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**Immune cell interplay in colorectal cancer prognosis**

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**Abstract**

The immune response to colorectal cancer has proven to be a reliable measure of patient outcome in several studies. However, the complexity of the immune response in this disease is not well understood, particularly the interactions between tumour-associated cells and cells of the innate and adaptive immune system. This review will discuss the relationship between

cancer associated fibroblasts and macrophages, as well as between macrophages and T cells, and demonstrate how each population may support or prevent tumour growth in a different immune environment.

**Key words:** Colorectal cancer neoplasms; Fibroblasts; Immune system processes; Macrophages; T lymphocytes

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**Core tip:** The outcome of patients with colorectal cancer is influenced by the complex local immune system. Understanding how multiple relationships between immune cells may affect tumour growth or elimination will be key in designing new therapies to treat this disease.

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**PERSPECTIVE**

Colorectal cancer (CRC) is the second and third most common cancer in women and men, respectively, worldwide<sup>[1]</sup>. In most cases, the disease occurs sporadically, but can also be caused by genetic predisposition or prior intestinal inflammation. While resection is often curative, approximately 45% of patients still die from the disease.

The recent introduction of successful immunotherapies against cancer, specifically checkpoint blockade antibodies, has increased attention on the immune response to tumours. These new treatments have provided opportunities for the development of new

immune-based therapies for less responsive tumours, such as CRC.

The complexity of the anti-tumour immune response is vast - not only are there multiple cells, these cells interact with each other, and are plastic so can change phenotype and function in response to inflammatory or suppressive signals from the tumour and tumour associated cells<sup>[2]</sup>. Understanding the relationships between cancer cells and immune cells is critical to understanding and, ultimately, manipulating the tumour immune microenvironment.

The importance of local immunity is particularly true in CRC where the immune response in the gut has been "trained" to ignore commensal microflora, and yet retain the ability to induce an attack against a pathogen. The ability of the gut to do this relies on a series of signals and interactions between bacteria, epithelial cells, and innate cells such as dendritic cells, monocytes and gut resident macrophages. In CRC, there are local adaptive immune cells such as effector T cells likely to have an antitumor effect, and regulatory or inflammatory T cells predicted to have a pro-tumour effect<sup>[3]</sup>.

Recent study of the immune response in CRC has resulted in the development of the Immunoscore, a means of measuring T cell infiltrate into CRCs<sup>[4]</sup>. The Immunoscore thus far has shown to be predictive of outcome and also superior to other methods for staging patients. Innate immune responses, particularly those involving tumour associated macrophages (TAMs), have been studied and data show that the frequency of these cells infiltrating the tumour can be associated with poor patient outcome, although this is controversial<sup>[5]</sup>.

Immune responses against colorectal tumours can be detected in early stage cancers, indicating that the immune system is capable of recognizing a tumour<sup>[6]</sup>. However, the tumour produces molecules that inhibit immune cell infiltration, that reduce activity of immune cells, or that change the phenotype of immune cells to a less effective anti-tumour function, ultimately allowing tumour outgrowth<sup>[7]</sup>.

The inflammatory immune environment underlying tumour initiation and progression in CRC has been reviewed extensively<sup>[8]</sup>, although much of the supporting data relies on animal models of colitis-induced cancer<sup>[9]</sup>. However, colitis-associated cancer accounts for only a small percentage (1%-4%) of CRC cases in humans<sup>[10]</sup>. The influence of inflammation mediated by immune cells in established familial or sporadic human CRC has been much less studied. In addition, new data demonstrate an impressive complexity of innate and adaptive immune cells<sup>[11]</sup>, suggesting that some associations with cancer progression may have been too simplistic in their interpretation.

This review will concentrate on the networks of innate and adaptive immune cells, and tumour-associated immune cells in established CRC, and how these interactions can influence subsequent patient outcome (Figure 1). Despite recent interest in the immunology of CRC, there are limited experimental

data studying the complexity of the immune response and the interactions between cancer cells and immune cells, particularly in humans. We will discuss (1) the interplay between the tumour stromal cells [particularly cancer-associated fibroblasts (CAFs)] and the macrophages infiltrating the tumour; and (2) the interactions between macrophages and T cells and how T cell populations may influence each other. We will attempt to describe the complexity and plasticity of these immune populations and discuss how they can be used to better understand the disease and to predict patient outcomes.

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## CANCER ASSOCIATED FIBROBLASTS AND TUMOUR ASSOCIATED MACROPHAGES - INNATE CELLS AND TUMOUR PROMOTION

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### *CAFs in CRC*

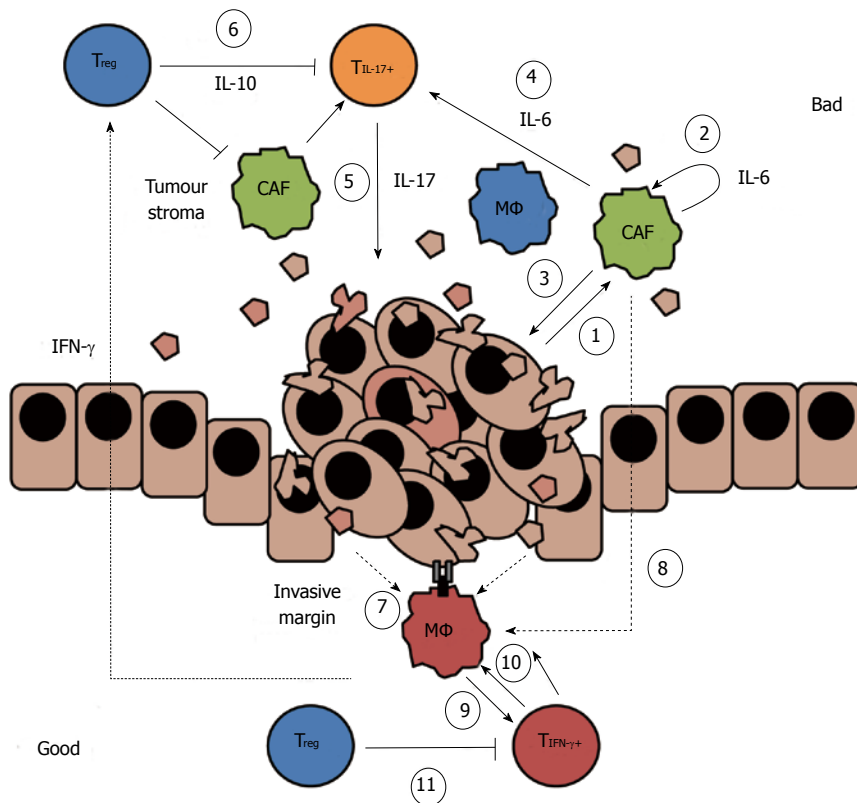
Fibroblasts are a key component of the connective tissue and are found embedded in the extracellular matrix (ECM). Fibroblasts have important roles in tissue homeostasis and remodelling. They produce multiple cytokines and can therefore modulate the immune microenvironment. Fibroblasts found in tumour stroma are referred to as CAFs.

The exact origin of CAFs is not clear. It has been proposed that they are cancer cells that have undergone an epithelial-mesenchymal transition<sup>[12]</sup>. Other research suggests that fibroblasts mature from fibrocytes that, in turn, have differentiated from monocytes<sup>[13]</sup> and thus have a similar haematopoietic lineage to macrophages. It is then not surprising that there is significant phenotypic overlap between CAFs and macrophages. CAFs do not express the immune cell marker CD45, however they can express CD68, a marker commonly used to differentiate macrophages<sup>[14]</sup>. Madar *et al*<sup>[15]</sup> hypothesised that CAFs were the result of convergent differentiation from any one of multiple pathways within the tumour microenvironment, and that CAF is a description of a functional state rather than a defined lineage.

CAFS may have a direct role in promoting CRC cell growth. Primary CAFs cultured from human colorectal tumours developed into distinct populations, some inducing a pro-migratory effect on CRC cells<sup>[16]</sup>. These pro-tumour CAFs had a distinct genetic signature with significant prognostic value. In addition, CAFs have been shown to promote metastases in CRC<sup>[17]</sup>.

### *CAF interactions promoting tumour growth*

Because of their role in tissue homeostasis, CAFs are able to promote tumour growth *via* similar pathways, including *via* inflammatory mediators consistent with the wound healing process. These pathways were reviewed recently<sup>[12]</sup>, so we will discuss the role of CAFs briefly, and focus on their influence on innate immune



**Figure 1 Immune cell interplay in established colorectal cancer.** CAFs and macrophages play an important role in promoting tumour progression in the stroma, mediated by IL-6 ("Bad"). Conversely, immune responses at the invasive margin, including macrophage and T cell compartments inhibit tumour growth ("Good"). (1): Unknown factors from colorectal tumours promote IL-6 production from CAFs; (2) IL-6 promotes further IL-6 production from CAFs as well as initiation of VEGF production; (3) IL-6, IL-17, VEGF and ECM modulators produced by CAFs promote growth, angiogenesis and invasion of colorectal tumours; (4) IL-6 produced by CAFs or stromal macrophages promotes T cell differentiation towards an inflammatory IL-17 producing phenotype; (5) IL-17 producing T cells promote colorectal tumour progression and are associated with poorer patient prognosis; (6) Tregs suppress the inflammatory IL-17 response; (7) Macrophages at the invasive margin are associated with improved prognosis; (8) IL-6 produced in the stroma enhances the anti-tumour phenotype; (9) Invasive margin macrophages are primed to induce good effector T cell responses; (10) IFN- $\gamma$ + effector T cells are associated with improved prognosis in CRC; (11) Tregs can inhibit effector anti-tumour T cell responses. CAFs: Cancer-associated fibroblasts; IL: Interleukin; VEGF: Vascular endothelial growth factor; ECM: Extracellular matrix.

cells. CAF-derived inflammatory mediators can both promote tumour growth and tumour invasion (Figure 1). An important inflammatory cytokine produced by CAFs in the regulation of wound healing, interleukin (IL)-6, is also associated with disease progression in CRC.

IL-6 in patient serum has been associated with poor patient prognosis in many cancers, including CRC<sup>[18]</sup>. IL-6 promotes cell survival and supports the production of vascular endothelial growth factor (VEGF) from both tumour and immune cells. VEGF was associated with enhanced tumour progression and poor patient prognosis in CRC<sup>[19]</sup>, likely through its role in angiogenesis<sup>[20]</sup>. CAFs produced more IL-6 than cancer cells, and CAF-derived IL-6 was increased in the presence of CRC cell lines<sup>[21]</sup>. In response to greater IL-6 production, CAFs up-regulated production of VEGF, leading to the proposal that the indirect effect of IL-6 on tumour growth *via* CAFs was more important than the direct effect of IL-6 on tumour cells<sup>[21]</sup>.

Other inflammatory mediators produced by CAFs also increase IL-6 production, including IL-1 $\beta$  and TNF $\alpha$ <sup>[21]</sup>. In patients, high plasma levels of the TNF $\alpha$  receptor, TNFR-2, were associated with an increased

relative risk of CRC<sup>[22]</sup>. Expression of both VEGF<sup>[23]</sup> and FSTL-1<sup>[24]</sup> (which enhances inflammatory cytokine and chemokine expression) was increased in CRC-associated CAFs. Chemotherapy, known to cause inflammation as cancer cells are killed<sup>[25]</sup>, resulted in increased numbers of active CAFs in a cohort of CRC patients<sup>[26]</sup>, and enhanced tumour growth in *in vitro* assays.

#### CAF recruitment of inflammatory cells

Fibroblasts both recruit, and are recruited by, monocytes/macrophages<sup>[12]</sup>. CAFs have been shown to recruit monocytes to the tumour microenvironment and thus may directly affect the local macrophage compartment. Indeed, Schellerer *et al.*<sup>[27]</sup> showed there were more Intracellular Adhesion Molecule-1<sup>+</sup> fibroblasts in tumour tissue than healthy bowel tissue from CRC patients, implying that cancer-associated cells have a higher affinity for monocytic cells. In an *in vitro* human breast cancer model, CAFs produced high levels of the chemokines CCL2 and CCL5 that attracted monocytes<sup>[28,29]</sup>. The production of these chemokines required IL-6, in a suggested IL-6-CCL2 auto-regulatory cycle<sup>[29]</sup>. CCL2 and CCL5 were also produced by tumour

cells as well as the recruited monocyte/macrophages, creating a positive feedback loop and generating an inflammatory tumour microenvironment<sup>[28]</sup>.

### TAMs in CRC

The prognostic significance of TAMs is controversial, particularly in CRC<sup>[30]</sup>. Macrophages are myeloid derived cells of the innate immune system. They are potent phagocytes and are involved in clearance of pathogens and cellular debris. They also initiate the adaptive response by functioning as antigen presenting cells (APCs). Macrophages reside in all tissues where they also maintain tissue integrity (reviewed in<sup>[31]</sup>). The phenotype and ontogeny of tissue resident macrophages varies between tissues. Some are freshly recruited bone marrow-monocyte derived macrophages, whereas others derive from the embryonic yolk sac (reviewed in<sup>[32]</sup>). In most adult tissue, however, resident macrophages are fetal liver derived. Both the ontogeny and microenvironment of resident macrophages influence their phenotype. As such, resident macrophage populations are often heterogeneous.

The phenotypic diversity of macrophages makes analysis of subpopulations challenging. A great deal of work has been undertaken assessing macrophage subsets using only one or two surface markers to determine function. However, a recent opinion suggests this approach to be misleading, due to the many causes of diversity<sup>[33]</sup>. Instead, multiple markers must be used to estimate the function of macrophage populations, or, where possible, primary functional data. It has been proposed that minimum reporting standards be introduced to allow better meta-analysis of macrophage data between research groups. This type of approach is paramount when assessing highly plastic macrophages, for example, human macrophages were shown to switch from anti-inflammatory to pro-inflammatory cytokine production within 24 h in response to IFN $\gamma$ , Granulocyte-Monocyte Colony Stimulating Factor and lipopolysaccharide *in vitro*<sup>[34]</sup>.

The link between macrophage infiltration and prognosis in CRC is still poorly understood. While some studies have shown a positive correlation between macrophage infiltration and patient prognosis, others have shown the opposite<sup>[30]</sup>. For example, Forsell *et al.*<sup>[35]</sup> demonstrated that a dense macrophage infiltration at the tumour invasive margin was associated with improved patient prognosis, and that macrophage inhibition of tumour spread and growth required direct cell-to-cell contact in an *in vitro* CRC model. In contrast, Kang *et al.*<sup>[36]</sup> demonstrated that intra-tumoural TAM count correlated with parameters of worse disease progression (depth of invasion, lymph node metastasis and stage). Using an *in vitro* co-culture macrophage and CRC cell lines these researchers also demonstrated that macrophages increased cancer cell invasiveness and migration. It may be that the conflicting data relating to the role of macrophages in CRC prognosis is due to inaccuracies of reporting culture conditions or a

lack of detailed phenotype<sup>[33]</sup>.

### Gut resident macrophages and CRC

Regular interaction between immune cells and microbes in the gut creates an immune environment that must be tightly regulated. Gut resident macrophages provide an important role in regulating this commensal barrier. These particular macrophages have an anergic phenotype; they destroy any bacteria that breach the epithelial barrier but do not initiate an immune reaction against them under homeostatic conditions<sup>[37,38]</sup>.

Unlike most tissue resident macrophage populations, gut resident macrophages are bone marrow derived<sup>[32,37]</sup>. Newly recruited monocytes undergo a conditioning process, mediated by the gut epithelia, that matures them into the resident anergic phenotype. However, upon acute inflammatory insult, such as that seen in inflammatory bowel disorders, this conditioning process becomes dysregulated, resulting in a mature macrophage population that acquires and maintains migratory and inflammatory characteristics<sup>[37,39]</sup>.

In the context of CRC, monocyte conditioning is unlikely to be modulated only by inflammation, but also factors actively produced by the tumour<sup>[40]</sup>, hypoxic conditions<sup>[41]</sup> and glucose starvation<sup>[28]</sup>. As a result, unique macrophage populations will exist depending strongly on the context of the local microenvironment. Hence, describing a homogeneous macrophage population in CRC can be misleading.

### TAMs promote an inflammatory pro-tumour environment

It is well documented that TAMs can promote tumour growth, both directly on tumour cells, and indirectly *via* cells in the tumour microenvironment (reviewed in<sup>[42]</sup>). The human monocytic cell line, THP-1, produced IL-6 in the presence of a colorectal cell line<sup>[43]</sup>, and macrophage-derived IL-6 induced expression of IL-6 by the HT29 CRC cell line<sup>[44]</sup>. TAMs also upregulated the expression of metalloproteinase (MMP)-2 and MMP-9 on cancer cells, molecules associated with lymph node metastasis<sup>[42,45]</sup>. TAM-derived IL-6 promoted STAT-3 mediated IL-10 production in CRC cells, a cytokine that has also been associated with poor patient prognosis<sup>[46]</sup>. In fact, p-STAT3 overexpression in the tumours of CRC patients is significantly correlated with tumour specific mortality<sup>[47]</sup>. Together, these studies demonstrate that TAMs and CAFs promote an environment to support tumour progression in CRC.

Macrophages have been shown to preferentially migrate to hypoxic regions of tumours<sup>[48]</sup>. In a mouse model of colitis-associated CRC, repression of hypoxia inducible factor 1 led to decreased macrophage infiltration in tumours<sup>[49]</sup>. Interestingly, under hypoxic conditions, macrophages can acquire a phenotype similar to that seen in macrophages involved in wound-healing role - a phenotype likely to promote tumour growth. More specifically, human macrophages in hypoxic conditions (0.5% oxygen) up-regulated expression of both VEGF and glucose transporter (GLUT)-1 compared

to normoxia<sup>[50]</sup>. GLUT-1 is the primary rate limiting glucose transporter in inflammatory macrophages<sup>[51]</sup>. Using transgenic RAW264.7 macrophages that stably overexpressed GLUT-1, it was shown that high glucose trafficking *via* GLUT-1 promoted a pro-inflammatory macrophage phenotype<sup>[51]</sup>. It is then possible to hypothesise that under hypoxic conditions such as those in a tumour, macrophages up-regulate GLUT-1 in an attempt to scavenge more glucose in a low glucose environment.

Beyond the production of inflammatory modulators, colorectal tumours also cause barrier defects, which allow for contact between immune cells and microbial products. Myeloid cells showed an increase in production of the inflammatory cytokine IL-23 under inflammatory conditions compared with homeostatic conditions in the APC<sup>min</sup> mouse model of CRC<sup>[52]</sup>. IL-23 stimulates and maintains IL-17 production from both tumour cells and T cells. In a mouse model of colitis associated CRC, IL-23- and IL-17-mediated inflammation disrupted the commensal microflora, and created a population of microbes that promoted tumour progression<sup>[53]</sup>. Furthermore, confocal microscopy of human CRC patient samples revealed that IL-17 production was not limited to T cells, but was also co-expressed with the myeloid cell marker, CD68<sup>[54]</sup>. These findings indicate that myeloid cells such as macrophages may be capable of producing IL-17 in CRC *in vivo*.

#### **Location of TAMs and influence on CRC prognosis**

A high infiltrate of macrophages at the invasive margin of colorectal tumours has been associated with improved patient prognosis<sup>[35]</sup>, and macrophages at the invasive margin of patients with CRC displayed characteristics of an anti-tumour phenotype<sup>[55]</sup>. These cells expressed the co-stimulatory molecules CD80 and CD86, and apoptotic signalling molecule FasL at greater levels than stromal macrophages. Moreover, macrophages have been closely associated with apoptotic cancer cells along the invasive margin<sup>[56]</sup> and, using cell lines, CRC TAMs have been observed to be highly phagocytic<sup>[57]</sup>. In an *in vitro* model of macrophage differentiation, with either human peripheral blood mononuclear cells or murine bone marrow derived macrophages, IL-6 promoted maintenance of the established macrophage phenotype, even when the original cytokine stimuli were removed<sup>[58]</sup>. Because macrophages themselves also produce IL-6, as well as respond to CAF-produced IL-6, they are especially sensitive to the conditioning signals in their immediate environment. For example, macrophages pre-exposed to IL-4/13, acquired a phenotype characterised by increased IL-10 production in response to IL-6. However, macrophages pre-exposed to IFN $\gamma$ , acquired a phenotype characterised by production of IL-1 $\beta$  and TNF $\alpha$  in the presence of IL-6. We propose that, in CRC, IL-6 both promotes and inhibits tumour growth *via* uniquely located macrophage populations (Figure 1).

#### **T cells and the anti-tumour immune response**

While considerable evidence on the role of T cells in preventing tumour growth in animal models has been acquired over decades, it was not until 2005 that a definitive role for T cells in CRC outcome was shown in patients<sup>[59]</sup>. Galon *et al.*<sup>[60]</sup> demonstrated, in 2006, that a high infiltrate of CD3<sup>+</sup> CD8<sup>+</sup> CD45RO<sup>+</sup> T cells at the invasive margin and the centre of the tumour was predictive of improved Overall Survival and Disease-Free Survival in a large cohort of people with CRC. Since then, these data have been confirmed by other groups, and have led to the introduction of the Immunoscore to quantify infiltrating T cells in clinical practice<sup>[61]</sup>.

The Immunoscore uses immunohistochemistry techniques to quantify the CD3<sup>+</sup> CD8<sup>+</sup> T cell infiltrate cell analysis at the centre of the tumour and at the invasive margin in people with CRC<sup>[4]</sup>. To date, the Immunoscore has proven to provide an accurate staging diagnosis as well as to predict patient outcome<sup>[62]</sup>. Although the Immunoscore is an improvement on the current staging methods for CRC, its efficacy may be hindered by the interference of T cell subsets that are not associated with good prognosis.

Although it remains clear that the infiltrate of CD3<sup>+</sup> CD8<sup>+</sup> CD45RO<sup>+</sup> T cells is associated with good patient prognosis in CRC, some T cell subsets have been associated with poor prognosis. Specifically, inflammatory CD4<sup>+</sup> T cells (Th17 cells), usually measured *via* production of the cytokine IL-17; and regulatory CD4<sup>+</sup> T cells (Tregs), often quantified by expression of the transcription factor, FoxP3; have been associated with both good and bad outcomes (reviewed in<sup>[63]</sup>). In addition, a low ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells is associated with improved outcome<sup>[64]</sup>. Interestingly, Väyrynen *et al.*<sup>[65]</sup> measured infiltrates of innate cells and adaptive cells in 117 CRC patients and found three parameters associated with Disease Free Survival at 24 mo: High infiltration of CD3<sup>+</sup> cells at the invasive margin and high infiltration of FoxP3<sup>+</sup> cells at the invasive margin and at the tumour stroma. Taken together, these findings indicate that CD8<sup>+</sup> T cells may be more effective than CD4<sup>+</sup> T cells in an anti-tumour immune response, or that beneficial CD4<sup>+</sup> T cell subsets are masked by subsets associated with poor outcome<sup>[64]</sup>. The phenotype of T cells resident in the tumour is controlled by the local cytokine environment, particularly APCs such as macrophages. The efficacy of the T cell response against the tumour is therefore dependent on interactions with other cells (Figure 1).

#### **Effective anti-tumour T cell responses**

T cells respond to specific antigens expressed by pathogens or tumours. These antigens are presented by a subset of immune cells, APCs, including dendritic cells and macrophages, but also non-immune cells such as epithelial cells or tumour cells. The T cell infiltrate in CRC is likely to be maximally effective if those cells are specific for tumour antigens.

Nagorsen *et al.*<sup>[66]</sup> used HLA tetramer analysis to show that tumour specific CD8<sup>+</sup> T cells in the blood were not correlated with improved clinical outcome in people with CRC or breast cancer, highlighting the need to study the tumour microenvironment. In a separate study, tumour-associated-antigen specific T cells were detected in 30%-40% of patients with CRC<sup>[67]</sup>. This study also showed that only a small subpopulation of infiltrating T cells could respond to tumour-associated antigens, indicating that not all infiltrating T cells were tumour-specific. Recently, Reissfelder *et al.*<sup>[68]</sup> proposed that a subpopulation of tumour antigen-specific T cells infiltrating the tumours of people with CRC was responsible for the prognostic impact of T cells shown by other studies.

Multiple studies in animals have shown that cytotoxic T cells, *via* IFN $\gamma$ , perforin and granzymes, can destroy established tumours. Gene cluster analysis of a large cohort of 602 patients with early stage CRC revealed that those patients with high CD8<sup>+</sup> and CD45RO<sup>+</sup> T cell infiltrates into the tumour also had increased expression of genes associated with anti-tumour responses compared with those patients with low CD8<sup>+</sup> and CD45RO<sup>+</sup> T cell infiltrates into the tumour<sup>[69]</sup>. The up-regulated anti-tumour gene signature included genes encoding for granzymes and perforin, as well as effector molecules such as IFN $\gamma$  and the related transcription factor T-bet. The expression of Granzyme B protein in tumours from CRC patients was also associated with improved survival<sup>[70]</sup>. These, and many other data, support a role for CD8<sup>+</sup> T cells and T cells producing the effector molecules IFN $\gamma$  and granzymes in eliminating CRC.

Effective T cells must become activated by interactions with APCs presenting antigen in the context of an appropriate cytokine milieu. TAMs were shown to express higher levels of the co-stimulatory molecule, CD80, than tumour stromal cells, indicating that these cells could activate T cells within the tumour<sup>[55]</sup>. In addition, using a multi-cellular tumour spheroid model, Ong *et al.*<sup>[71]</sup> showed that TAMs up-regulated the expression of CD25 and IFN $\gamma$  in T cells better than *in vitro* macrophages did. They also showed that the frequency of TAMs in human CRC tumours correlated with the frequency of infiltrating IFN $\gamma$ -producing T cells *in vivo*. These data indicate that TAMs may be able to promote effector T cell responses within the tumour microenvironment (Figure 1). We propose that effective anti-tumour immunity is determined by TAM-T cell interactions occurring at the invasive margin in CRC.

### **Th17 cells, inflammation and cancer**

Inflammatory T cells [defined here as IL-17-producing (or Th17) cells] are important in antimicrobial responses in the gut (reviewed in<sup>[72]</sup>). The acquisition of an IL-17-producing phenotype occurs when naïve T cells are activated in the presence of IL-6, IL-1 $\beta$ , TGF $\beta$  and IL-23; the maintenance of the phenotype is regulated by these same cytokines. Inflammatory IL-17 responses involve production of cytokines (especially IL-17) that recruit

monocytes and neutrophils to sites of inflammation<sup>[73]</sup>. These innate cells in turn produce the same cytokines to promote ongoing Th17 responses<sup>[74]</sup>.

IL-17 production in CRC has been associated with low Disease-Free Survival and Overall Survival<sup>[75]</sup> but the exact role of Th17 cells in CRC is not understood. Liu *et al.*<sup>[54]</sup> showed that Th17 induced production of VEGF in CRC cell lines *in vitro*, which decreased T cell production of IFN $\gamma$  and Granzyme B. This study also showed that in human CRC tumours, high expression of IL-17 correlated with high VEGF expression. VEGF expression has been inversely correlated with CD8<sup>+</sup> CD45RO<sup>+</sup> T cell infiltrate in tumours of CRC patients<sup>[69]</sup>.

### **Th17 cells indirectly affect tumour growth via CAFs**

CAFs may be activated *via* microbial products that cross the compromised epithelial barrier and promote IL-23 secretion<sup>[52]</sup>, further supporting Th17 responses. Using a mouse model of CRC, Numasaki *et al.*<sup>[76]</sup> showed that tumour cells engineered to express IL-17 led to increased production of angiogenic factors, including VEGF, not only by tumour cells, but also by CAFs. Th17 responses may therefore directly aid in the inflammatory responses of innate cells in CRC.

### **Th17 cells directly promote tumour growth**

Liu *et al.*<sup>[54]</sup> showed that IL-17 was increased in tumour tissue compared to healthy bowel tissue in a cohort of CRC patients, and that it was strongly correlated with overall survival. IL-17 added to human CRC cells *ex vivo* stimulated glucose metabolism by the tumour cells<sup>[77]</sup>. IL-17 promoted tumour growth through a STAT3-mediated pathway in CRC patients<sup>[78]</sup>; this result has also been shown in other models of cancer<sup>[79]</sup>. Together, these data indicate that the presence of intra-tumoural IL-17 may support tumour angiogenesis *via* VEGF and IL-6, and directly promote tumour cell proliferation (Figure 1).

### **Tregs and IL-10 controlling immunity**

Regulatory T cells (Tregs) suppress inflammatory responses in the healthy gut and regulate normal immune responses by inhibiting proliferation and activity of effector T cells. Induced Tregs acquire a suppressive phenotype in the presence of cytokines such as TGF $\beta$ ; the regulatory phenotype is characterised by up-regulation of the transcription factor FoxP3 and the production of IL-10, amongst other cytokines (reviewed in<sup>[80]</sup>). Dysregulated immune responses of the gut, for example inflammatory bowel diseases, are often typified by a high infiltrate of Tregs. In the presence of excess inflammatory cytokines from innate and adaptive immune cells, particularly IL-6, Tregs can convert into IL-17 inflammatory cells, or maintain their regulatory function while co-producing IL-17 (reviewed in<sup>[81]</sup>). Conversely, Treg differentiation can also inhibit the generation of Th17 cells.

In many human cancers an accumulation of Tregs is associated with poor patient outcome, presumably

by suppressing effector T cell responses against the tumour<sup>[63]</sup>. Controversially, in CRC, Tregs have been associated with both good and poor outcomes for patients<sup>[82]</sup>. It is possible that because Tregs suppress other T cells, they could impair the function of anti-tumour effector cells as well as pro-tumour inflammatory Th17 cells.

Using a complex library of tumour associated antigen-polypeptides, tumour-antigen specific Tregs were identified in the blood of CRC patients<sup>[83]</sup> providing evidence that these cells have the potential to inhibit specific anti-tumour immune responses. Therefore, the nature of the tumour immune microenvironment may influence the action of infiltrating Tregs.

### **Tregs suppress anti-tumour immune responses**

Tumour-specific Tregs isolated from ovarian tumours suppressed effector CD8<sup>+</sup> T cell production of IFN $\gamma$  *in vitro* after stimulation with tumour antigen<sup>[84]</sup>. The infiltrate of Tregs correlated with poor patient prognosis. In CRC patients with recurrent disease, specific T cell responses to the tumour antigens CEA and 5T4 were also suppressed<sup>[85]</sup>. In the same study, tumour specific Tregs and effector T cells were required to have the same specificity in order for Tregs to suppress the T cell response. Indeed, in an independent study, while tumour-antigen specific Tregs were identified in the tumours of CRC patients, the specificity of the majority of these cells was distinct from that of the effector and memory T cells in the same patients<sup>[83]</sup>. By depleting Tregs *ex vivo* in culture, only the effector anti-tumour T cells with the same specificity as the Tregs were increased.

The mechanism of Treg mediated suppression in tumour environments is not clear. In a mouse model of transplantable CRC using CMT93 cells, TAMs were able to recruit CCR6<sup>+</sup> Tregs to the tumour *via* production of the chemokine CCL20<sup>[86]</sup>. The infiltrate of Treg cells was associated with tumour development. Similarly, in breast cancer patients, the infiltrate of CCR6<sup>+</sup> Tregs into the tumour was inversely correlated with IFN $\gamma$  production from tumour infiltrating CD8<sup>+</sup> T cells<sup>[87]</sup>. Using flow cytometry, the authors showed that CCR6<sup>+</sup> Tregs, but not CCR6<sup>-</sup> Tregs were associated with poor survival in breast cancer patients. This leads us to hypothesise that, in CRC, tumour-antigen specific Treg populations are actively recruited to the tumour by TAMs and inhibit the anti-tumour immune response, leading to poor prognosis of patients.

### **Tregs suppress pro-tumour T cells**

Tregs recovered from blood of CRC patients were shown to inhibit the proliferation of Th17 cells sorted from blood and to suppress IL-17 production<sup>[88]</sup>. It is possible, therefore, that an accumulation of Tregs in the tumour of some CRC patients suppresses the inflammatory Th17 cell response rather than the anti-tumour effector response, leading to improved patient outcome.

### **Role for IL-10 in regulating tumour immune responses**

Tregs are characterised by production of IL-10, a multifunctional cytokine generally believed to support anti-inflammatory immune responses. CRC patients had elevated levels of serum IL-10, and IL-10 remained high in those patients who had recurrent disease following tumour resection<sup>[89]</sup>. However, it has become clear that treatment of cancer with IL-10 could lead to improved anti-tumour responses (reviewed in<sup>[90]</sup>). In human CRC, the amount of IL-17 was inversely correlated with the amount of IL-10 produced<sup>[91]</sup>. Interestingly, it has been shown that IL-10 mediated suppression of IL-17 responses was dependent on type-I IFN signalling<sup>[92]</sup>. Further, Mumm *et al.*<sup>[93]</sup> showed that IL-10 production induced the production of IFN $\gamma$  and granzymes from human effector CD8<sup>+</sup> T cells *in vitro*. Together these data suggest that IL-10 production from Tregs may, in fact, inhibit pro-tumour inflammatory responses as well as promote anti-tumour immune responses. Phase 1 clinical trials have now begun in advanced solid tumours using recombinant human IL-10 as a therapy (<https://clinicaltrials.gov/show/NCT02009449>).

## **CLINICAL RELEVANCE**

### **Experimental limitations**

Studying the immune response to CRC is difficult because of the complexity of both the gut immune response and the tumour microenvironment. As with most human studies, much of what has been studied has been observational and compounded by individual patient variation and individual tumour variation. The vast majority of CRC cases in humans are sporadic and the mutations that lead to tumour initiation and progression, and therefore immune responses, differ from person to person. Further, while animal models of CRC have provided useful information, their ability to truly mimic human disease is limited (reviewed in<sup>[94]</sup>). The two most commonly used models represent colitis-associated CRC (1%-4% of human CRC) or APC<sup>min</sup> mice representing familial CRC (about 20% of human CRC)<sup>[95]</sup>. We (and others<sup>[96,97]</sup>) have developed orthotopic surgical murine models of CRC that result in a tumour immune microenvironment more similar to that seen in sporadic human CRC than other mouse models. It is possible these models may be used to test new immune-based interventions.

### **Checkpoint blockade in CRC**

Two new immune-based drugs have recently been introduced in the treatment of cancer - anti-CTLA-4 (ipilimumab) and anti-PD-L1/anti-PD-1 (nivolumab or pembrolizumab). Both types of drugs act to prevent the tumour-mediated suppression of effector T cell responses, and have been successful in melanoma (reviewed in<sup>[98]</sup>). However, both checkpoint blockade drugs have shown much less success in CRC<sup>[99-102]</sup>. The reasons behind this are unclear but it has been

shown that many colorectal tumours do not express PD-L1, the ligand for PD-1. Therefore, if the suppressive effect of PD-L1 on anti-tumour T cells is absent, then therapy targeting the PD-1 pathway is unlikely to be successful<sup>[101]</sup>. However, it has recently been shown that microsatellite instability (MSI) high CRC tumours (15% of CRC tumours that have mutations in mismatch repair genes and are more immunogenic) expressed more PDL1 than MSI low tumours, indicating that checkpoint blockade may be more successful in the MSI high subset of CRC patients<sup>[103]</sup>. Clinical trials using anti-PD1 therapy in such a subset of patients are now underway to exploit this possibility.

### Adoptive T cell therapy in CRC

Adoptive cell therapy (ACT) has been trialled in CRC to some success. Karlsson *et al.*<sup>[104]</sup> used *ex vivo* T cells (recovered from tumour-draining lymph nodes) of CRC patients as a therapy. No side effects were observed and complete responses were seen in 4 out of 9 patients with metastatic disease. A Phase II trial is currently being undertaken to further test ACT in patients with metastatic CRC (<https://clinicaltrials.gov/ct2/show/NCT01174121>). The use of genetically engineered tumour-antigen specific T cells has been less successful in CRC. T cells genetically engineered to target carcinogenic embryonic antigen (CEA) caused a measurable decrease in serum CEA levels in 4/4 CRC patients treated but also induced severe colitis in all patients<sup>[105]</sup>, consistent with studies in other cancers. Targeting neo-antigens in tumours and individualising therapy may be the way forward in ACT of CRC.

## CONCLUSION

Recent technological breakthroughs have allowed the analysis of single cells, providing enormous amounts of data on the immune system (reviewed in<sup>[11]</sup>). These data provide novel insights into the function and complex connectivity of immune cells. This new network approach to studying immunology is likely to transform our understanding of the immune microenvironment of individuals with CRC. The immune response to CRC in humans is complex and involves a panoply of cells interacting with each other and the tumour. Patient outcome is unlikely to be accurately predicted by measuring one immune parameter independently. Moreover, any new immune-based therapies will need to take into account the pro- as well as anti-tumour activities of specific innate and adaptive immune cells.

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