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Gut Microbes Drive T Cell Infiltration Into Colorectal Cancers And Influence Prognosis

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Immune cells have an important effect of the prognosis of human colorectal cancer (CRC). Infiltration of CRC tissue by cytotoxic CD8 T cells, T helper 1 (Th1) cells, CXCR5+ follicular T helper cells (Tfh) and Foxp3+ T regulatory cells (Tregs) is associated with improved patient survival and a favorable clinical outcome (Nat Rev Cancer 2012;12;298-306). In this study, Cremonesi et al aimed to identify the nature of chemotactic factors promoting CRC infiltration by these T cell populations and the stimuli responsible for inducing their expression in the CRC microenvironment.

The investigators analyzed gene expression of a variety of T cell markers in 62 CRC and corresponding tumor free colonic tissues, to study the composition of T cell populations within these tissues. They found that CRC tissues were only minimally infiltrated by Th2 cells, as indicated by undetectable levels of IL4 and minimal expression of IL5 and IL13. However, Th17 and Treg cell markers were highly expressed in CRC cells in comparison to control tissues, whilst CD4 T-helper cell and TfH cell markers were slightly reduced. Unsupervised hierarchical analysis of the data resulted in CRC samples being clustered into 3 main groups based on expression of T cell markers - cluster high (overexpression of most T cell markers), cluster het (heterogeneous expression of T cell markers) and cluster low (downregulation of all T cell markers). In the cluster high group, a specific panel of chemokine genes was found to be significantly upregulated. Furthermore, specific highly significant correlations were noted between T cell markers and chemokine genes in CRC samples, indicating particular chemokines could drive T cell recruitment into CRC tissues.

The investigators also studied chemokine receptor profiles of T cells in CRC samples in comparison with control tissues or peripheral blood mononuclear cells (PBMCs). In the group with overexpression of T cell markers, chemokine genes were found to be significantly upregulated, indicating certain chemokines could drive T cell recruitment into CRC tissues. Specifically, they found that CCR5, CXCR3 and CXCR4 were highly expressed on CD8, CD4 and FoxP3 T cells in both cancer and control tissues. However, significantly higher proportion of CCR5+ and CXCR4+ T cells were detected in cancer tissues in comparison with PBMCs.

Further interrogation of the data, enabled the investigators to identify particular chemokine signatures for each CRC T cell subset i.e. mainly CCR5-binding ligands (CCL3, CCL4, CCL5, CCL8), CXCR3-binding ligands (CXCL9 and CXCL10) and a CXCR4-binding ligand (CXCL12) for cytotoxic T lymphocytes; CCR4 ligands (CCL17, CCL22), CCR5-binding ligands (CCL3, CCL5), CXRC3-binding ligands (CXCL9, CXCL10) and CXCL12 for Th1 cells; CCR4 ligands (CCL17, CCL22), CXCL12, CCL5 and CXCL9 for Treg cells; CXCL13 for Tfh cells; and CCL20 and CCL17 for Th17 cells. The authors concluded from these data that the overexpression of the chemokines they identified associates with the infiltration of beneficial T cell populations and improved prognosis.

Gene expression analysis of chemokines in CRC samples identified that tumor cells were the main source of T-cell recruiting chemokines including CCL3, CCL4, CCL5 and CXCL10 (which bind to receptors on cytotoxic T cells and Th1 cells) but not CCL7, CCL8, CCL11, CCL13, CCL27 and the Th17 recruiting chemokines CCL17 and CCL22. Crucially, authors also noticed that *in vitro* cultured CRC cell lines expressed far fewer chemokine genes in comparison to primary tumor cells, suggesting that chemokine expression may require other environmental factors present in the *in vivo* setting.

The authors hypothesized that this environmental signal may potentially arise from the gut microbiota, given that it has been previously demonstrated that commensal bacteria and/or their products can translocate across CRC epithelial tissues and thus come into direct contact with CRC cells (Science 2012; 338:120-3). The authors exposed CRC cells from cell lines and CRC organoids to TLR agonists. This resulted in upregulation of constitutively expressed chemokine genes including CCL20, CXCL9 and CXCL10 and de novo expression of additional chemokine genes, including CCL3, CCL4, CCL5 and CCL22. Several chemokine genes were also induced on exposure of CRC cells to particular bacterial species known to be enriched in CRC tissues; *Fusobacterium nucleatum*, *Bacteroides fragilis* and *Escherichia coli*.

To determine whether such effects were also present in the *in vivo* setting, chemokine expression was measured in CRC tumor xenografts generated by injecting human CRC cells into NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ). These mice, also called NOD scid gamma mice, are a type of immunodeficient mouse that lack mature T cells, B cells and natural killer (NK) cells. The injection of tumor cells was performed either intra-peritoneally or intra-cecally. Interestingly, they noticed that intracecal tumors contained significantly higher levels of a range of chemokines compared to intraperitoneal controls - CCL5 (70-fold increase), CCL20 (19-fold increase), CXCL10 (12-fold increase) and CXCL11 (3-fold increase). Furthermore, levels of these chemokines significantly reduced following treatment with antibiotics and correlated with bacterial load. By adoptively transferring CSFE (carboxyfluorescein diacetate succinimidyl ester)-labelled tumor infiltrating CD4+ and CD8+ T lymphocytes, into these tumor-xenograft bearing mice, the authors observed that these tumor-infiltrative lymphocytes homed to intracaecal xenografts to a much greater extent than intraperitoneal xenografts. Hence, taken together, these experiments suggest that the commensal gut microbiota is a major factor in inducing chemokine expression in CRC cells which subsequently determines T cell infiltration into tumor tissues.

To characterize microbiota-chemokine-T cell relationship in clinical CRC samples, the investigators sought to identify significant biological correlations. Although higher quantities of gut bacteria were observed in highly T cell infiltrated CRC samples in

comparison to poorly infiltrated tumors, total bacterial load (as assessed by 16S analysis) only weakly correlated with individual chemokine expression in contrast to the observations in tumor xenografts. Instead, analysis of individual bacteria genera in highly infiltrated and poorly infiltrated CRC tumor samples, revealed significant correlations between specific bacteria and T cell markers and chemokine gene expression. *Lachnospiraceae* and *Ruminococcaceae*, both part of the *Firmicutes* phylum, correlated strongly with CCR5 and CXCR3 binding chemokines. *Bacteroides* and *Proteobacteria*, in particular *Methylobacteriaceae*, also significantly correlated with the expression of all prognostically favorable T cell markers and corresponding chemokines. The authors concluded that the expression of chemokines by human CRC cells is associated with the abundance of specific bacteria within the tumors.

Comment

The importance of immune cells in cancer pathogenesis has become increasingly appreciated. CRC, in particular, has become a paradigmatic tumor for understanding the complex role of immune cells in cancer.

It is now recognized that the developing CRC resides within a rich microenvironment comprised of a complex array of immune cell populations including T lymphocytes, B lymphocytes, macrophages, dendritic cells, NK cells, and mast cells, which are found either within the tumor core (TC), the invasive margin (IM) or in tertiary lymphoid structures. This is collectively referred to as the “immune contexture” (Nat Rev Cancer 2012;12:298–306).

The clinical significance of these immune cells in human CRC was first noted in the 2000s, when it was demonstrated that the type, density and location of immune cells within tumor samples are a better predictor of prognosis than the established histopathological Dukes’ staging system (Science 2006;313:1960–1964). A strong immune cell reaction in both the TC and IM, comprising of CD8 cytotoxic T cells and CD45RO memory T cells, correlated with favorable prognosis regardless of cancer stage, whilst a poor immune reaction in both regions correlated with poor prognosis even in those with minimally invasive (Stage 1) tumors (Science 2006;313:1960–1964, J Clin Oncol 2009;27:5944–5951). This led to the development of a novel, immune scoring system for CRC, based on memory and cytotoxic T cell markers, which was found to be superior to the Dukes’ staging in predicting recurrence as well as survival (J Clin Oncol 2009;27:5944–5951, J Clin Oncol 2011;29:610–618). More recent studies have revealed that other components of the immune contexture including T regulatory cells, B cells, NK cells, macrophages, as well as endothelial cells and fibroblasts, also correlate with CRC prognosis.

In parallel to these developments, a major transcriptomic analysis identified that the vast majority of CRCs fall into four distinct consensus molecular subtypes: CMS1

(tumors exhibiting high microsatellite instability (MSI) due to mutations in genes encoding DNA mismatch-repair proteins), CMS2 (tumors with high chromosomal instability (CIN) and activation of the Wnt and MYC pathways), CMS3 (tumors with KRAS mutations and disruption of metabolic pathways) and CMS4 (tumors with a mesenchymal phenotype and frequent CpG island methylator phenotype (CIMP)) (Nat Med 2015;21:1350-6). This has prognostic implications, as CMS4 is associated with the worst disease-free survival and overall survival, and both CMS1 and CMS4 with poor survival after recurrence (Ann Onc 2018; 29(Suppl 8):viii18).

Using a transcriptome-based computational method, these molecular and immune based classifications of CRC were recently integrated (Clin Cancer Res 2016;22:4057-4066). It was found that unlike CMS2 and CMS3 CRC subtypes, CMS1 and CMS4 subtypes displayed a strong immune and inflammatory contexture. CMS1 contained higher abundances of cytotoxic T cells, whilst CMS4 had higher expression of B cells, myeloid cells, fibroblasts and endothelial cells. In addition, CMS1 subtype cancers expressed high levels of T cell attracting chemokines (including CXCL9, CXCL10, CXCL16), Th1 cytokines (IFNg and IL15), immune checkpoints (e.g. CTLA4, PD1) and MHC class 1. CMS4 subtype, however, expressed high levels of myeloid chemokines (e.g. CCL2), complement components, angiogenic factors (VEGFB, VEGFC, and PDGFC), and immunosuppressive molecules (TGFB1, TGFB3, LGALS1, and CXCL12). CMS2 and CMS3 subtypes were relatively devoid of immune cell populations.

These findings demonstrate that CRC is a heterogeneous disease, comprising of distinct molecular and immune signatures, requiring different therapeutic strategies. For instance, CMS1 subtype cancers are most likely to respond to checkpoint inhibitor therapy such as pembrolizumab (an anti-PD1 antibody), whilst CMS4 subtype cancers may require a combination of anti-angiogenic, anti-TGF-beta and checkpoint inhibitor therapies (Curr Opin Immunol 2016;39:7-13).

In this study, Cremonesi et al, aimed to further characterize the chemokine signals that drive favorable T cell populations into the CRC microenvironment and identify the cellular sources of these chemokines, and the potential underlying stimuli responsible for inducing their expression. They found chemokine genes expressed by CRC cells in response to gut microbiota-derived stimuli are mainly responsible for the infiltration of favorable immune cell populations into the CRC microenvironment. This is an exciting discovery, as it could potentially lead to the addition of an entirely new strategy in our armamentarium against CRC – one which involves modulation of the CRC immune contexture by targeting the gut microbiota.

In the past decade, evidence of the involvement of bacterial populations during tumor progression was identified using metagenomic tools (Nat Genet 2002; 30: 141-142, PLoS ONE 2011;6: e 19838.doi:10.1371). This approach demonstrated that a number of bacteria are involved in the pathogenesis of CRC including

Fusobacterium nucleatum, *Bacteroides fragilis* and *Escherichia coli*. *F. nucleatum* is an anaerobe which is highly invasive (Infect Immun 2000; 68:3140-3146, Gut 2011; 60:34-40), with pro-inflammatory characteristics (Infect Immun 2000;68:2907-2915, Cytokine 2009; 46:201-210) and known to be present in CRC specimens. *F. nucleatum* has been proposed to promote the pathogenesis of CRC by a variety of mechanisms including creating a pro-inflammatory environment with increased levels of TNF and NF-κB, the activation of β-catenin signaling and reducing T cell activation (World J Gastrointest Oncol 2018;10:71-81). *B. fragilis* has been found to be an independent predictor of three year survival from CRC (Oncotarget 2016;7:46158-72). It secretes a toxin which is a metalloprotease that cleaves E-cadherin, thereby activating the Wnt pathway (JCI 2014;124:4166-72). *E. coli* have been found to frequently colonize CRC and have been reported to have mutagenic effects (World J Gastroenterology 2014;20:6560-6572, Cell, Host and Microbe 2014;15:317-28). Some gut bacteria metabolize bile salts into procarcinogenic secondary bile acids whereas bacteria fermentation of complex carbohydrates into short fatty acids is anticarcinogenic (Nat Rev Microbiol 2014;12:661). Biofilms, which are polybacterial communities encased in a polymeric matrix, are found in only 15% of healthy patients but in 100% of right-sided colorectal cancers. The colonic mucosa under biofilms have decreased E-cadherin, and increased IL-6, Ki-67 and phospho-stat3, suggesting biofilms play a procarcinogenic role (PNAS 2014;111:18321).

In the current study, a range of bacteria including in particular *Lachnospiraceae* and *Ruminococcaceae* were associated with the expression of T cell recruiting chemokines. *Fusobacteria* which have previously been associated with poor prognosis were found to evoke T cell recruiting chemokines in this study, suggesting that they may in some circumstances be associated with a good prognosis. Clearly more detailed studies of individual bacteria grouping are required to define the specific circumstances in which they encourage recruitment of favorable T cell populations. The potential effect of gut bacteria on the other cell populations comprising the CRC immune contexture also need to be investigated.

In conclusion, the intestinal microbiota is being found to play an increasingly important role in determining the prognosis of a range of cancers including CRC. The gut microbiota is already known to be a key determinant of checkpoint inhibitor therapy (Gastroenterology 2018;154:2068-70). These new observations that a range of gut bacteria can promote T cell infiltration into CRC which could confer a good prognosis, opens another exciting avenue for future treatments.