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Tumour immune microenvironment biomarkers predicting cytotoxic chemotherapy efficacy in colorectal cancer

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ABSTRACT

The role of the local tumour and stromal immune landscape is increasingly recognised to be important in cancer development, progression and response to therapy. The composition, function, spatial orientation and gene expression profile of the infiltrate of the innate and adaptive immune system at the tumour and surrounding tissue has an established prognostic role in colorectal cancer (CRC). Multiple studies have confirmed that a tumour immune microenvironment (TIME) reflective of a type 1 adaptive immune response is associated with improved prognosis. There have been significant efforts to evolve these observations into validated, histopathology-based prognostic biomarkers, such as the Immunoscore. However, the clinical need lies much more in the development of predictive, not prognostic, biomarkers which have the potential to improve patient outcomes. This is particularly pertinent to help guide cytotoxic chemotherapy use in CRC, which remains the standard of care. Cytotoxic chemotherapy has recognised immunomodulatory activity distinct from its antimitotic effects, including mechanisms such as immunogenic cell death (ICD) and induction/inhibition of key immune players. Response to chemotherapy may differ with regard to molecular subtype of CRC, which are strongly associated with immune phenotypes. Thus, immune markers are potentially useful, though under-reported, predictive biomarkers. In this review, we discuss the impact of the TIME on response to cytotoxic chemotherapy in CRC, with a focus on baseline immune markers, and associated genomic and transcriptomic signatures.

INTRODUCTION

The tumour immune microenvironment (TIME) has an important role in mediating cytotoxic drug response and resistance, as illustrated by the differences in efficacy between in vitro, ectopic tumour mouse models and humans.[1] The TIME is extremely complex in colorectal cancer (CRC), reflecting genomic, host immunity and environmental (including microbiome) diversity.[2] The immune visibility and susceptibility of CRCs can vary widely, and explain differential prognosis. The baseline TIME may facilitate immune evasion through low antigenicity, paucity of immune effectors or immunosuppressive mechanisms, which may contribute to primary resistance to chemotherapy. However, it is hypothesised that immunostimulatory chemotherapy may overcome these deficits specifically to improve prognosis, or conversely be redundant in an optimally infiltrated tumour. There is a significant clinical need to identify biomarkers of response to the standard cytotoxics used in CRC—the antimetabolites (5-fluorouracil (5-FU) and capecitabine), platinum derivatives (oxaliplatin) and topoisomerase inhibitors (irinotecan). This review will summarise the key literature and studies that focus on baseline, pretreatment TIME histopathological markers as potential predictive and prognostic biomarkers in patients with CRC receiving cytotoxic chemotherapy. Biomarkers relevant to radiotherapy and novel immunotherapies are outside the scope of this review.

TIME ASSESSMENT IN CRC

The TIME is composed of various infiltrating cells of the innate and adaptive immune system and their associated mediators. Immune cells can be identified in the core of the tumour ($_{CT}$), both in intraepithelial cancer cell nests, or the tumour stroma ($_{CS}$); at the invasive margin ($_{IM}$), and in organised tertiary lymphoid structures ($_{TLS}$) distant from the tumour[3] (figure 1). This nomenclature will be used in the review to identify biomarker location where identified in respective papers. The cell type, location, density and functional orientation are all relevant for prognostication. Peritumoural infiltrates can be assessed on H&E-stained slides, using semiquantitative validated scoring systems including the Klintrup-Mäkinen (KM) grade[4] and the Jass score.[5] Multiplex immunohistochemical (IHC) techniques in clinically annotated tumour slides, to identify specific immune cells based on surface markers, is currently one of the key assessments of the TIME. Whole slides can be assessed, or tissue microarray techniques used to allow high throughput of samples. Cell density estimation can be performed manually, or assessed through digital image analysis[6] and machine learning algorithms to allow objective quantification, although scoring methodology varies widely. Advances in RNA sequencing, proteomics and single-cell technologies are also increasingly used to assess the TIME. Techniques such as CIBERSORT[7] and MCP-counter[8] can estimate the abundance of immune infiltrate in the tumour using the gene expression data from bulk tissues. Mass cytometry provides data at the individual cell level, and single-cell RNA sequencing allows profiling and classification of individual immune cells.[9] Tumour heterogeneity

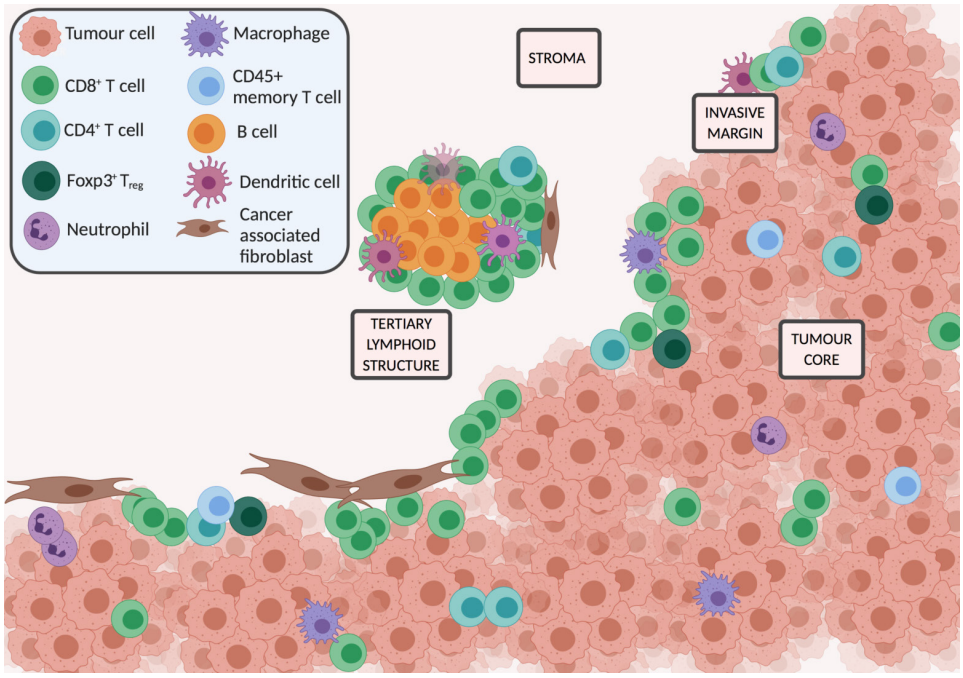

Figure 1 Key cells and locations in the tumour immune microenvironment.

and sampling issues add complexity to the use of biopsy-driven TIME biomarkers. Key cell types analysed using IHC techniques are listed in table 1, in addition to a summary of their known prognostic and predictive associations.

IMMUNOMODULATORY MECHANISM OF ACTION OF CYTOTOXIC CHEMOTHERAPY

Many chemotherapeutic agents, including oxaliplatin, fluoropyrimidines and irinotecan, have local and systemic immunomodulatory effects beyond their cytostatic mechanisms.[10–12] Preclinical models demonstrate that chemotherapy can augment immune responses directly by activation of immune effector cells (eg, production of interferon (IFN)γ) or inhibition of immunosuppressive factors (such as circulating regulatory T cells (T_{regs})[13]), or act on tumours directly to increase antigenicity,[14 15] immunogenicity[15] or susceptibility to immune attack through other mechanisms.[12 16] A small repertoire of chemotherapeutics, including oxaliplatin, can generate a specific mechanism of cell death, termed 'immunogenic cell death' (ICD), whereby release of specific danger signals from dying tumour cells stimulates a dendritic cell (DC)-mediated, cytotoxic T-helper 1 (T_h1) response to eradicate residual tumour cells.[17–19] Platinum cytotoxics can cause DC maturation,[14] downregulate immune checkpoints and thus increase $CD8^+$ T cell activation.[20 21] In vivo, fluoropyrimidines selectively deplete immunosuppressive myeloid-derived suppressor cells (MDSCs),[22] although have also been associated with a pro-tumour T_h17 response.[23 24] The immunogenicity of irinotecan is less certain, although in vivo work has reported influence on T_{reg} and MDSC infiltration,[25] and upregulation of tumour PD-L1.[26] For clinical correlation, patients receiving neoadjuvant (preoperative) 5-FU/oxaliplatin show increased infiltration of $CD3^+$,[27 28] natural killer (NK) and $CD8^+$ cells[29] in resected liver metastases compared with patients undergoing upfront surgery. Neoadjuvant fluoropyrimidines increase the density of $CD3^+_{CS}$ and $CD8^+_{CS}$ cells in patients with resected rectal cancer compared with pretreatment biopsies.[30 31]

TIME BIOMARKERS

Inflammatory infiltrate

Increased tumour inflammatory infiltrate is strongly associated with improved survival,[32] although most studies do not specify survival by subgroups based on chemotherapy utilisation. For those studies that do, an increased infiltrate seems to confer a positive prognostic advantage in patients receiving chemotherapy, mirroring the trend in the untreated population. A higher KM grade (more florid infiltrate at invasive margin) is associated with improved overall survival (OS) in patients receiving adjuvant chemotherapy (unspecified regimes)[33 34] and FOLFOX (infusional 5-FU and oxaliplatin) chemotherapy.[35 36] Tumour-infiltrating lymphocyte (TIL) density $_{CT\ and\ IM}$ was not prognostic in stage II/III patients receiving adjuvant 5-FU plus oxaliplatin regimes[37]; however, increased primary TIL density was associated with improved response rates (79% vs 48%, p=0.025) to doublet chemotherapy (oxaliplatin or irinotecan based) in patients with metastatic disease.[38] This is notable as the primary tumour TIME appeared to impact on response rates at distant metastatic sites. Morris *et al*[39] reported a significant survival benefit with adjuvant 5-FU chemotherapy versus observation in stage III patients (n=1156) with peritumoural TILs present (HR 0.22, p<0.001) which was not evident in patients with absent TILs (HR 0.84, p=0.29). This suggests a possible predictive role, with 5-FU being more efficacious in patients with pre-existing immune recognition; however, non-standardised methods were used to identify TILs in this study which may impact validity.

$CD3^+/CD8^+$ T cells

The predominant infiltrating immune cells in CRC are T lymphocytes, identified by the generic $CD3^+$ surface marker. Cytotoxic $CD8^+$ T lymphocytes recognise tumour antigen presented by MHC class I molecules, thus providing the key antitumour immune response. High density of $CD3^+$ and $CD8^+$ T cells in the core tumour and invasive margin are well established as a positive prognostic marker in the majority of CRC studies.[32]

Table 1 Primary tumour prognostic and predictive IHC-based TIME biomarkers in patients receiving chemotherapy

Immune biomarker	Location	Prognostic role in early stage patients receiving adjuvant chemotherapy (regime)	Prognostic role in stage IV patients receiving palliative chemotherapy (regime)	Predictive role or differential biomarker prognostic role by treatment group
Specific immune cell				
$CD3^+$	CT	Most studies - î density=positive prognostic assoc ► Improved OS[36 40 42 43] and DFS[41] (5-FU) ► Improved DFS (FOLFOX)[44 45] Few studies – no association ► OS (unspecified regimes)[46] and DFS (5-FU+/-bevacizumab)[47]	î density=positive prognostic assoc ► Improved OS (unspecified regimes)[55]	Possible negative predictive role (adjuvant chemotherapy unspecified) ► î density (vs low density)=improved OS in observation group (not chemotherapy group)[46]
	IM	î density=mixed findings ► No association DFS[48] or OS[40] (5-FU) ► Improved DFS[44 45] and OS[49] (FOLFOX)	î density=positive prognostic assoc ► Improved OS (unspecified regimes)[55]	
$CD8^+$	CT (CS)	Most studies - î density=positive prognostic assoc ► Improved OS[42 46 50] and DFS[47 51] (5-FU) ► Improved DFS[44 45 52] (FOLFOX) Few studies – no association ► OS (5-FU)[40]	î density=mixed findings ► Improved OS (oxal/irinotecan+5-FU)[54] ► No assoc OS (unspecified regime[55] and FOLFOX)[56]	No predictive role (adjuvant 5-FU) ► î density=improved OS in patients treated with and without adjuvant chemotherapy[46]
	IM	Most studies - î density=positive prognostic assoc ► Improved OS (5-FU)[53] ► Improved DFS (FOLFOX,[45] CAPOX)[52] Few studies – no association ► OS (5-FU,[40] unspecified regime)[71]	No prognostic assoc ► OS (unspecified regime)[55]	Possible positive predictive role (adjuvant 5-FU) ► î density (vs low density)=OS benefit greater for patients treated with adjuvant chemotherapy>observation[53]
$CD4^+$	CT		î density=mixed findings ► Improved OS (unspecified regimes)[55] ► No association OS (FOLFOX)[56]	
	IM		î density=positive prognostic assoc ► Improved OS (unspecified regimes)[55]	
Immunoscore ($CD3^+$ and $CD8^+_{CT+IM}$)	0–4	High score=positive prognostic assoc ► Improved DFS high-risk stage II (5-FU)[65] ► Improved DFS stage III (FOLFOX)[45 63] ► Improved OS stage III (5-FU,[33 62] variable regimes)[64]		Positive predictive role stage III (various adjuvant regimes) ► High IS (2-4)=DFS benefit with adjuvant chemotherapy (vs low IS 0–1—no benefit)[64] Not predictive stage II (adjuvant 5-FU)[65]
$Foxp3^+$ (T_{reg})	CT	î density=mixed findings ► Improved OS (5-FU)[40 42 46 50] ► No association DFS (5-FU)[41] ► Worse DFS/OS (unspecified regime)[70]	î density=mixed findings ► Improved OS (FOLFOX,[56] 5-FU+oxaliplatin or irinotecan)[54] ► No association OS (unspecified regime)[55]	Mixed findings ► Not predictive (adjuvant 5-FU)[46] ► Possible negative predictive role (adjuvant chemotherapy unspecified) – î density (vs low density)=worse OS/DFS in adjuvant chemotherapy group (not observation group)[70]
	IM	No prognostic association ► DFS/OS on multivariate analysis (unspecified regime)[71]	No prognostic association ► OS (unspecified regime)[55]	Possible negative predictive role (adjuvant chemotherapy unspecified) ► î density (vs low density)=improved OS in observation group (not chemotherapy group)[71]
$CD66b^+$ (TAN)	CT	î density=positive prognostic association ► Improved DFS/OS (5-FU,[74] unspecified regime)[70]		Mixed findings ► Possible negative predictive role (adjuvant chemotherapy unspecified) – î density=no OS/DFS benefit from adjuvant chemotherapy (vs observation); vs low density—possible OS/DFS detriment with adjuvant chemotherapy[70] ► Positive predictive role (adjuvant 5-FU) – î density (vs low density)=improved DFS in adjuvant chemotherapy group; vs worse DFS in observation group[74]
	IM	î density=positive prognostic association ► Improved DFS (5-FU)[75]		Possible positive predictive role (adjuvant 5-FU) ► î density (vs low density)=improved DFS in chemotherapy group only (not observation group)[75]
$CD68^+$ (general TAM marker)	CT	No prognostic assoc stage II ► DFS/OS (5-FU)[77]	î density=negative prognostic association ► Worse OS (unspecified regimes)[55]	
	IM	î density=positive prognostic association ► Improved DFS (5-FU)[75]	No prognostic association ► OS (unspecified regime[55]	Possible positive predictive role (adjuvant 5-FU) ► î density (vs low density)=improved DFS in chemotherapy group (not observation group)[75]
$CD163^+$ (M2 polarised TAM)	CT	î density=negative prognostic association ► Worse DFS/OS (unspecified regime)[70]	î density=negative prognostic association ► Worse OS (unspecified regimes)[55]	Possible negative predictive role (unspecified adjuvant regime) ► î density (vs low density)=worse DFS in chemotherapy group (not observation group)[70]
	IM		No prognostic association ► OS (unspecified regime)[55]	
CD206+ (M2 polarised TAM)	CT	î density=negative prognostic association ► Worse DFS/OS (5-FU)[77]		Possible positive predictive role (adjuvant 5-FU) ► î ratio $CD206^+$:$CD68^+$ = improved DFS with adjuvant chemotherapy (vs low ratio—no benefit)[77]
$CD45RO^+$ (memory T cell)	CT	î density=positive prognostic association ► Improved OS (5-FU)[42 43]	î density=positive prognostic association ► Improved OS (oxal/irinotecan+5-FU)[54]	

Continued

Table 1 Continued

Immune biomarker	Location	Prognostic role in early stage patients receiving adjuvant chemotherapy (regime)	Prognostic role in stage IV patients receiving palliative chemotherapy (regime)	Predictive role or differential biomarker prognostic role by treatment group
	IM	î density=positive prognostic association ► Improved OS (unspecified regime)[71]		No predictive role (adjuvant chemotherapy unspecified) ► î density=improved OS in patients treated with and without adjuvant chemotherapy[71]

CS, core tumour stroma; CT, core tumour; DFS, disease-free survival; 5-FU, 5-fluorouracil; IHC, immunohistochemical; IM, invasive margin; OS, overall survival; TAM, tumour-associated macrophage; TAN, tumour-associated neutrophil; TIME, tumour immune microenvironment; TLS, tertiary lymphoid structures.

However, location is relevant—tumours demonstrating a paucity of $CD8^+$ cells in the tumour core, and lacking the activation markers granzyme-B and IFNγ, have been termed 'infiltrated excluded' with worse survival outcomes.[1] The prognostic associations in chemotherapy-treated patients are less well reported. Retrospective studies have confirmed a positive survival association of increased density $CD3^+_{CT}$ in patients receiving single agent 5-FU[40–43] and FOLFOX,[44 45] although some groups have found no relationship.[46 47] $CD3^+_{IM}$ was not prognostic for single agent 5-FU chemotherapy,[40 48] and this may reflect the phenomenon of the 'infiltrated excluded' tumour discussed above, which could impact on 5-FU efficacy. In contrast, increased density of $CD3^+_{IM}$ did correlate with improved disease-free survival (DFS) in a large prospective phase III trials of patients receiving adjuvant FOLFOX±cetuximab (an epidermal growth factor receptor monoclonal antibody).[44 45 49] It is possible that the addition of oxaliplatin to 5-FU may influence the prognostic impact of invasive margin T cells. Increased density of $CD8^+_{CS}$ was positively prognostic in patients with early stage disease receiving adjuvant 5-FU single agent chemotherapy,[42 46 50 51] ±bevacizumab,[47] and high $CD8^+_{CT/CS\ and\ IM}$ was associated with improved DFS in patients receiving oxaliplatin doublet adjuvant chemotherapy.[44 45 52] Some studies have reported that the relative survival benefit of adjuvant 5-FU chemotherapy is much greater for patients with increased density of $CD8^+_{CT}$ compared with patients with low density,[53] supporting Morris *et al*'s findings,[39] and suggesting fluoropyrimidines may be more efficacious when a pre-existing T_h1 response is present. However, a treatment interaction has not been confirmed by other groups.[46] $CD8^+_{CT}$ as a prognostic marker in stage IV patients has shown contradictory results (see table 1).[54–56] Multiple studies[30 57 58] have correlated high pretreatment $CD3^+$ and $CD8^+$ cell density on rectal biopsy with increased response rates to neoadjuvant therapy and improved survival, although this has not been replicated in all reports,[31 59] and outcomes are mediated by the effects of radiotherapy and are thus outside the scope of this review.

Immunoscore

The Immunoscore (IS) was designed as a digitally quantified IHC assessment of $CD8^+_{CT+IM}$ and memory T cell ($CD45RO^+_{CT+IM}$) densities added to produce a cumulative score.[60] It has been validated to show prognostic ability superior to the traditional tumour/node/metastasis (TNM) staging system,[61] with high scores conferring superior survival. $CD3^+$ later replaced $CD45RO^+$ due to superior antibody performance[3] (figure 2). Its validity as a prognostic marker in patients receiving adjuvant 5-FU[33 62] and FOLFOX[45 63] chemotherapy has been reported, but its role as a predictive marker is less clear. In a recent multinational trial of stage III patients, those with a low IS (0–1) did not benefit from adjuvant chemotherapy (various regimes), whereas those with IS 2–4 did, and the magnitude of the survival benefit was greater the higher the IS.[64] In high-risk stage II disease, high IS was prognostic, but not a predictive discriminator of 5-FU benefit.[65]

Interestingly, in an analysis of stage III patients in the IDEA collaboration (3 months vs 6 months of adjuvant FOLFOX), patients with IS 2–4 had a significantly improved DFS with 6 months vs 3 months of FOLFOX (HR 0.53, p=0.0003), whereas patients with IS 0–1 did not derive a significant DFS benefit with extended treatment (HR 0.84, p=0.27).[63] This suggests again possible futility of doublet regimes, irrespective of cumulative dose, in immune-excluded disease and a dose-dependent benefit of oxaliplatin regimes in tumours with a baseline cytotoxic T lymphocyte response.

$CD4^+$/$Foxp3^+$ T cells

$CD4^+$ helper T cells, which aid tumour immune responses by activation of signalling to facilitate $CD8^+$ T cell-mediated cell death, can exert both T_h1 responses which promote antitumour effects with good prognostic association,[66] and T_h2 responses which are tumourigenic. $CD4^+_{CT}$ as a prognostic marker has shown positive association in only half of the studies it has been assessed, and in no studies of $CD4^+_{IM}$.[32] Adjuvant studies referencing chemotherapy are lacking. Increased primary tumour $CD4^+_{CS\ and\ IM}$ was prognostic in some studies of stage IV patients receiving mixed palliative regimes[55] but not in other cohorts

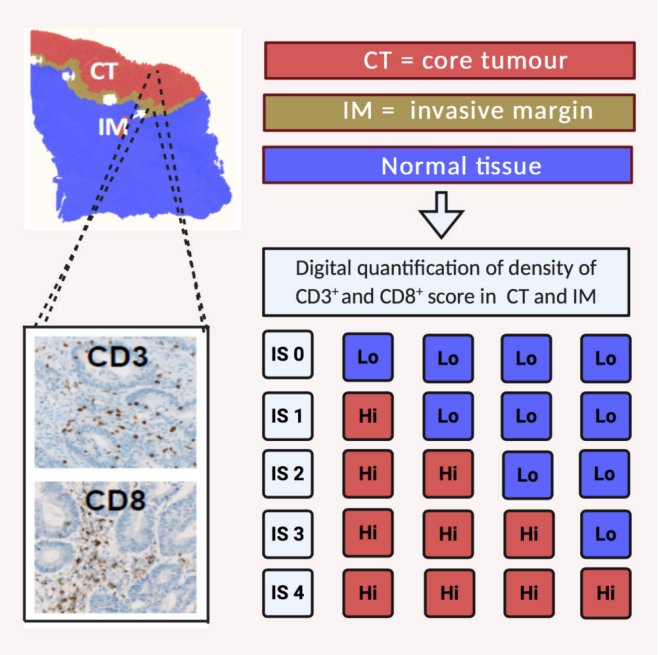

Figure 2 The Immunoscore (IS) is based on the numeration of two lymphocyte populations ($CD3^+$ and $CD8^+$) in the CT and IM. Density of cells is determined using an image analysis workstation. Each marker in a specified region is categorised as 'Hi' or 'Lo' based on predetermined cut-off values. Patients are stratified according to a score IS 0 to IS 4 based on the total number of 'Hi' densities observed in the four regions.

receiving oxaliplatin regimes.[56] T_{regs} constitute a specific subtype of $CD4^+$ T cell, identified by immunoprofile $CD25^+Foxp3^+$, and have general immunosuppressive functions, although this can vary depending on marker expression.[67] Meta-analyses of prognostic studies in CRC has reported a positive association with cancer-specific survival[68] (CSS) and OS[69] which is in contrast to other tumour types. High density of $Foxp3^+_{CT/CS}$ has been associated with improved OS in some cohorts receiving adjuvant 5-FU chemotherapy,[40 42 46 50] and palliative oxaliplatin,[56] but not others.[41 70 71] Some cohorts have suggested that increased $Foxp3^+$ density may confer a positive prognostic association only in untreated patients, and not in patients receiving chemotherapy,[70 71] although true predictive studies are required.

Tumour-associated neutrophil

Tumour-associated neutrophils (TANs), identified by their markers $CD11b^+$, $CD66^+$ and $Ly6G^+$, are less populous than other cells, and subsets can be either tumour-suppressive or supportive depending on TGF-β and IFN-γ signalling.[72] Prognostic studies have reported conflicting results. In stage I-III patients (n=1008), high TAN ($CD66^+_{CT}$) density conferred an excellent prognosis, and no benefit from adjuvant chemotherapy, whereas low density conferred worse prognosis and poorer survival in patients receiving adjuvant chemotherapy.[70] However, this unexpected result may be a reflection of treatment bias and lack of adjustment for tumour stage and necrosis, which are associated with TAN density.[73] In a contradictory smaller cohort of stage III patients, high TAN_{CT} density was reported as a negative prognostic marker in patients undergoing surgery (DFS HR 3.0, p=0.07). However, this impact was mitigated by the use of adjuvant 5-FU, whereby patients with high TAN_{CT} had improved prognosis.[74] High $CD66b^+_{IM}$ was also a positive prognostic and predictive marker in stage III patients receiving adjuvant 5-FU.[75] Contradictory results may also be explained in this and other studies by variations in methodology, including different prognostic associations depending on assessment in invasive margin or core tumour,[73] and variable marker categorisation.

Tumour-associated macrophages

Tumour-associated macrophages (TAMs), often identified by the non-specific $CD68^+$ monocyte lineage marker, are broadly grouped into two phenotypes. The classically activated (M1) type (surface markers iNOS, $CD86^+$, $CD169^+$) that stimulate antitumour immune responses, and the alternatively activated (M2) type (surface markers $CD163^+$, $CD206^+$, $CD204^+$) that enhance tumour progression and suppress immune response (eg, NK and T cell mediated killing).[76] Increased $CD68^+_{IM}$ density was a positive predictive marker of 5-FU benefit in a small cohort, and in a companion in vitro study, 5-FU and M1-macrophages showed synergistic impact on cell death in CRC cell lines.[75] $M2_{CT}$ infiltration has been reported as negatively prognostic in several studies of patients treated with systemic chemotherapy.[55 70 77 78] In vitro work has suggested that M2 macrophages confer resistance to 5-FU,[79] and some studies suggest a negative predictive relationship.[70] Feng *et al*[77] reported high $CD206^+$:$CD68^+$ ratio (increased proportion of M2 macrophages) was a marker for poorer DFS and OS in stage II disease, although also predicted a significant survival benefit from adjuvant 5-FU-based chemotherapy versus observation (DFS HR 0.42, p=0.003) which was not present in the better prognostic group with a low ratio (HR 0.99, p=0.99). Oxaliplatin plus trifluridine/tipiracil (an antimetabolite) depletes M2 macrophages, resulting in higher $CD8^+$ infiltration and better therapeutic efficacy.[80] Further exploration in patient cohorts using differential chemotherapy regimes is required.

$CD45RO^+$ T cells

Central and effector memory T cells (characterised by $CD45RO^+$ marker) drive secondary immune responses post exposure to primary antigens. Meta-analyses suggest a positive prognostic association for increased density of these cells both in the core tumour and invasive margin.[32] High density of primary tumour $CD45RO^+_{CT}$[42 43] and $CD45RO^+_{IM}$[71] is an independent prognostic factor for improved OS in early stage disease patients receiving 5-FU. High density was associated with better survival in patients with stage IV CRC undergoing adjuvant oxaliplatin or irinotecan chemotherapy post-curative intent resection (estimated 3-year survival 62% vs 27%, p=0.007).[54] High $CCR7^+_{CS}$ (used to identify $CD8^+$ naïve and central memory T cells) was associated with improved OS in patients receiving palliative oxaliplatin-based regimes.[81]

Gamma delta (γδ) T cells

Gamma delta T cells are a rare subset of predominantly mucosal $CD8^-CD4^-$ T cells with a broad functional role in cytokine (IFN-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-17) and chemokine (RANTES, IP-10, lymphotactin) production, cytolysis and coordination of antigen presentation.[82] In vivo studies show that ICD-inducing chemotherapy causes a rapid invasion of γδ T lymphocytes prior to the invasion of $CD8^+$ T cells, and that in TCR $\delta^{-/-}$ mice, the therapeutic efficacy of chemotherapy was reduced.[83] Increased expression of γδ T cells has been associated with improved DFS in patients with CRC.[84] While results from CRC cohorts receiving chemotherapy are under-reported, a series (n=463) of patients with gastric cancer receiving adjuvant 5-FU chemotherapy suggest a significant survival advantage of chemotherapy versus observation if infiltrating γδ T cells were increased.[85]

B cells

B cells also recognise tumour antigens, produce tumour-specific antibodies and are identified through $CD19^+$, $CD20^+$ and $CD78^+$ markers. High $CD20^+_{CS}$ has been associated with better prognosis in CRC,[86] as has the presence of TLSs, which contain concentrated B cells.[87] However, $CD20^+_{CT\ or\ IM}$ was not prognostic in patients receiving adjuvant FOLFOX.[45]

Immune checkpoints

Multiple stimulatory and inhibitory immune checkpoints, crucial for self-tolerance, and co-opted by tumours to evade immunosurveillance, have been identified in the TIME. One such checkpoint, programmed death-ligand 1 (PD-L1), is predominantly derived from the immune infiltrate,[88] not tumour cells, in CRC. Immunodeficient murine xenograft models of PD-L1 knockout tumours display resistance to oxaliplatin,[89] which contrasts with models in other tumour types. In early stage patients receiving 5-FU chemotherapy, high tumour PD-L1 was not prognostic in some studies,[40 42] although negatively impacted on DFS in another stage III cohort receiving adjuvant chemotherapy.[90] In contrast, PD-L1 expression on immune infiltrating mononuclear cells was associated with longer DFS. Dunne *et al*[91] reported that in stage III CRC (n=201), $PD\text{-}L1_{low}$ tumours conferred a significant DFS benefit from adjuvant chemotherapy versus observation (adjusted HR 0.44, p=0.0062), and the use of adjuvant chemotherapy was able to overcome the negative prognostic impact of

low PD-L1. However, in contrast, PD-L1$_{high}$ expression resulted in inferior DFS post adjuvant chemotherapy versus observation (unadjusted HR 4.95, 95% CI 1.10 to 22.35, p=0.02), although the significance was lost on multivariate analysis. This is one of the first series to suggest a possible detrimental effect of chemotherapy in tumours which overexpress PD-L1.

Immune markers associated with microsatellite instability

Tumours harbouring microsatellite instability (MSI) have defects in the DNA mismatch repair system (dMMR), and thus display a hypermutable phenotype. The differential improved survival in patients with early stage dMMR tumours has been extensively reported and is partly attributable to increased immune stimulation in these tumours due to the increased neoantigen load. MSI tumours have a dense infiltration of CD8$^+$ cells,[92] a T$_h$1 cytokine response, and also overexpress many inhibitory immune checkpoints (including PD-1, PD-L1, CTLA-4, LAG-3 and IDO).[93] However, MSI tumours are more chemoresistant to 5-FU than microsatellite stable (MSS) lines in preclinical models.[94] The relative survival benefit from FOLFOX compared with 5-FU is much greater in stage II-III dMMR patients compared with pMMR,[95] suggesting possible resistance to 5-FU alone. However, in a recent report from the FoXTROT trial, dMMR colon cancers showed significantly reduced pathological response rates to neoadjuvant oxaliplatin doublet chemotherapy than pMMR,[96] and clinical progression through this chemotherapy regime was more common in dMMR than pMMR rectal cancers (29% vs 0%, p=0.0001).[97] In contrast, in the metastatic setting, MSI status did not affect response rates to palliative FOLFOX chemotherapy.[98] Regarding irinotecan therapy, in both cell lines and tumour xenografts, dMMR tumours are more sensitive to irinotecan than MMR proficient (pMMR) lines.[99 100] In a small retrospective cohort, response rates to palliative 5-FU plus irinotecan were much higher in MSI than MSS disease (57% vs 10%, p=0.009),[101] and DFS was longer in MSI tumours receiving an irinotecan containing regime in a separate cohort.[102] In a large adjuvant phase III trial, only patients with dMMR tumours received a DFS benefit from adding irinotecan to 5-FU,[103] but this MSI/treatment interaction was not confirmed in another similar trial.[104] The relevance of immunological variation on chemotherapeutic response in the context of genetic alterations is largely unknown.

Immunogenic Cell Death markers

ICD is the cornerstone of the immunomodulatory action of oxaliplatin and associated markers are potential predictive biomarkers (figure 3). DC activation is a key step in ICD. However, identification of DCs, which show functional diversity and heterogeneous activation states, can be challenging and markers are variably reported between studies and may account for conflicting results reporting both good[105–109] and bad[110] prognostic association. In vivo studies have demonstrated that blockade of surface calreticulin exposure[111] and HMGB1-dependent TLR-4 signalling,[112] both key steps in ICD, severely compromised the cytotoxicity of oxaliplatin chemotherapy.

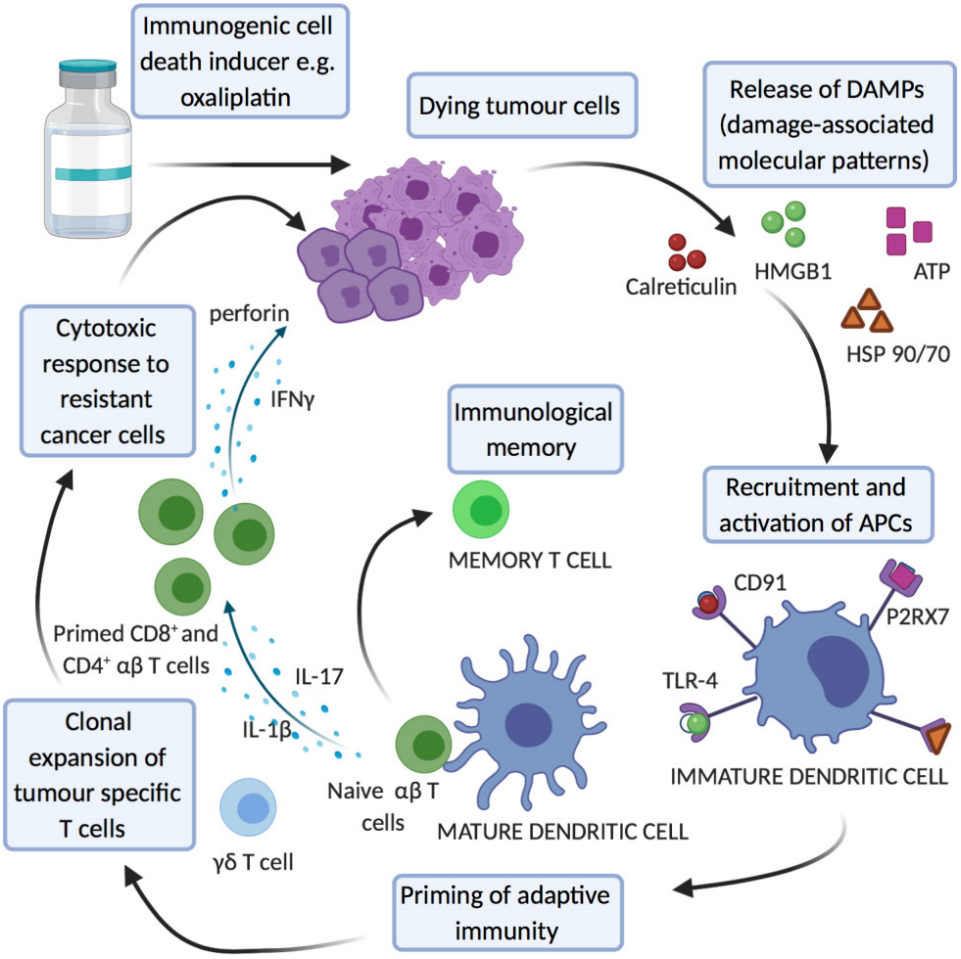

Figure 3 Key cells and pathways in immunogenic cell death as potential predictive biomarkers. IFNγ, interferon; IL, interleukin.

Stromal calreticulin expression is associated with infiltration of CD45RO$^+$ cells and improved OS in univariate analysis in patients receiving adjuvant 5-FU.[113]

Stromal markers

The tumour stroma plays a direct and indirect role in modulating response to immunomodulatory chemotherapy. De novo drug resistance may occur from environment-mediated phenomena, where cancer cells are protected from treatment-induced apoptosis by 'barriers', including either soluble secreted factors or cell-adhesion-mediated mechanisms.[114] The tumour:stroma percentage is a validated prognostic marker, with increased stromal percentage associated with poorer prognosis, including in chemotherapy-treated patients.[115] Cancer-associated fibroblasts (CAFs) are a heterogeneous group of fibroblast-like cells that release certain cytokines, growth factors and proinflammatory factors. In vitro cell line studies suggest CAFs trigger a JAK/STAT pathway signalling cascade that leads to reduced response rates to oxaliplatin and 5-FU,[116] and stromal CAF-derived conditioned medium primed the growth of cancer stem cells after treatment with 5-FU and oxaliplatin, thus increasing their inherent chemoresistance.[117] High CAF infiltration is associated with worse DFS in adjuvant-treated patients[118] and associated-induced expression of their surrogate markers smooth muscle actin and survivin have been related to worse survival in 5-FU[119] and oxaliplatin-treated advanced patients.[120]

Genomic markers and transcriptomic profiles

Recent advances in high-throughput gene testing technology have led to the development of some molecular signatures for chemotherapy prediction. Increased expression of infiltrating immune cells, as identified by CIBERSOrT, showed a trend to improved overall survival in patients receiving chemotherapy.[121] Multiple classifications of CRC, based on molecular transcriptomic data, have been proposed in recent years, and unified into the Consensus Molecular Subtypes (CMS). This incorporates gene expression profiles from the tumour, stroma and immune cells to differentiate four groups (CMS1-4) and are highly correlated with immune cell infiltration patterns.[122] The CMS1 subgroup (MSI-like) is enriched for genes coding for CD8$^+$ and CD68$^+$ cells, T-cell attracting chemokines, TLSs and Th1 cytokines. The CMS4 subgroup (mesenchymal) is enriched for expression of genes encoding CD8$^+$ cells, MDSCs, T_{regs}, T_h17^+ cells, angiogenic factors and immunosuppressive molecules (eg, TGFβ1). Both CMS2 (canonical) and CMS3 (metabolic) subgroups exhibit low-immune and low-inflammatory signatures. In a retrospective taxonomy study, only CMS2 and 3 subgroups derived a benefit from adjuvant chemotherapy (unspecified) in stage III disease, with CMS4 showing a trend to benefit.[123] Song *et al*[124] used an alternative transcriptomic classifier (CRCA) to examine patients in the NSABP-07 trial (adjuvant FOLFOX vs 5-FU), and reported only patients with an 'enterocyte' subtype (with immune features similar to the 'cold' CMS2) derived a benefit from the addition of oxaliplatin, with a significant interaction test. The same group repeated the analysis using patients enrolled on the MOSAIQ trial (adjuvant CAPOX vs capecitabine) but did not find any association,[125] which may be due to different fluoropyrimidine use or oxaliplatin schedule, which have shown different interactions in other immune biomarker studies.[36] CMS1 patients have worse OS with FOLFIRI-based regimes compared with the other CMS subtypes in the FIRE-3 trial[126]; however, they also show improved OS with the addition of bevacizumab in the metastatic setting.[127] Published studies suggest a trend to 5FU/oxaliplatin resistance in CMS4 (or similar classifier) patients, both in the adjuvant[127 128] and metastatic setting,[129] where first-line irinotecan regimes showed better response rates and survival.[130 131]

IMMUNE INFILTRATE IN RESECTED METASTASES

Several reports have assessed the prognostic and predictive impact of the TIME from resected metastases, predominantly liver metastases,[132] which appears to correlate with the primary tumour. However, many of the studies include patients receiving neoadjuvant therapy, which can alter the immune infiltrate substantially. Metastatic disease has a different immunological milieu which is defined by tumour immune evasion. Liver metastases with pretreatment high Immunoscore (and high CD3$^+$, CD8$^+$, and CD20$^+$ cells[133]) are associated with increased response to chemotherapy (p=0.009) and improved DFS and OS.[55] The type of postoperative chemotherapy/adjunct did not impact survival. However, a high IS is not tantamount to excellent prognosis in this setting (as opposed to with early stage disease) as most patients relapsed after surgery. The authors showed the density of CD8$^+$:CD20$^+_{CT+IM}$ to be an additional strong prognostic discriminator. A high 'density score' (based on a cumulative density of CD3$^+$, CD8$^+$ and granzyme B in liver metastases) was also reported by Halama *et al*[134] to have significant prognostic ability in stage IV patients receiving any regime of chemotherapy (HR OS 0.06, p<0.01).

CONCLUSIONS

Here, we have reviewed CRC studies focusing on the TIME and found that the prognostic ability of these markers in CRC is mediated in the context of chemotherapy, and true predictive studies are under-reported. While prognostic biomarkers have been used as a surrogate for predictive markers, with an assumption that patients with 'poor' prognosis will gain a greater absolute benefit from chemotherapy, this may be untrue, especially if the biomarker is also a marker of therapy resistance. Nevertheless, current reports indicate that the relative benefit of 5-FU chemotherapy may be enhanced in the context of some pre-existing CD8$^+$/CD3$^+$ infiltration in core tumour, but may be unnecessary or importantly even detrimental in the milieu of a highly inflamed TIME. CRCs with high immunosuppressive pathways may also be more resistant to oxaliplatin doublets. Furthermore, chemotherapy may improve prognosis in cancers driven by specific immune cell populations, such as TAMs and TANs. The emerging move

Take home messages

- The tumour immune microenvironment has an important role to play in mediating cytotoxic chemotherapy response and primary resistance.
- Many chemotherapy agents used in colorectal cancer have local and systemic immunomodulatory effects.
- Baseline tumour immune cells, including T cell subsets, tumour-associated neutrophils and macrophages, may represent potential predictive biomarker predicting response and resistance to cytotoxic chemotherapy.
- Prospective trials using standardised validated markers, such as the Immunoscore, are required to rationalise the use of adjuvant chemotherapy and target different palliative chemotherapy regimes and adjuncts to patients more likely to respond.

to standardise assessment of TILs/IHC-based markers in CRC reporting has the potential for more robust prospective trials. Such trials are needed to develop better clinical biomarkers for therapy benefit and cytotoxic effects of chemotherapy. Patients likely having adverse effects may require de-escalated or even no therapy, and some may require alternative or combination agents, which have shown early promise.[135] Significant research and development is in progress with regard to such adjuncts, which include various combination approaches with synergistic benefits[136] and novel immunotherapies, to improve precision medicine in the future.

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