosuppression through secretion of cytokines and chemokines including TGF-β and PGE2 [136]. TAM derived tumor growth factor-β (TGF-β) has a strong immunosuppressive effect, altering response to anti PD-1/PD-L1 immunotherapy via inhibition of T cell activation and reducing CD8+ T cell infiltration of the TME. This has been shown to result in decreased overall tumor PD-L1 expression and affect response to anti-PD-1/PDL1 immunotherapies.

TAMs, along with altering the entire tumor PDL1 expression, express PDL1 on their surface. The functional significance and clinical relevance of high vs low PDL1 expression of TAMs remains unclear, with conflicting results.

Liu et al [137] reported a significant correlation of PDL1+ CD68+ macrophages; overall tumor PDL1 expression and CD8+ T cell infiltration in

NSCLC patients. They identified high macrophage PDL1 expression was associated with improved overall survival, while high tumor cell PDL1 expression was not [137]. A further study of 475 treatment naïve lung adenocarcinoma patients found high PDL1+ TAM or tumor cell expression was associated with improved survival following adjuvant chemotherapy [138].

Similarly, PDL1+ TAMS were found to be immunostimulatory, promote CD8+ T cell proliferation, and correlate with improved recurrence free and overall survival in patients with breast cancer [139, 140]. The role of PDL1 status of TAMs in colorectal cancer has been less well-described, Elomaa et.al report moderate correlation of PDL1+ TAMs to M1-like pro-inflammatory phenotype [141].

The aim of this work was to:

i) compare the PDL1 expression of the three distinct, THP-1 monocyte derived macrophage phenotypes (M0, M1 vs M2); and
ii) study the change in macrophage PDL1 expression following co-culture with colon cancer cell lines.

ii) Results

Gene Expression

At baseline in single culture, M1-like macrophages had the highest PDL1 gene expression (∆CT=15.54, [95%CI:15.33;15.75] n=16) compared to both M0 macrophages (∆CT=22.84, [95%CI:22.53;23.16] n=18) and M2-like macrophages (∆CT=20.24, [95%CI: 19.91;20.77] n=12), p<0.001 for all. **(Figure 18)** PDL1 gene expression was significantly upregulated in M1 (pro-inflammatory) macrophages, compared to M0 (untreated) macrophages [mean fold-change (FC)=185.6 ± 79.6].

Macrophage PDL1 expression following co-culture with cancer cells lines is shown in **Figure 19 and Table 11.** Macrophage PDL1 gene expression decreased following co-culture with HT29 and Hct116 colon cancer cell lines in both M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophage phenotypes. The effect was much less pronounced in the M2 phenotype, with changes in ∆CT representing only a 2-fold decrease in gene expression compared to 13-fold and 10-fold downregulation in M1 macrophage with the HT29 and Hct116 cell lines, respectively.

Figure 18

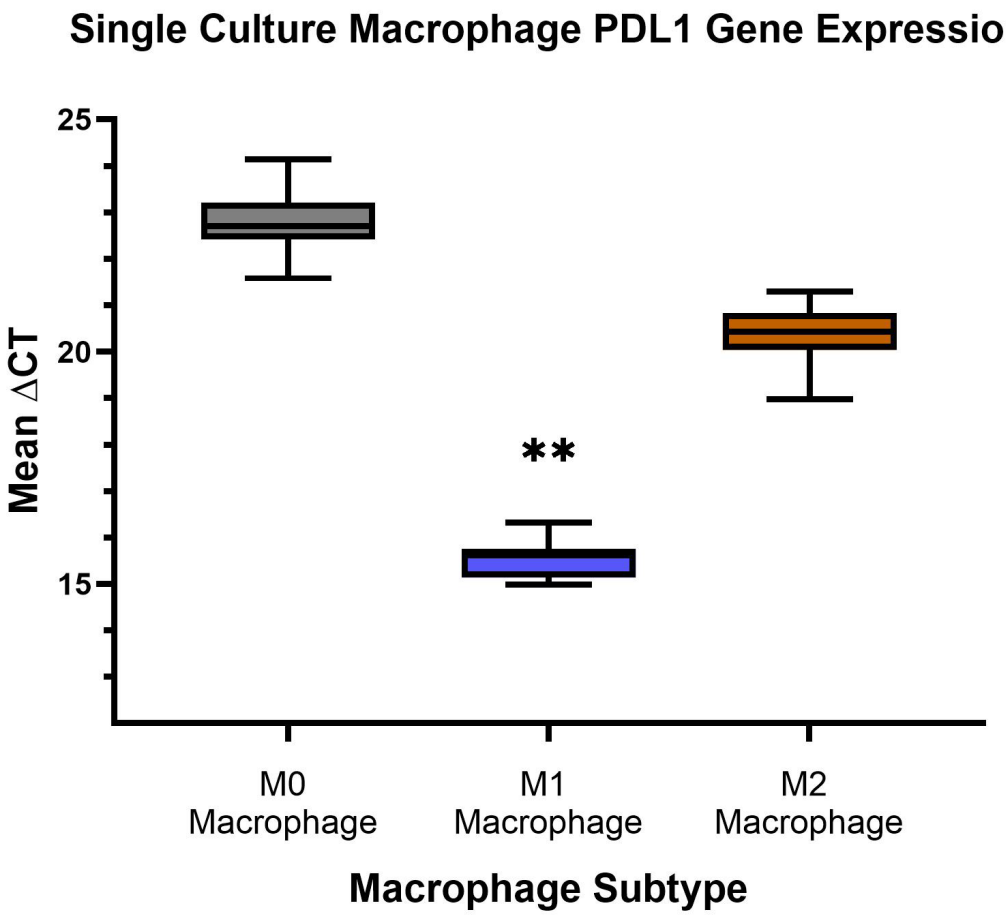


Figure 18: *Macrophage PDL1 gene expression expressed as ΔCT. M1 Macrophages (n=16) have significantly higher PDL1 expression compared to M0 (n=18) and M2 macrophages (n=12). ** = p<0.001.*

Figure 19

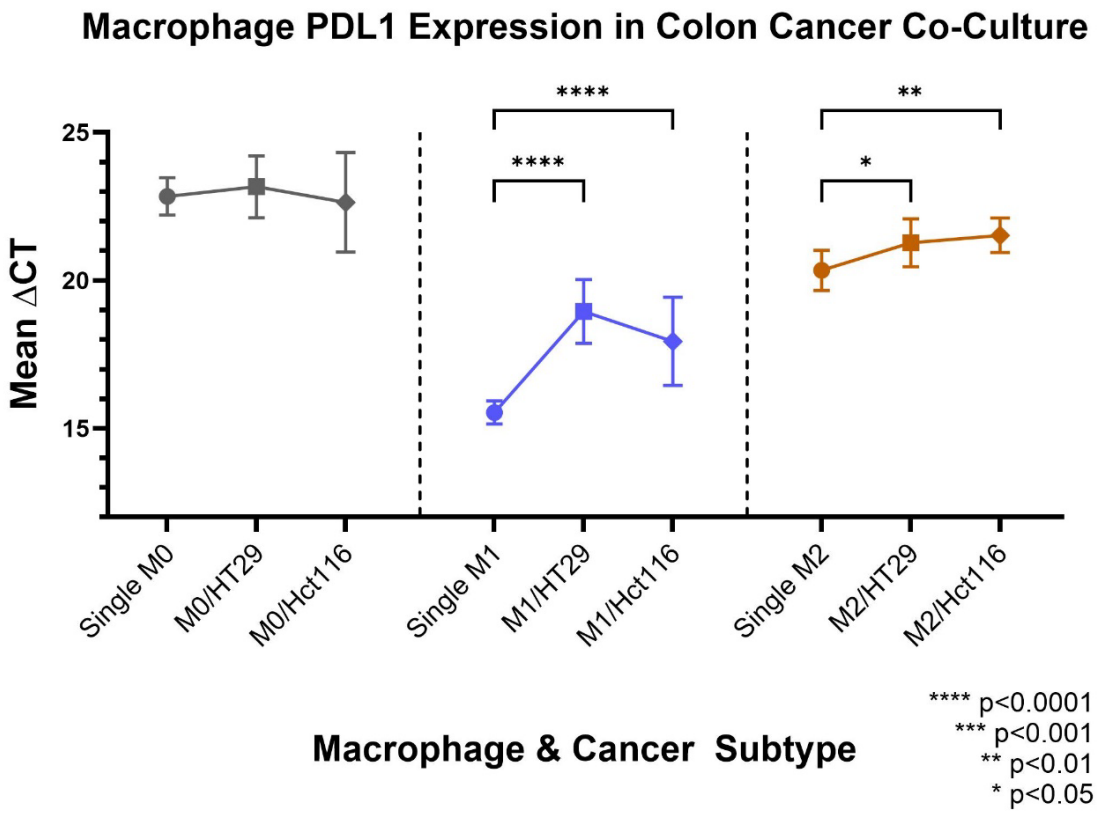


Figure 19 *Macrophage PDL1 gene expression in single culture (macrophage alone) and following co-culture with HT29 or Hct116 Colon Cancer cell lines for 24 hours. Results expressed as mean ΔCT ± SD One-way ANOVA performed with a post hoc Benjamini–Hochberg correction to control the false discovery rate at 5%.*

Table 11: Macrophage PDL1 Expression

	Single Culture		HT29 (MMRp) Co-Culture				Hct116 (MMRd) Co-Culture				Overall P value
	Δ CT	SD	**Δ CT**	SD	**P value [a]**	**FC ± SD**	**Δ CT**	SD	**P value [a]**	**FC ± SD**	
M0	**22.84**	*0.63*	**23.17**	*1.04*	0.5839	1.01 ± 0.59	**22.64**	*1.68*	0.7943	1.08 ± 0.64	0.2816
M1	**15.54**	*0.39*	**18.95**	*1.08*	<0.0001	-13.38 ± 8.05	**17.94**	*1.49*	<0.0001	-10.89 ± 5.19	<0.0001
M2	**20.34**	*0.68*	**21.27**	*0.81*	0.0449	-2.15 ± 0.95	**21.53**	*0.59*	0.0055	-2.45 ± 0.91	<0.01

Table 11: Macrophage PDL1 Expression by macrophage phenotype in single (macrophage alone) vs co-culture model. CT= Cyle Threshold, SD = Standard Deviation, FC = Fold Change. P values calculated using ΔCT values, [a] compared to single culture. One-way ANOVA performed with a post hoc Benjamini–Hochberg correction to control the false discovery rate at 5%.

Cell Surface Protein Expression

PDL1 cell surface protein expression was also measured via flow cytometry and compared across single and co-culture model as described above. Throughout single and co-culture models <1% of M0 and M2 macrophages expressed PDL1 on their cell surface, (n = 12 for all groups).

At baseline, 45.5% ± 8.4 of M1 macrophages expressed PDL1 (n = 10) with a median fluorescence intensity (MFI) of 20.79 ± 3.47 (n = 10). As seen with PDL1 gene expression, cell surface protein expression was also reduced following cancer cell co-culture. Hct116 (MMRd) co-culture resulted in a reduction of M1 macrophages expressing PDL1 to 30.6 ± 13.7% positive (MFI = 19.6 ± 2.95) and following co-culture with HT29 (MMRp) 23.7± 8.6%, (MFI 18.6 ± 2.42) $p<0.05$ for both. **(Figure 20)**

Figure 20

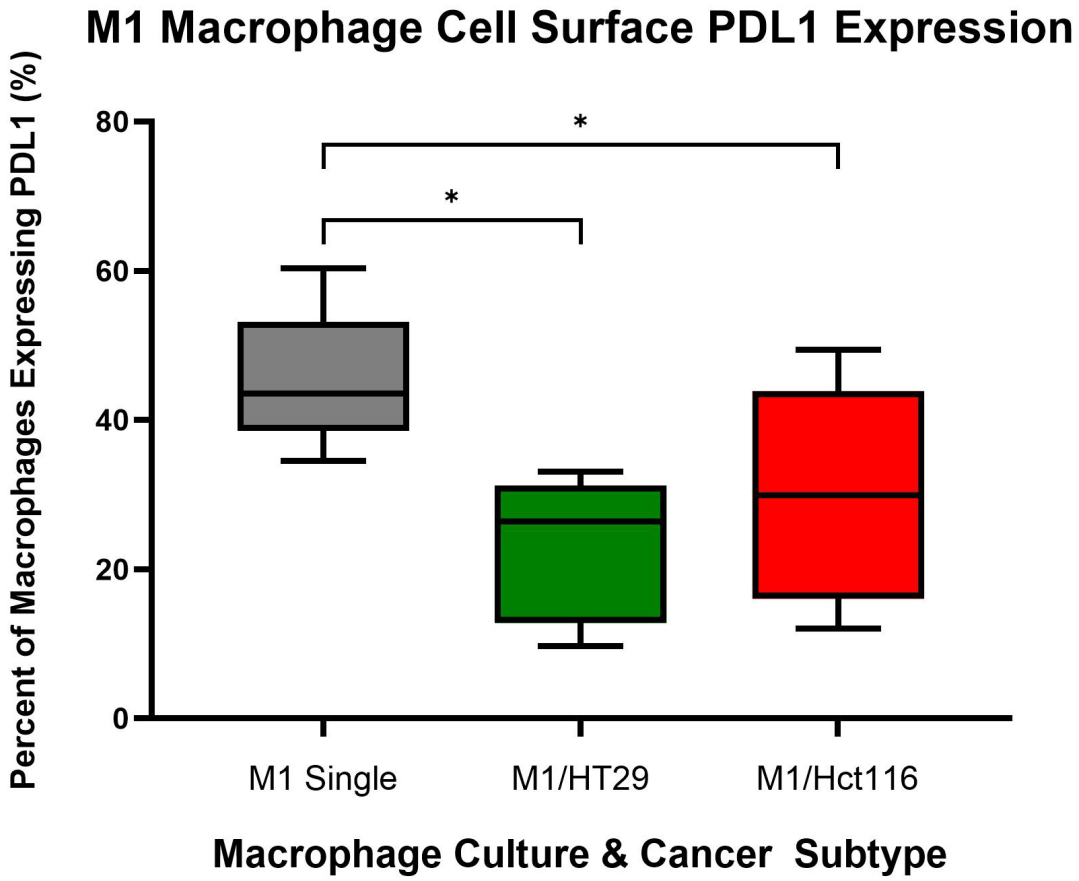


Figure 20: *Cell surface PDL1 protein expression on M1 macrophages in single culture (macrophages alone) vs co-culture with colon cancer cell lines HT29 (MMRp) and Hct116 (MMRd). N=10. *p<0.05. One-way ANOVA performed with a post hoc Benjamini–Hochberg correction to control the false discovery rate at 5%.*

iii) Discussion

The results of this project suggest that in our THP-1 derived M0, M1 and M2 macrophages , PDL1 is expressed most significantly in M1 macrophages. While there was evidence of gene expression in M0 and M2 macrophages,, this translated to minimal (<5%) cell surface PDL1 expression, as measured by flow cytometry in all experimental conditions.

PDL1 expression on macrophages within the TME, may play a significant role in contributing to the overall PDL1 status of the tumor, and therefore its response to immunotherapy. There are very few studies on cell line macrophage PDL1 expression These results are agreement, in part with Lai et al., who also reported higher PDL1 expression in M1 polarized THP-1 macrophages, however their M2 macrophages did also express PDL1, but to a lesser extent [142].

Cell line models do not recapture the complex interplay of the TME; particularly the relationship between T-cell induction of macrophage PDL1 expression. There are limitations to the clinical translation of these results; however' it is known, in several cancer types, that TAM-PDL1 expression can predict outcomes, including response to treatment [137, 141, 143-145], therefore the role that TAM PDL1 expression plays in MSI vs MSS CRC warrants further study. Future goals of this research overall, includes the single cell sequencing of MMRd/MSI vs MMRp/MSS tumors and comparison of macrophage populations. This would include identification of different populations of macrophages

depending on their PDL1 expression and how these overlap with the tumor MSS status as well as the macrophage polarization state.

e). PERIPHERAL BLOOD MONONUCLEAR CELL INFLAMMATORY PROFILES AND PDL1 EXPRESSION IN CRC

i) Introduction

As described, the tumor microenvironment particularly immune cell infiltration and macrophage phenotype, differs in MMR deficient and proficient CRC. While there are many competing factors that alter the polarization of macrophages within the tumor, whether they differ in their pre-cursor circulating form is not yet known.

There are differing opinions regarding the origin of tumor associated macrophages. The traditional model is that of bone marrow derived macrophages, entering circulation as myeloid derived monocytes, before becoming incorporated into the tumor and differentiating into their final state as tumor associated macrophages in response to local tumor and systemic stimuli [146, 147]. While there is also consideration that they originate from existing tissue-resident macrophages originating from embryonic precursors. These macrophages are derived from the fetal liver and yolk sac, exist in tissue and become incorporated into tumor, independent of circulating monocytes [148, 149]. There is likely an interplay of both mechanisms and subtypes of macrophages in the incorporation into the tumor microenvironment [150-152].

The differences our study has identified in the phenotype and polarization of TAMs of MMRd/MSI-H vs MMRp/MSS CRC tumors, and the effect the polarization has on PDL1 expression, led us to the aim of this work:

i) to compare the inflammatory profiles and PDL1 expression of peripheral blood mononuclear cells (PBMCs) in the plasma of patients with MMRd/MSI-H vs MMRp/MSS colorectal cancer.

Patient Demographics

A total of 16 patients with colorectal cancer, for which blood was available, were included in this analysis (MMRp/MSS n=10, MMRd/MSI-H, n= 6). Gender was equal across both groups, although the MMRd/MSI-H group were slightly older. Patient demographics can be found in **Table 12**

Table 12: Colon Cancer Patient Demographics.

Patient/ Cancer Characteristic		MMRp/MSS Number,	%	MMRd/MSI-H Number,	%
Patients	**N,** *%*	**10**	*62.5*	**6**	*37.5*
Age	**Mean** *(SD)*	**61.8** *(15.6)*		**73.2** *(23.2)*	
Gender					
	Female	**5**	*50.0*	**3**	*50.*
	Male	**5**	*50.0*	**3**	*50.0*
Ethnicity					
	White	**9**	*90.0*	**6**	*100.0*
	Asian	**1**	*10.0*	**0**	
	African American	**0**		**0**	
Location					
	Right Colon	**6**	*60.0*	**5**	*83.3*
	Left Colon	**4**	*40.0*	**0**	
	Rectum	**0**		**1**	*16.7*
Overall Stage					
	1	**1**	*10.0*	**1**	*16.7*
	2	**4**	*40.0*	**4**	*66.7*
	3	**4**	*40.0*	**1**	*16.7*
	4	**1**	*10.0*	**0**	
MMR gene mutation		**N/A**		**MLH1**	5
				PMS2	5
				Unknown	1

Table 12 *Demographics of patients included in comparison of inflammatory profiles and PDL1 expression of PBMCs of patients with MMRp/MSS vs MMRd/MSI-H CRC*

Results

Gene expression of inflammatory markers, cytokines and PDL1 were measured and grouped based on their inflammatory status, either pro-inflammatory (IL-6, TNFα, IL-1β, CD80, CD86, CCL3, IL27, CXCL9, CXCL10) or anti-inflammatory (MRC1, IL-10, IL8, TGF-β, PPARγ). Results comparing the two groups are shown in **Table 13.** Analysis of the results revealed no significant difference between the inflammatory markers, or PDL1 expression of PBMCs of patients with MMRd/MSI-H and MMRp/MSS CRC.

Table 13: Peripheral Blood Mononuclear Cell Gene Expression in Colon Cancer Patients.

	Gene	MMRd/MSI-H Mean ΔCT (SD)	MMRp/MSS Mean ΔCT (SD)	P value
Pro-inflammatory Markers	**IL-6**	23.31 (1.83)	23.89 (2.75)	0.515
	TNFα	17.26 (2.06)	18.52 (2.79)	0.278
	IL-1β	17.00 (2.97)	17.31 (2.98)	0.515
	CD80	22.86 (0.90)	22.43 (0.67)	0.329
	CD86	17.64 (1.86)	17.67 (1.19)	0.588
	CCL3	16.50 (3.73)	16.40 (3.89)	0.914
	IL-27	21.63 (1.50)	20.81 (1.10)	0.515
	CXCL9	22.32 (3.47)	23.19 (2.50)	0.448
	CXCL10	21.32 (2.28)	22.06 (1.94)	0.329
Anti-Inflammatory Markers	**IL-10**	23.15 (1.60)	23.16 (1.24)	0.745
	MRC1	23.91 (1.26)	24.87 (0.71)	0.129
	PPARγ	24.04 (1.22)	23.90 (0.67)	0.828
	TGFβ	12.43 (0.56)	12.58 (0.73)	0.515
	IL-8	15.52 (4.68)	16.28 (4.19)	0.588
Immune Checkpoint Protein	**PDL1**	19.97 (0.50)	20.84 (2.25)	0.515

Table 13: *Gene expression (mean ⊿CT; SD) of pro- and anti-inflammatory markers in the PBMCs of patients compared between MMRd/MSI-H and MMRp/MSS CRC*

ii) Discussion

Gene expression of macrophage markers and inflammatory cytokines did not differ in the PBMCs of patients with MMRp vs MMRd colorectal cancers.

We hypothesized that given the differences in the TME, patients with MMRd/MSI-H CRC may have PBMCs of a more pro-inflammatory nature, and may have increased PDL1 expression, which could potentially serve as a marker for responsiveness to anti-PD01 immunotherapy. Results however, suggest that differences in macrophage phenotypes in MMRd/MSI-H vs MMRp/MSS CRC likely develop as they become part of the tumor microenvironment, and not prior, in their monocyte form.

A significant limitation to this work however is the small sample size for each of the tumor categories. It is also important to note that 5 of the 6 MMRd/MSI-H cancers were sporadic, only one patient had Lynch Syndrome. In order to fully understand whether differences in macrophage polarization and PDL1 expression of the TME in MMRd/MSI-H originate from their monocyte precursor form, further study evaluating patients with inherited germline mutations in MMR genes (Lynch Syndrome) is warranted, and an important future step of this work.

CHAPTER VIII: MACROPHAGE MECHANISMS OF PDL1 EXPRESSION AND PATIENT SURVIVAL

a) Overview of Results so far:

The immune cell composition analysis of the tumor microenvironment of CRC suggests that MMRd/MSI-H cancers contain a small, but potentially clinically relevant, increased M1 macrophage phenotype compared to MMRp/MSS. This increase in M1 macrophages was found to correlate to cancers with increased PDL1 expression.

The implications of M1 polarization of macrophages and cancer PDL1 expression were further confirmed through in vitro studies as described. We found that M1-like, cell line derived macrophages, could significantly increase the PDL1 expression of four different colon cancer cell lines. Crucially, this increase was evident in two different MMRp/MSS CRC cell lines, which are PDL1 negative at baseline. These cancers are clinically PDL1 negative and respond poorly to anti-PD-1/PDL1 immunotherapy.

In our cell line model, M1-like macrophages exhibited high PDL1 expression, in contrast to both non-polarized (M0) and anti-inflammatory, M2 phenotypes. We also noted no difference in PDL1 expression of the circulating monocytes of our MMRp/MSS vs MMRd/MSI-H CRC patients, supporting the

thought, that macrophage behavior, activation and potential to alter PDL1 expression are influenced by the neighboring cells and surrounding environment once incorporated into the tumor. While the association of macrophage phenotype and PDL1 expression can be seen, to utilize this knowledge and use it to alter PDL1 expression in a future, clinically relevant context, we next sought to identify and study cancer related, PDL1 expression pathways, which may identify a mechanism by which M1 macrophages alter PDL1 expression.

b) Genomic Analysis

i) Patient Demographics and Tumor Characteristics.

Genomic analysis was performed using RNA-seq data collected from colorectal cancer patients from i) The Cancer Genome Atlas (TCGA) n= 598, ii) the European-Phenome-Genome Atlas (EGA) n=69 and iii) University of Louisville patients (n=20). A total of 687 patients were included: (MMRd/MSI-H n=97 (14.1%) vs MMRp/MSS n= 590 (85.9%).

An overview of available clinical and pathological features of the patients included in the genomic studies from University of Louisville, and The Cancer Genome Atlas are found in Table 14 and 15 below.

Table 14: Patient Demographics and Tumor Characteristics (Louisville)

Patient/Tumor Characteristic		MMRp/MSS		MMRd/MSI-H	
		Number	**%**	**Number**	**%**
Patients	N, *%*	**30**	*68.0*	**14**	*32.0*
Age	Mean (SD)	**62.0**	(13.1)	**70.4**	(16.9)
BMI	Mean (SD)	**28.9**	(7.9)	**25.9**	(13.1)
Gender					
	Female	**11**	*36.7*	**9**	*64.2*
	Male	**19**	*63.3*	**5**	*35.7*
Location					
	Right Colon	**9**	*30.0*	**8**	*57.1*
	Left Colon	**13**	*43.3*	**2**	*14.3*
	Rectum	**8**	*26.7*	**3**	*21.4*
Overall Stage					
	1	**3**	*10.0*	**3**	*21.4*
	2	**14**	*46.7*	**10**	*71.4*
	3	**10**	*33.3*	**1**	*7.2*
	4	**1**	*3.3*	**0**	*0*

Table 15: Patient Demographics and Tumor Characteristics (TCGA)

Patient/Tumor Characteristic		MMRp/MSS		MMRd/MSI-H	
		Number	%	Number	%
Patients	N, %	**508**	*86.5*	**79**	*13.5*
Age	Mean (SD)	**65.7**	(12.4)	**69.0**	(14.1)
Gender					
	Female	**230**	*45.3*	**45**	*57.0*
	Male	**278**	*54.7*	**34**	*43.0*
Ethnicity					
	White	**238**	*46.9*	**41**	*51.9*
	African American	**57**	*11.2*	**8**	*10.1*
	Asian	**11**	*2.2*	**1**	*1.3*
	Other	**1**	*0.2*	**0**	*0.0*
	N/A	**201**	*39.6*	**29**	*36.7*
Location					
	Colon	**355**	*69.9*	**79**	*100*
	Rectum	**153**	*30.1*	0	0
Overall Stage					
	1	**81**	*15.9*	**19**	*24.1*
	2	**173**	*34.1*	**43**	*54.4*
	3	**161**	*31.7*	**13**	*16.5*
	4	**79**	*15.6*	**3**	*3.8*
	N/A	**14**	*2.8*	**1**	*1.3*
T stage					
	Tis	**1**	*0.2*		
	1	**14**	*2.8*	**4**	*5.1*
	2	**87**	*17.1*	**15**	*19.0*
	3	**350**	*68.9*	**50**	*63.3*
	4	**55**	*10.8*	**10**	*12.7*
	N/A	**1**	*0.2*	**0**	*0*
N Stage					
	0	**270**	*53.1*	**63**	*79.7*
	1	**132**	*26.0*	**12**	*15.2*
	2	**103**	*20.3*	**4**	*5.1*
	N/A	**3**	*0.6*	**0**	*0*
M Stage					
	0	**371**	*73.0*	**64**	*81.0*
	1	**76**	*15.0*	**3**	*3.8*
	N/A	**61**	*12.0*	**12**	*15.2*

Tables 14 and 15: *Patient and tumor characteristics for University of Louisville and TCGA patient cohorts. Data presented as n, % or mean, (SD). Staging based on AJCC 8th Ed. BMI= kg/m^2*

ii) Differential Expression and Functional Annotation Analysis

To identify target genes and key pathways that regulate PDL1 expression in MMRd/MSI cancers, and the contribution of M1 macrophages. We further analyzed the results of the differential gene expression described above.

Genes that were differentially expressed in MMRd/MSI-H vs MMRp/MSS tumors were identified. A total of 762 genes were upregulated and 445 were down regulated. In addition to the immune checkpoint proteins that were differentially expressed (PDL1, PD-1, PAG3 and FGL1 discussed previously in **Chapter VII.a**), we focused on immune related genes, macrophage markers and genes known to be crucial in relevant canonical pathways. Of note several M1 markers were significantly upregulated including chemokines CCL3 and CCL4 (Log_2FC=1.32 and 1.4, respectively); CXCL9 and CXCL10 (Log_2FC=1.47; 1.46). IDO1 and IFN gamma were both 2-fold upregulated, and as expected MLH1 was significantly downregulated.

Functional annotation analysis was performed and reported several key pathways in which the differentially expressed genes were enriched. This included Antigen Presentation, PDL1/PDL1 immunotherapy pathway and the macrophage activation pathway. (**Figure 21**)

Figure 21: Significant Involved Pathways of the Functional Annotation Analysis.

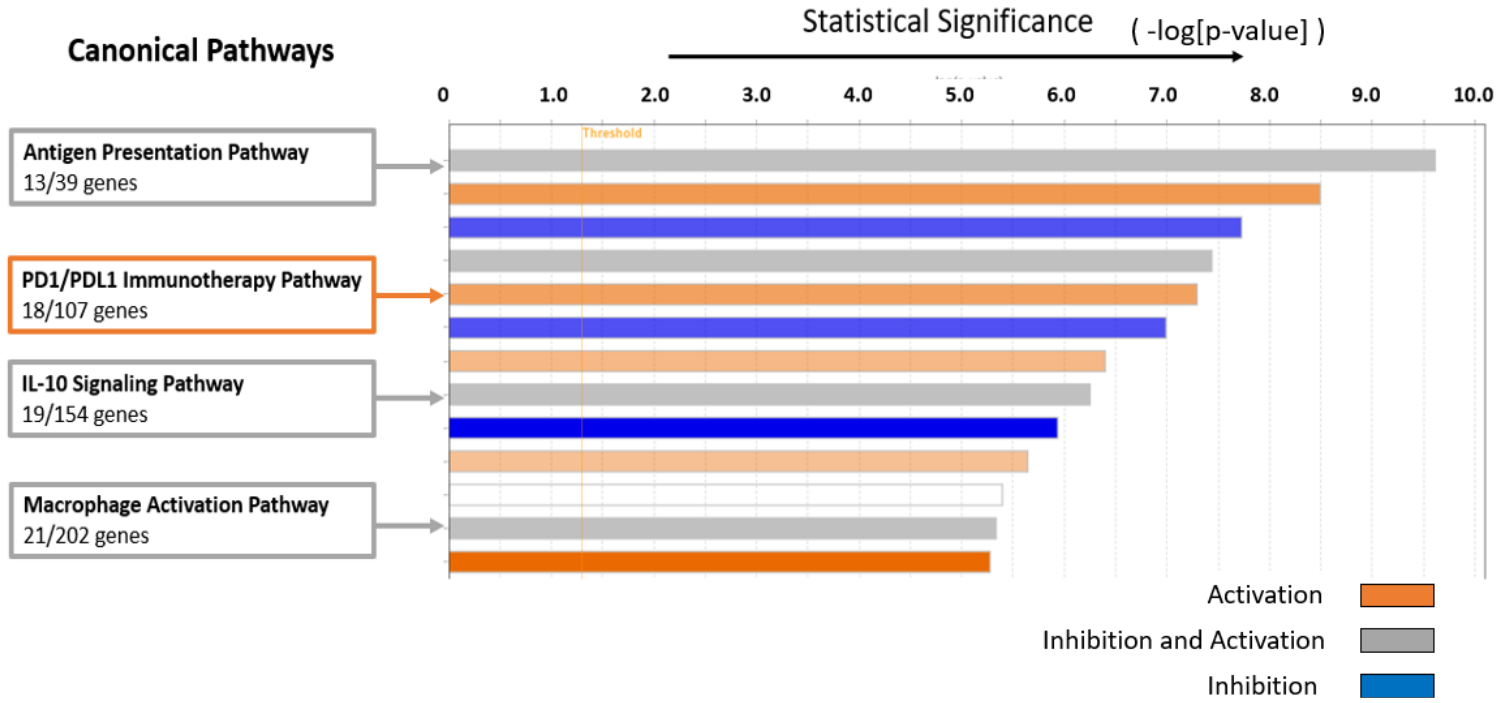


***Figure 21**: Relevant functional pathways in which differentially expressed genes were significantly enriched.*

iii) Pathway Analysis and Gene Network Creation

Following identification of differentially expressed: macrophage related genes, those involved in immune checkpoint protein expression and cancer progression, these were combined with significant canonical functional pathways, and with the aid of Ingenuity Software, a gene network was created. (**Figure 22**)

Figure 22: Macrophage- PDL1 Expression- Gene Network.

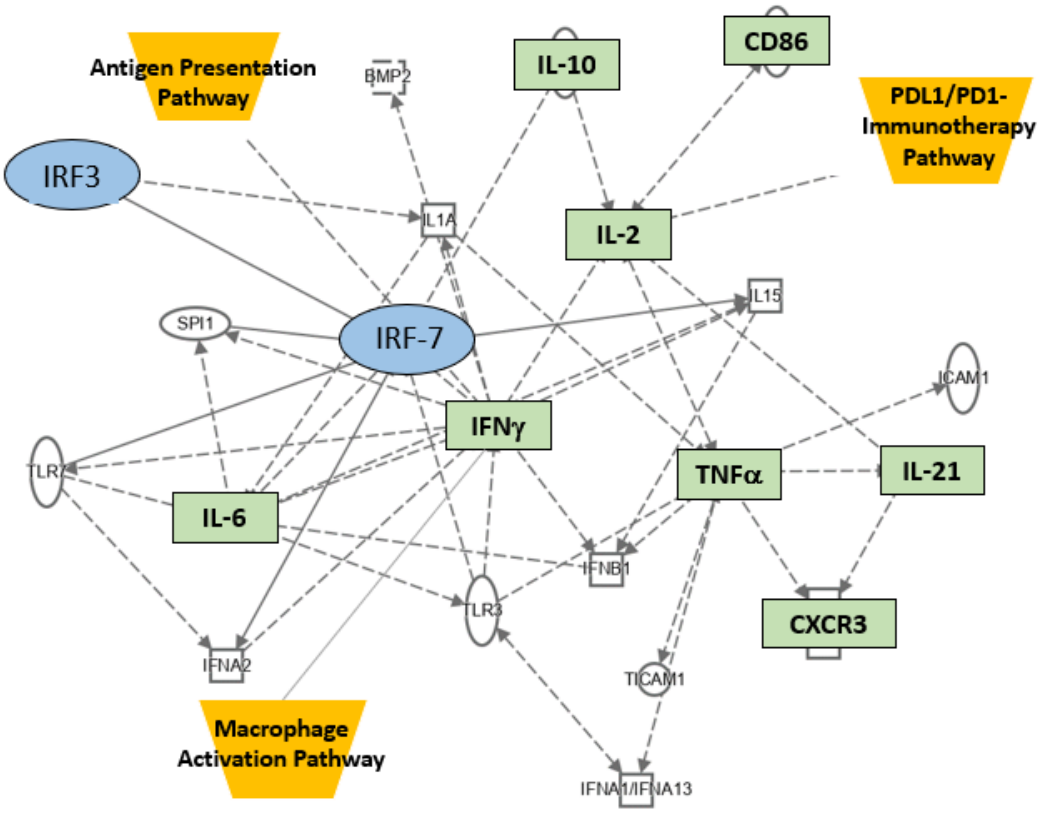


Figure 22: *Gene network demonstrating some of the differentially expressed and associated target genes relating antigen presentation, macrophage activation and PDL1-immunotherapy pathways: IRF: Interferon-Regulating Factor. Created using Qiagen Ingenuity Pathway Analysis.*

Of interest IL6 and TNFα both play a central role in this network. As described in our in vitro model and results, both these cytokines are hallmarks of the M1 pro-inflammatory macrophage phenotype. CXCR3 is the receptor ligand of CXCL9 and CXCL10 which are also pro-inflammatory cytokines produced by macrophages and the CXCR3/CXCL9/CXCL10 axis has been described in mechanisms of colon carcinogenesis. IFNγ and interferon regulating factors are key mediators and inducers of PDL1 expression via the JAK/STAT pathway in cancer cells.

c) The Role of IFNγ

The role of IFNγ in the regulation of the immune system is extensive and complex; some of its primary functions includes the priming of classically activated (pro-inflammatory) macrophages and promotion of host defense mechanisms [153-155]. It is mainly produced by Natural Killer and CD4+ T-cells and along with its role in classic macrophage activation, it plays a pivotal role in cancer cell PDL1 expression. T cells, upon recognition of tumor antigens, release interferons, which in turn induce PDL1 expression via activation of the JAK/STAT pathway in cancer and other cells of the TME. This has been extensively researched and described in the context of melanoma, and several other cancers.

To study whether a similar effect was seen in colon cancer, we performed a dose-time response experiment, treating our 4 colon cancer cell lines with 0, 10, 100 or 500ng/ml for progressive time periods between 3 to 48 hours.

Figure 23 demonstrates the flow cytometry results showing the upregulation of PDL1 in all four cancer cell lines. The IFNγ induced PDL1 expression followed a similar pattern to the increase seen following the co-culture of cancer cells with M1 macrophages (**Figure 17**); however, the response in the MMRp/MSS cancer cell lines was more pronounced with IFNγ treatment.

Gene expression measurement of the IFNγ treated colon cancer cell lines also revealed, as expected, an upregulation in PDL1 at all time points and doses (data not shown).

Figure 23: Colon Cancer Cell Line PDL1 Expression following IFNγ Treatment.

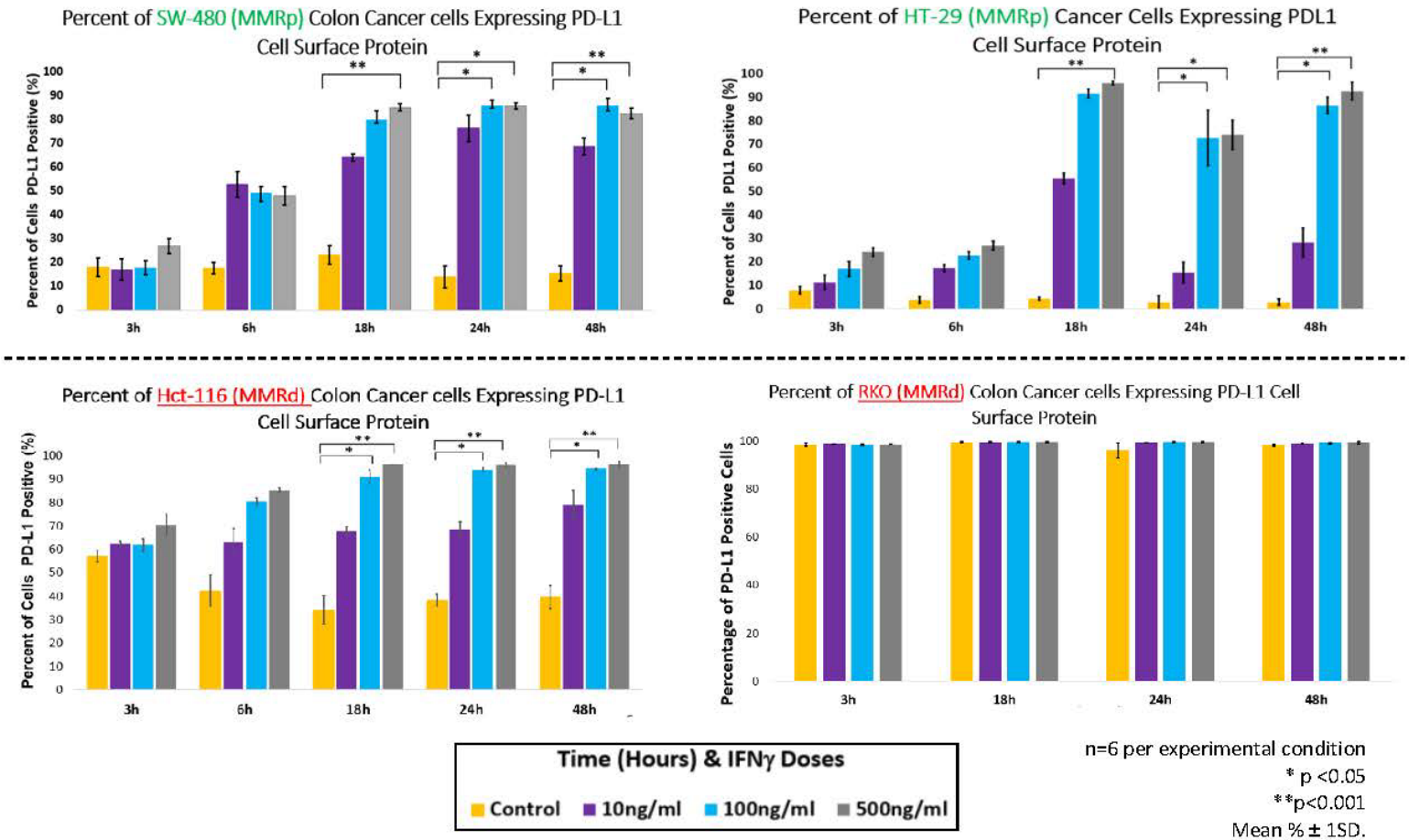


Figure 23: *Cancer cell line cell surface PDL1 Protein expression following IFNγ treatment at 0, 10, 100 vs 500ng/ml at 3, 6, 18, 24 and 48 hours of treatment. Measure via flow cytometry, 10,000 gated events minimum, n=6 for each time point and dose.*

i) Macrophage IFNγ production

Although not a primary source of IFNγ several studies have shown inducible production of IFNγ by macrophages following stimulation by IL-12 and IL-18 [156-158]. While macrophages are unlikely to be the source of the up regulation of IFNγ found in the tumors of MMRd/MSI-H vs MMRp/MSS CRC, demonstrated in the differential gene expression analysis; it was found that IFNγ expression was strongly correlated with M1 macrophage infiltration (R =0.571) and as expected, with PDL1 gene expression (R = 0.651, p<0.0001 both.)

While we initially hypothesized that the M1-macrophage induced, increased cancer cell line PDL1 expression could be due to macrophage IFNγ production given the absence of T cells in our co-culture model, this was found not to be the case. Gene and protein expression analysis of our THP-1 polarized M1 macrophages, while demonstrating successful polarization to an M1 phenotype, demonstrated **no** IFNγ gene or protein expression at all LPS treatment times and doses, despite successful induction of PDL1 in co-cultured cancer cells.

Despite the lack of IFNγ produced by our cell line M1 macrophages, they did induce a significant upregulation of several genes of the JAK/STAT pathway in co-cultured cancer cell lines along with PDL1 expression **(Table 16).** This suggests that the JAK/STAT pathway mechanism of PDL1 expression could be induced by other macrophage-related cytokines in the absence of IFNγ.

Table 16: Cancer Cell Expression of JAK/STAT Genes following M1 Macrophage Co-Culture

		Gene of Interest: Single vs M1 Co-Culture							
Cancer Cell Line		**JAK1**		**JAK2**		**STAT1**		**STAT3**	
		Single	**M1**	Single	**M1**	Single	**M1**	Single	**M1**
HT29	**ΔCT**	15.03	**14.56**	21.07	**20.33**	15.04	**12.87**	17.12	**16.29**
	SD	*0.9670*	*0.3521*	*0.7603*	*0.4098*	*0.6391*	*0.5409*	*0.5331*	*0.2129*
SW480	**ΔCT**	15.36	15.01	20.76	20.46	15.60	**14.12**	18.23	**17.39**
	SD	*0.1809*	*0.1663*	*0.4404*	*0.5860*	*0.1958*	*0.4430*	*0.2383*	*0.1231*
Hct116	**ΔCT**	14.89	**14.43**	19.60	19.53	15.51	**14.03**	17.53	**16.82**
	SD	*0.7226*	*0.4170*	*0.7030*	*0.8827*	*0.5570*	*1.042*	*0.5589*	*0.5176*
RKO	**ΔCT**	14.64	14.43	19.55	19.85	15.93	**14.90**	17.71	17.16
	SD	*0.3369*	*0.3069*	*0.1101*	*0.3622*	*0.2506*	*0.9180*	*0.3196*	*0.4304*

Table 16: *Mean ΔCT and SD of cancer cell lines gene expression of JAK/STAT pathway genes, in single culture and M1 macrophage co-culture. Grey shaded and boldface indicate significant upregulation compared to single culture (control). p <0.05. Green shading represents MMRp/MSS cell lines and red represents MMRd/MSI-H.*

d) Macrophages, PDL1 and the CXCL9/CXCL10/CXCR3 Axis.

Chemokines CXCL9 and CXCL10 are primarily expressed by monocytes, endothelial cells, fibroblasts, and under stimulation by IFNγ, also by cancer cells. The CXCL9/CXCL10/CXCR3 axis has been shown to exhibit two different functions in the TME. It plays a role the regulation of migration, activation and differentiation of immune cells, but has also been found to alter tumor progression and metastases via recruitment of tumor infiltrating lymphocytes and myeloid derived suppressor cells. [159-161].

Our results showed that chemokines CXCL9 and CXCL10 were significantly upregulated in MMRd/MSI-H CRC. (Log_2FC=1.47; 1.46 respectively). Spearman Correlation analysis using our genomic data found that both CXCL9 and CXCL10 were among the genes most highly correlated with M1 macrophage infiltration of the TME. Significant correlation to M1 infiltration was present in CRC overall, (CXCL9, R = 0.83; CXCL10 R= 0.84) and when considering tumors by MMR status separately: CXCL9: R=0.84 MMRp/MSS and R=0.68 MMRd/MSI-H, CXCL10: R=0.827 MMRp/MSS and R= 0.763 MMRd/MSI-H ($p<0.001$ for all).

Upregulation of CXCL9 and CXCL10 gene expression was also seen in the THP-1 polarized M1 macrophages vs untreated M0s: CXCL9: ΔCT: 18.61 ± 0.6 vs 29.16 ± 2.2 and CXCL10: ΔCT: 12.03 ± 0.5 vs 24.03 ±1.7, $p<0.0001$. These differences in gene expression translate to a fold-change of 2980 in CXCL9 and a 4300-fold increase in CXCL10.

Lastly, along with macrophage correlation, both chemokines showed a moderate correlation with PDL1 gene expression in RNAseq analysis of our 687 CRC samples (Spearman Coefficient CXCL9: R= 0.74, CXCL10: R= 0.79 p <0.001)

Chemokines CXCL9 and CXCL10 are primarily expressed by monocytes, endothelial cells, fibroblasts, and under stimulation by IFNγ, also expressed on cancer cells. The CXCL9/CXCL10/CXCR3 axis functions in two manners in the TME. It plays a role the regulation of migration, activation and differentiation of immune cells, but has also been found to promote tumor progression and metastases via recruitment of Th2 Tregs and myeloid derived suppressor cells.

Increased CXCL9 and CXCL10 expression has been associated with patient survival in a number of cancers[162]. This has been hypothesized to be due to their recruitment of tumor infiltrating lymphocytes. Brogner et al. found that increased expression almost doubled overall survival in ovarian cancer (HR 0.41 and 0.46 respectively) [163]. The results from our analysis support this and also

in vivo melanoma studies identified correlation between CXCR3-induced T cells at the tumor site and anti-PD-1 treatment effect. Anti-PD-1 failed to shrink the tumor in CXCR3 knock out mice, suggesting that the CXCL9/CXCL10/CXCR3 axis is required for effective anti-PD-1 therapy response [164].

Similar results were reported in a breast cancer xenograft model, which reported that CTLA-4/PD-1 blockade was CXCR3 dependent. The study went on to further study the CXCL9/CXCL10 and CXCR3 source across the immune cells of the TME and identified that CXCL9/CXCL10 was predominantly expressed by macrophages. Zhang et al investigated the role of this axis in gastric cancer. They demonstrated using an in vivo model of CXCL9 and CXCL10 treated gastric cancer cell lines, an upregulation of PDL1 via STAT and PI3k pathways. They also reported a correlation between PDL1 gene expression and CXCR3 in gastric cancer tissues. [159]

Several studies have evaluated the relationship between CXCL9/CXCL10/CXCR3 axis and anti-cancer therapies including immunotherapy /immune checkpoint protein targets. This is an area of significant ongoing pre-clinical research with potentially promising results which may aid our understanding of limitations to current immunotherapy.

e) Macrophage Phenotype and CRC Patient Survival.

Survival analysis was performed comparing high vs low tumor M1 macrophage fraction. Results revealed that higher M1 fraction was associated with increased overall patient survival in all cases of CRC (N=588, p=0.062, **Figure 24a**) and interestingly the survival benefit was also seen in the MMRp/MSS CRC patients (N= 509, p = 0.00093, **Figure 24b).** Survival was also improved in patients with high expression of macrophage produced cytokines CXCL9 (p= 0.0069) and CXCL10 (p=0.003) which is in agreement with the studies previously described. **Figure 24c and 24d**.

To analyze whether the survival benefit was associated with the macrophages polarized to M1 phenotype, and not just overall high macrophage infiltration; we compared survival in high vs low M2, and found higher M2 macrophage had, as expected a worse overall survival (p= 0.0011). The most significant macrophage-associated difference in survival was in patients with highest M1:M2 macrophage ratio (p= 0.00093). This supports the thought that increasing M1 macrophage infiltration, relative to M2 macrophage, and not just overall macrophage density is the important difference in survival. This encourages the concept of “reprogramming” or “re-polarizing” the M2 macrophages to an M1 phenotype in which there are ongoing studies and is an important next step and future direction of this work.

Figure 24: Patient Survival Analysis

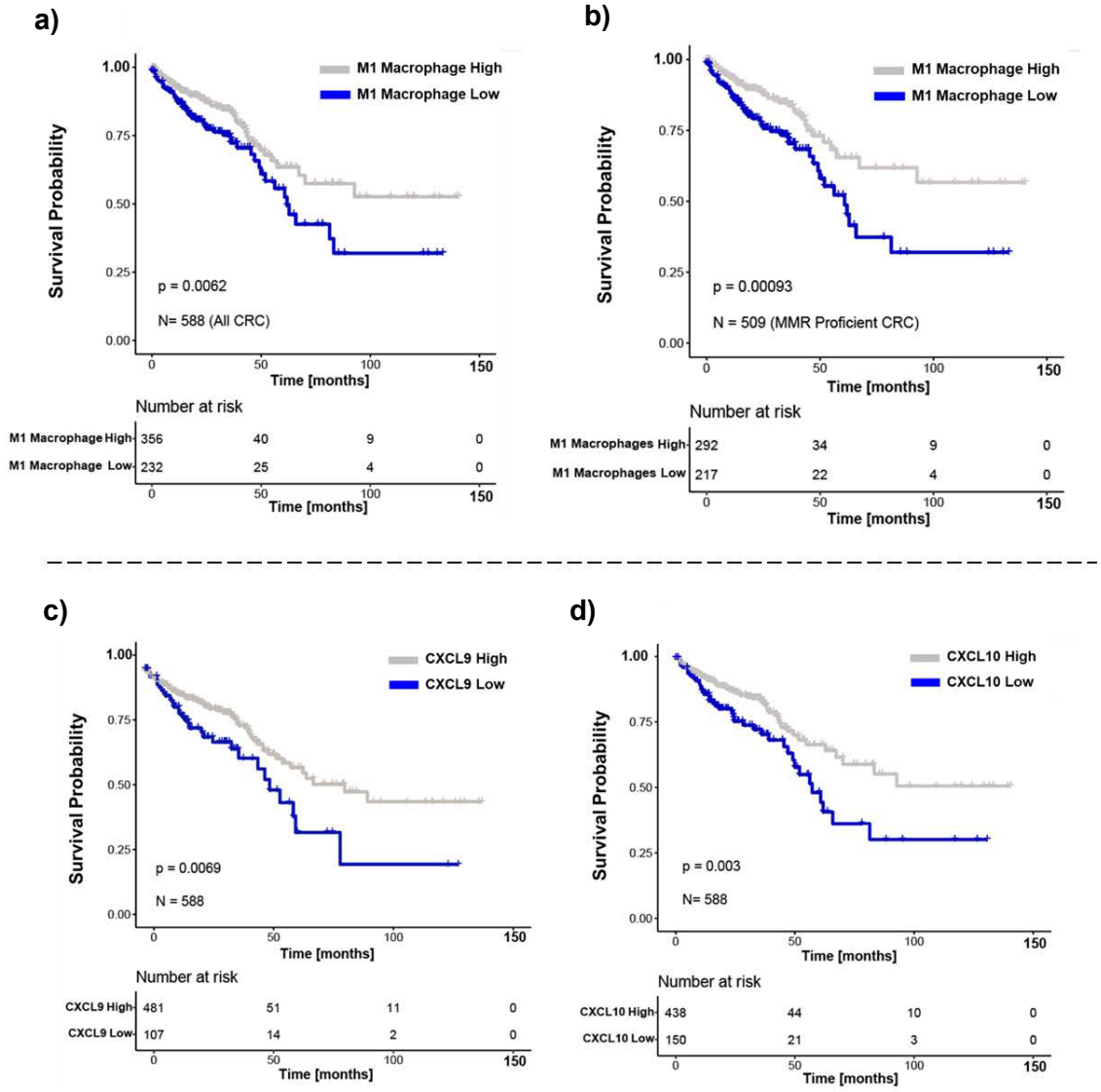


Figure 24: *Overall patient survival and* ***a)*** *All CRC M1 macrophage fraction,* ***b)*** *MMR proficient CRC M1 macrophage fraction,* ***c)*** *All CRC CXCL9 expression* ***d)*** *All CRC CXCL10 expression. Overall survival was compared using Kaplan-Meier survival curves and log-rank test.*

CHAPTER IX: CONCLUDING REMARKS AND FUTURE DIRECTIONS

The immune cells of the tumor microenvironment in colorectal cancer differ significantly in MMRd/MSI-H vs MMRp/MSS tumors. There is an overall increased infiltration and macrophages appear to exist in a more pro-inflammatory, classically activated phenotype. An increase in M1 macrophages within the tumor microenvironment correlated with tumors expressing higher PDL1.

To confirm these findings, in vitro studies enabled us to induce PDL1 expression in colon cancer by the combining of colon cancer cell lines with M1 polarized macrophages. Importantly, this observation was seen in MMRp/MSS cancers as well. These cancers are traditionally PDL1 negative, and do not respond to immunotherapy. We also demonstrated, in our model of THP-1 derived macrophage phenotypes that M1 macrophages themselves, had the highest PDL1 expression. This may be a contributing factor to the tumor overall increased PDL1 expression in M1-macrophage rich environments.

We found no differences in PDL1 expression or inflammatory profiles of the circulating monocytes of CRC patients with proficient vs deficient mismatch repair systems. This supports the rationale, that macrophage phenotype and PDL1 expression are altered once they are incorporated into the tumor, and not as circulating pre-cursor monocytes.

Genomic analysis led to the identification of target genes that overlapped in the PDL1 expression /macrophage activation /antigen presentation pathways. This led to further in vitro studies to identify a pathway in which macrophages may alter PDL1 expression. Given the knowledge of the role of T cell produced IFNγ in the upregulation of PDL1 in cancers; and its central role in the pathways of our genetic analysis, we initially explored this as a mechanism of macrophage induced PDL1 expression. IFNγ treatment of our cancer cell lines significantly upregulated PDL1 expression, as expected. However, our THP-1 derived M1 macrophages did not produce IFNγ. This suggested IFNγ activation alone could not account for the observation of M1 macrophages inducing cancer cell line PDL1 expression, so alternative mechanisms were explored.

The CXCL9/CXCL10/CXCR3 axis was identified as a potential pathway of macrophage induced PDL1 expression. CXCL9 and CXCL10 gene expression was upregulated in MMRd/MSI-H tumor samples; their expression correlated strongly with M1 infiltration of the tumor microenvironment, and their gene expression was significantly upregulated in our M1 polarized macrophages.

Despite the absence of IFNγ, our M1 macrophages induced increased gene expression in the JAK/STAT pathway genes, notably JAK1, JAK2, STAT1 and STAT3. This pathway is well established as the mechanism for IFNγ induced PDL1 expression, but these results suggest it can be activated by alterative pro-inflammatory macrophage induced cytokines, such as CXCL9 and CXCL10.

It is however important to note, several human and mouse studies have demonstrated the ability of macrophages to produce IFNγ and as such it should not be entirely discounted as a potential contributor to macrophage induced, cancer PDL1 expression. It is crucial to remember the isolated cancer-macrophage interaction we observe in cell line models does not recapture the complex dynamic relationship of the entire TME, and further studies into the role of IFNγ and its relationship with macrophages, and PDL1 expression in an in vivo model are warranted.

Future directions of this work will aim to study the relationship of macrophage phenotype and PDL1 expression in a mouse model, and current work is ongoing evaluating the effect of systemic IFNγ on cancer cell PDL1 expression in a xenograft mouse model. Future extension of this work would be to study the combination of IFNγ and anti-PDL1 immunotherapy, in a xenograft mouse model and the role of the CXCL9/CXCL10/CXCR3 axis in altering its response.

Another avenue under consideration for future work in this field, is to study the effect of re-programming of TAMs in established or early tumors to a re-polarized, M1 phenotype. Doing so, offers the potential to convert MMRp/MSS cancers to a more “immune hot”, pro-inflammatory phenotype which may induce successful response to immunotherapy.

In conclusion, these results suggest that M1 macrophages in the tumor microenvironment can induce PDL1 expression in colon cancer providing a potential treatment target for mismatch repair proficient CRC, which at present do not respond to immunotherapy. While IFNγ continues to play an important role in PDL1 expression, PDL1 can be induced via the JAK/STAT pathway by alternative mechanisms such as the CXCL9/CXCL10/CXCR3 axis.

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
APC	Adenomatous Polyposis Coli
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B (Braf) Oncogene
BRCA1	Breast Cancer Gene 1
CCL3	Chemokine Ligand 3
CD80	Cluster Of Differentiation 80
CD86	Cluster Of Differentiation 86
CIN	Chromosomal Instability
CRC	Colorectal Cancer
CT	Cycle Threshold
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
CXCL10	CXC Motif Chemokine Ligand 10
CXCL9	CXC Motif Chemokine Ligand 9
EGFR	Epidermal Growth Factor Receptor
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
FC	Fold Change
FDA	U.S. Food And Drug Administration
FGL1	Fibrinogen Like 1
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
ICI	Immune Checkpoint Inhibitor
IFNγ	Interferon-Gamma
IL-10	Interleukin 10

IL-13	Interleukin 13
IL-1B	Interleukin 1β
IL-27	Interleukin 27
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
JAK	Janus Kinase
KRAS	Kristen Rat Sarcoma Viral Oncogene Homolog
LAG-3	Lymphocyte Activation Gene 3
LOH	Loss Of Heterozygosity
LPS	Lipopolysaccharide
MLH1	Mut L Protein Homolog 1
MMR	Mismatch Repair
MRC1	Mannose Receptor C-Type-1
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
MSI-H	Microsatellite Instability-High
MSS	Microsatellite Stable
NCSLC	Non-Small Cell Lung Cancer
OS	Overall Survival
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PD-1	Programmed Death- 1
PDL1	Programmed Death Ligand-1
PFS	Progression Free Survival

PMA	Phorbol 12-Myristate 13-Acetate
PMS2	Postmeiotic Segregation Increased 2
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
SD	Standard Deviation
SEER	Surveillance, Epidemiology, And End Results
STAT	Signal Transducer And Activator Of Transcription
STK11	Serine/Threonine Kinase 11
TGFB	Peroxisome Proliferator-Activated Receptor Gamma
TIM3	T-Cell Immunoglobulin And Mucin Domain 3
TME	Tumor Microenvironment
TNFα	Tumor Necrosis Factor A
TNM	Tumor, Nodal, Metastases
TP53	Tumor Protein P53 Gene
VEGF	Vascular Endothelial Growth Factor

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QUALIFICATIONS:

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AWARDS/PRIZES:

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Association of Laparoscopic Surgeons Great Britain and Northern Ireland, Annual Meeting,

1st Prize: Poster Presentation: **Efficiency of Minor Operation Lists** *Liverpool May 2018*
Association of Surgeons Great Britain and Northern Ireland, International Surgical Congress

GRANTS:

Awarded the Cynthia Anne Getz Award for Lynch Syndrome Research through the Brown Cancer Center, University of Louisville.

PUBLICATIONS:

Peer Reviewed Publications

1. **Macleod A,** Parks MA, Cook CN, Petras RE, Galandiuk S. **Long-term behavior and functional outcomes of ileal-pouch anal anastomosis in inflammatory bowel disease with changing phenotype.** Surgery. 2023 Oct 4:S0039-6060(23)00516-0. PMID: 37802743.
2. **Macleod A**, Scheurlen KM, Burton JF, Parks MA, Sumy MSA, Gaskins JT, Galandiuk S. **Systemic adiponectin levels in colorectal cancer and adenoma: a systematic review and meta-analysis**. Int J Obes (Lond). 2023 Oct;47(10):911-921. PMID: 37626126.
3. **Macleod A**, Kavalukas SL, Scheurlen KM, Galandiuk S**. State-of-the-art surgery for Crohn's disease: Part II-colonic Crohn's disease and associated neoplasms.** Langenbecks Arch Surg. 2022 Nov;407(7):2595-2605. PMID: 35729401.
4. Scheurlen KM, Snook DL, Littlefield AB, George JB, Parks MA, Beal RJ, **MacLeod A**, Riggs DW, Gaskins JT, Chariker J, Rouchka EC, Galandiuk S. **Anti-inflammatory mechanisms in cancer research: Characterization of a distinct M2-like macrophage model derived from the THP-1 cell line.** Cancer Med. 2023 Nov 30. PMID: 38037545.
5. Scheurlen KM, Parks MA, **Macleod A**, Galandiuk S. **Unmet Challenges in Patients with Crohn's Disease**. J Clin Med. 2023. PMID: 37685662;

6. Scheurlen KM, **MacLeod A**, Kavalukas SL, Galandiuk S. **State-of-the-art surgery for Crohn's disease: part III-perianal Crohn's disease**. Langenbecks Arch Surg. 2023 Mar 30;408(1):132. PMID: 36995518
7. **Macleod A,** Abdulhannan P, Walker J, Wood T, Painter J, Kipling M. (2019). ***Colonoscopic miss rate for colorectal cancer: a district general hospital experience***. International Surgery Journal. doi:10.18203/2349-2902.isj20194150.
8. Bradley A, Sami S, Hemadasa N, **Macleod A**, Brown LR, Apollos J. **Decision analysis of minimally invasive management options for cholecysto-choledocholithiasis**. Surg Endosc. 2020 Dec;34(12):5211-5222. doi: 10.1007/s00464-020-07816-w. Epub 2020 Jul 24. PMID: 32710213.
9. Bradley A, Sami S, N G H, **Macleod A,** Prasanth M, Zafar M, Hemadasa N, Neagle G, Rosindell I, Apollos J. **A predictive Bayesian network that risk stratifies patients undergoing Barrett's surveillance for personalized risk of developing malignancy**. PLoS One. 2020 Oct12;15(10):e0240620. doi: 10.1371/journal.pone.0240620. PMID: 33045017; PMCID: PMC7549831
10. H Javanmard-Emamghissi, M Hollyman, H Boyd-Carson, B Doleman, A Adiamah, J N Lund, S Moler-Zapata, R Grieve, S J Moug, G M Tierney, The COVID: HAREM (Had Appendicitis and Resolved/Recurred Emergency Morbidity/Mortality) Collaborative Group, ***Antibiotics as first-line alternative to appendicectomy in adult appendicitis: 90-day follow-up from a prospective, multicentre cohort study***, *British Journal Of Surgery*, Volume 108, Issue 11, November 2021, Pages 1351–1359, https://doi.org/10.1093/bjs/znab287 (Collaborative Author)

In Press Book Chapter:

Macleod A, Galandiuk S. Chapter 35. Anal Cancer. In: **Major Complications of Female Pelvic Surgery: A Multidisciplinary Approach**. Hoffman, Bochner B, Hull T (Eds.) Springer, New York. In Press.

Accepted Abstracts:

Macleod A, Chariker J, Rouchka E, Feagins K, Galandiuk S. **The development of Crohn's Disease in a Familial Adenomatous Polyposis Cohort: An Analysis of Shared Genetic Variants**

- Accepted for poster presentation: American Gastroenterological Association, Digestive Diseases Week, Washington May 2024

Manuscripts in Progress

1. Scheurlen K, Chariker J, Beal RJ, **Macleod A,** Kanaan Z, Parks MA, Hall CC, Rutledge CF, Snook D, Hallion J, Gaskins J, Rouchka E, Galandiuk S. Adiponectin induces Itaconate production in Colorectal cancer. *Ca Res.*
2. Joshua I*, Polk HC Jr*, **Macleod A**, Eichenberger MR, Gardner SA, Schuschke D, Galandiuk S. Current Status of Physiology PhD Graduates. *Am. J. Physiol. Gastroenterol. Liver.*
3. **Macleod A,** Feagins K, Chariker J, Rouchka E, Galandiuk S. Research Letter: Coexisting Familial Adenomatous Polyposis/ Crohn's disease. *Gastroeneterol*.
4. Feagins K, **Macleod A,** Jones A, Alfieri T, Hinkebein E, Polk HC, Galandiuk S. Familial Adenomatous Polyposis and Crohn's Disease: Case Series and Systematic Review. *Clin. Gastroenterol & Hep.*
5. **Macleod A,** Hinkebein E, Alfieri T, Snook D, Beal RJ, Galandiuk S. M1 Polarization of THP-1 Monocyte Cell Line. *Experimental Cell Research*
6. **Macleod A,** Littlefield A, Seraphine C, Jaganathan V, Markert J, Galandiuk S. Macrophages Polarization in Colorectal Cancer and Immunotherapy: Systematic Review. *Ca Med*
7. **Macleod A,** Chariker J, Littlefield A, Markert J, Galandiuk S. Gene-Network/Pathway Analysis- M1 Polarization and Immune Checkpoint Protein Expression. *Seminars in Cancer Biology*
8. Jones A, Scheurlen K, **Macleod A**, Simon HL, Galandiuk S Obesity and Inflammatory Factors in the Development of Early Onset Colorectal Cancer. *Cancer.*

PRESENTATIONS:

1.	Long Term Behavior and Functional Outcomes of Ileal-Pouch Anal Anastomosis (IPAA) in Inflammatory Bowel Disease with Changing Phenotype	Oral	**Central Surgical Association**, Cleveland Ohio	07/23	1st Author + Presented
2.	M1 Macrophages and Immune Checkpoint Protein Expression of Colorectal Cancer using RNA Sequencing	Oral	**Academic Surgical Congress**, Washington DC	02/24	Supervisor
3.	Monocyte to M1 Macrophage Cell Line Polarization	Oral	**Academic Surgical Congress**, Washington DC	02/24	Supervisor
4.	Macrophage PDL1 Expression and the Effect of Colon Cancer Co-Culture	Oral	**Academic Surgical Congress,** Washington DC	02/24	Supervisor
5.	Colorectal Cancer Programmed Death Ligand-1 (PDL1) Expression in a Macrophage Co-culture Model.	Oral	**Academic Surgical Congress**, Washington DC	02/24	Supervisor
6.	Peripheral Blood Mononuclear Cell Inflammatory Profiles in MMRp and MMRd Colorectal Cancer	Oral	**Academic Surgical Congress**, Washington DC	02/24	Supervisor
7.	Macrophage Polarization and Mismatch Repair Status in Colorectal Cancer	Oral	**Academic Surgical Congress,** Washington DC	02/24	Supervisor
8.	Upregulation of Programmed Death Ligand-1 (PDL1) expression in HT-29 Colon Cancer Cell Line	Oral	**Academic Surgical Congress**, Houston Texas	02/23	Supervisor
9.	Programmed Death-Ligand 1 Expression in SW-480 Colon Cancer Cells Treated with Interferon Gamma	Oral	**Academic Surgical Congress,** Houston Texas	02/23	Supervisor
10.	Inflammatory Gene Expression as Screening Markers for Early Onset Colon Cancer: A Pilot Study	Oral	**Academic Surgical Congress**, Houston Texas	02/23	Supervisor
11.	Robotic Perineal Hernia Repair	Video/Oral	**Association of Laparoscopic Surgeons of Great Britain and Ireland**, Manchester UK	12/18	1st Author + Presented

12. Colonoscopic Miss-Rate for Colorectal Cancers: A DGH Experience	Poster	European Colorectal Congress, St Gallen, Switzerland.	12/17	1st Author + Presented
13. Efficiency of Minor Operation Lists	Oral Poster	Association of Surgeons of Great Britain and Ireland, Liverpool, UK	05/18	1st Author + Presented
14. Patient Involvement in Surgical Approach to Inguinal Hernia Repair	Poster	Association of Surgeons in Training, Belfast, UK	03/19	1st Author + Presented
15. Well-Leg Compartment Syndrome in Robotic Surgery	Poster	Association of Laparoscopic Surgeons of Great Britain and Ireland, Manchester UK	12/18	1st Author + Presented
16. Utility of Pelvic USS in Lower Abdominal Pain	Poster	Association of Surgeons of Great Britain and Ireland,, Glasgow, UK	05/17	1st Author + Presented
17. FY1 Rota Redesign: Improving Training & Patient Safety	Poster	8th National Medical Education Conference, NES, Edinburgh, UK	05/18	1st Author
18. Laparoscopic Cholecystectomy: Are we maintaining Day-Case Rates?	Poster	Association of Laparoscopic Surgeons of Great Britain and Ireland Telford, UK	05/19	1st Author + Presented
19. WhatsApp with Surgical Training	Poster	Association of Surgeons in Training , Edinburgh, UK	04/18	2nd Author Not Presented

CONTRIBUTION TO COLLABORATIVE RESEARCH:

p-ALICE:- UK Multi-centre Prospective Cohort Study: Audit of Laparoscopic Common Bile Duct Exploration- Local Hospital lead/collaborator *January-June 2021*

The COVID:HAREM Collaborative (Had Appendicitis and Resolved/Recurred Emergency Morbidity/Mortality. Local Hospital Lead/Collaborator *March-June 2020*

NOSTRA Collaborative: Management of T1 Rectal Cancers in the North East *Aug 2018-2019*

- Local Data Collection

QUALITY IMPROVEMENT/AUDITS

Laparoscopic Cholecystectomy Day Case Rates: What about the "Hot Gallbladder"	*Sunderland Royal Hospital, UK*	March 2019
Open Inguinal Hernia Repair Day Case Rates	*Sunderland Royal Hospital, UK*	March 2019
Umbilical Hernia Repair Day Case Rates	*Sunderland Royal Hospital, UK*	March 2019
Review and Re-design of FY1 Rota: Improving Patient Safety and Training	*Hairmyres Hospital, Glasgow, UK*	April 2018
Colonoscopic Miss Rate for Colorectal Cancer	*Sunderland Royal Hospital, UK*	Dec 2017
Patient involvement in the Choice of Surgical Approach to Inguinal Hernia Repair	*Sunderland Royal Hospital, UK*	Feb 2019
WhatsApp™ with Surgical Training	*Hairmyres Hospital, Glasgow, UK*	Feb 2018
Efficiency of Minor Operation Lists: A DGH Experience	*Hairmyres Hospital, Glasgow, UK*	May 2018
Utility of Pelvic USS in Lower Abdominal Pain	*Crosshouse, Glasgow, UK.*	May 2017

CLINICAL COURSES:

Basic Upper GI Endoscopy Course	JETS/JAG. Ninewells, Dundee, UK	06/2021
Introduction to Clinical Research Skills	RCS (England)/Dukes Club	05/2019
Clinical Research in the NHS	Health Education/CRN North East	09/2018
GCP: Good Clinical Practice	NIHR (National Institute Health Research)	12/2018
Advanced Surgery Cadaver Skills	RCPSG	12/2019
Surgical Skills Training Programme	RCS(Eng)	08/2019
CCrISP(Care of Critically Ill Surgical Patient)	RCS (England)	12/2018
GI Anastomosis Skills	RCPSG (Glasgow)	10/2018
Core Skills Laparoscopic Surgery	RCS (Edinburgh)	06/2018
Basic Surgery Cadaver Skills	RCPSG (Glasgow)	11/2017
Radiology for Surgeons	RCS (Edinburgh)	11/2017
ATLS	RCS (England)	02/2017
Basic Surgical Skills	RCPSG (Glasgow)	11/2016
Human Factors in Surgery	Health Education North East	03/2018

TEACHING:

Teaching Courses:

Reviewers Guild, Disease of Colon and Rectum		2021-2022
Teach the Teacher Course for Doctors.	Oxford Medical Glasgow	02/2019
Training the Clinical Anatomy Trainer	Doctors Academy Edinburgh	12/2016

Course Faculty

Core Trainee, Stoma Creation Course	Kirklands Medical Education Centre	03/2021
ST3 Interview Course	Ninewells Hospital, Dundee	01/2020
ST3 Interview Course	RCPSG, Glasgow	02/2020
Student Surgical Skills Course	Dumfries & Galloway Royal Infirmary	02/2020
Regional Nurse Practioner Training Day	University Hospital Hairmyres	03/2017

MANAGEMENT/LEADERSHIP:

ASCRS Annual Meeting Abstract Review Committee Member		2023-2024
Reviewer: Diseases of Colon and Rectum (11 reviews to date)		2022-present
Colorectal M&M/Audit Meeting Coordinator.	Sunderland Royal Hospital, UK.	Aug 18- Feb 19
Urology on call Rota Coordinator	Sunderland Royal Hospital, UK.	Aug-Dec 2017
FY1 General Surgical Rota Re-Design	University Hospital Hairmyres	Aug 2016-2017