ORIGINAL ARTICLE

Gut microbiota modulate T cell trafficking into human colorectal cancer


ABSTRACT
Objective  Tumour-infiltrating lymphocytes (TILs) favour survival in human colorectal cancer (CRC). Chemotactic factors underlying their recruitment remain undefined. We investigated chemokines attracting T cells into human CRCs, their cellular sources and microenvironmental triggers.

Design  Expression of genes encoding immune cell markers, chemokines and bacterial 16S ribosomal RNA (16SrRNA) was assessed by quantitative reverse transcription-PCR in fresh CRC samples and corresponding tumour-free tissues. Chemokine receptor expression on TILs was evaluated by flow cytometry on cell suspensions from digested tissues. Chemokine production by CRC cells was evaluated in vitro and in vivo, on generation of intraperitoneal or intracecal tumour xenografts in immune-deficient mice. T cell trafficking was assessed on adoptive transfer of human TILs into tumour-bearing mice. Gut flora composition was analysed by 16SrRNA sequencing.

Results  CRC infiltration by distinct T cell subsets was associated with defined chemokine gene signatures, including CCL5, CXCL9 and CXCL10 for cytotoxic T lymphocytes and T-helper (Th)1 cells; CCL17, CCL22 and CXCL12 for Th1 and regulatory T cells; CXCL13 for follicular Th cells; and CCL20 and CCL17 for interleukin (IL)-17-producing Th cells. These chemokines were expressed by tumour cells on exposure to gut bacteria in vitro and in vivo. Their expression was significantly higher in intracecal than in intraperitoneal xenografts and was dramatically reduced by antibiotic treatment of tumour-bearing mice. In clinical samples, abundance of defined bacteria correlated with high chemokine expression, enhanced T cell infiltration and improved survival.

Conclusions  Gut microbiota stimulate chemokine production by CRC cells, thus favouring recruitment of beneficial T cells into tumour tissues.

INTRODUCTION
Infiltration by immune cells heavily impacts on clinical outcome in human colorectal cancer (CRC). High densities of cytotoxic CD8+ T cells, IFN-g expressing T-helper 1 cells (Th1), CXCR3+ follicular T-helper cells (Tfh) and, surprisingly, Foxp3+ regulatory T cells (Tregs) are associated with prolonged patients’ survival. Consistent with positive role of T-helper cells, expression of HLA class II antigens was also reportedly associated with favourable clinical course. In contrast, prognostic significance of interleukin (IL)-17-producing T-helper cells (Th17) is controversial: their presence within CRC tissues was reported to correlate either with poor or improved prognosis. In addition, innate immune cells were also shown to predict clinical outcome. Tumour infiltration by CD16+ myeloperoxidase + myeloid cells, mostly consisting of activated neutrophils, is an independent predictor of favourable prognosis. Notably, infiltration by neutrophils or natural killer (NK) cells was also found to increase favourable prognostic significance of cytotoxic CD8+ T cells.

Chemotactic factors driving these cell populations into CRC tissues remain largely undefined. Expression of defined chemokines, including CXCL14, CXCL10 and CXCL16 and CX3CL1 was reported to correlate with high densities of tumour-infiltrating lymphocytes (TILs) and to predict favourable clinical outcome. However, putative responding cell subsets within immune infiltrating cells have not been carefully characterised. Furthermore, chemokine sources and microenvironmental stimuli leading to chemokine production within CRC tissues were not addressed so far.

On intestinal tumourigenesis, gut commensal bacteria translocate across altered epithelia and stimulate immune cells infiltrating lamina propria to release proinflammatory cytokines. However, whether gut flora-derived microbial stimuli also promote production of chemotactic factors was not evaluated yet. Here we investigated chemokine–chemokine receptor network underlying T cell infiltration into CRCs.

MATERIALS AND METHODS
Clinical specimen collection and processing
Clinical specimens were collected from consenting patients undergoing surgical treatment at Basel University Hospital, St. Claraspital in Basel and Ospedale Civico di Lugano, Switzerland (Swiss Clinical specimens were collected from consenting patients undergoing surgical treatment at Basel University Hospital, St. Claraspital in Basel and Ospedale Civico di Lugano, Switzerland (Swiss
In CRC, gut microbiota translocate across the neoplastic epithelium, but whether this phenomenon may impact on the production of chemokines recruiting immune cells has not been assessed so far.

What are the new findings?
- CRC infiltration by favourable immune cell populations is associated with expression of defined chemokine genes, that is, mainly CCL5, CCL9 and CXCL10 for CTLs; Th1, CCL17, CCL22 and CXCL12 for Th1 and Tregs; CXCL13 for Th17; and CCL20 and CXCL17 for Th17.
- Most chemokine genes identified may be expressed by CRC cells in response to stimulation with gut microbiota-derived stimuli.
- In tumour xenografts, bacterial loads correlate with chemokine gene expression levels and extent of T cell infiltration.
- In clinical CRC samples, abundance of defined bacteria families correlates with expression of specific chemokine genes, extent of T cell infiltration and improved prognosis.

How might it impact on clinical practice in the foreseeable future?
- Our findings reveal the ability of gut microbiota to induce in CRC cells the production of chemotactic factors recruiting T cell populations of favourable prognostic significance. This knowledge might pave the way towards the development of innovative treatments aimed at modifying the gut flora to promote CRC infiltration by beneficial immune cells.

In vivo experiments
In vivo experiments were approved by Basel and Zürich Cantonal Veterinary offices.

Tumour xenografts were generated in 8-week-old NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) on intraperitoneum (i.p.) and intracutaneous (i.c.) injection of LS180 cells (see online supplementary methods for full details). From day 10, a randomised group of i.c.-injected mice was treated with Ampicillin sodium salt (1 g/L, Amresco) and Vancomycin Hydrochloride (0.2 g/L, Bio Basic Canada), administered in drinking water. Tumours were harvested on day 31 and assessed for chemokine expression.

To evaluate T cell migration, at day 29, tumour-bearing mice were adoptively transferred with CFSE-labelled CD4+ and CD8+ T cells, previously isolated from primary CRC specimens and expanded in vitro (5 × 10^6 T cells/subset/mouse). Two days later, frequencies of transferred TILs were evaluated in cell suspensions of digested tumours by flow cytometry.

Details of mice, tumour cell injection protocols and TIL isolation are provided in online supplementary methods.

16SrRNA gene sequencing and analysis
Bacterial flora was analysed based on 16SrRNA sequencing. Total RNA was purified from 27 CRC and tumour-free tissue samples (German cohort) and from tumour xenografts (5 i.p. and 5 i.c.) and reverse transcribed. V4 region of the bacterial 16S rRNA gene was amplified by PCR. Amplicons were sequenced on the Illumina MiSeq platform. Sequences were analysed by USEARCH software (V8.1.1861). Differential operational taxonomic unit (OTU) analysis of normalised abundance counts was performed by DESeq2 software (V1.12.4). Libraries, sequencing and data analysis were performed by Microsynth AG (Balglach, Switzerland) as detailed in online supplementary methods.

Statistical analysis
Methods used to evaluate statistical significance of results are detailed in figure legends.

RESULTS
CRC infiltration by T cells is associated with overexpression of defined chemokines
We analysed expression of genes encoding immune cell markers, including CD3 for total T cells, CD4 for T-helper cells, CD8 for cytotoxic T lymphocytes (CTLs), T-bet and IRF-1 for Th1, IL-4, IL-5 and IL-13 for Th2, IL-17 for Th17, CXCR5 for Th and Foxp3 for Tregs, in 62 CRC and corresponding tumour-free colonic tissues (figure 1A). All T cell markers were expressed, except for IL-4, which was undetectable in all samples (data not shown), and IL-5 and IL-13, which were expressed only in a few samples, thus suggesting that CRC infiltration by Th2 cells in CRC is marginal. IL-17 and Foxp3 gene expression was significantly increased in tumours as compared with control tissues (p<0.0001), whereas expression of CD4 and CXCR5 genes was slightly reduced (p<0.05). Expression of all T cell subset markers, except IL-17, significantly correlated with that of CD3 and CD4. Furthermore, CD8, IRF-1 and CXCR5, were significantly associated with expression of T-bet and Foxp3 (see online supplementary table S4).

Normalisation of immune cell marker expression in tumour samples versus corresponding tumour-free colonic tissues
revealed a consistent subgroup of samples displaying upregulation of one or more T cell markers in (online supplementary figure S1). On unsupervised hierarchical analysis, samples clustered in three main groups: one characterised by overexpression of most T cell markers (cluster ‘high’), a second displaying heterogeneous expression (cluster ‘het’) and a third characterised by downregulation of all T cell marker genes (cluster ‘low’, figure 1B). Analysis of different clusters revealed a panel of chemokine genes significantly upregulated in highly but not in poorly infiltrated tumours (figure 1C). Furthermore, significant correlations between expression of genes encoding individual chemokines and specific immune cell markers were observed (table 1), suggesting that these chemokines might be involved in T cell recruitment into tumour tissues.

**TILs express receptors binding overexpressed chemokines**

We then analysed chemokine receptor profiles of CD8+, CD4+ (Foxp3−) effector T cells and CD4+ Foxp3+ Tregs, in freshly excised CRC specimens as compared with control tissues or PBMCs from patients or HDs (figure 2 and online supplementary figure S2). All three subsets in tumours and control tissues were largely positive for CCR5, CXCR3 and CXCR4, whereas lower cell fractions expressed CCR3, CCR6 and CCR10. Remarkably, significantly higher percentages of CCR5+ and CXCR4+ T cells were detected in tissues as compared with PBMCs (online supplementary figure S2). Considerable fractions of CD4+ T cells and Tregs also expressed CCR4. Furthermore, within CD4+ T cells, a small subset of CXCR5+ cells was detectable, confirming tumour infiltration by Tfh. This cell subset, however, did not show significant expression of additional chemokine receptors. Chemokine receptor profiles on Th1 and Th17 cells could not be properly assessed, since the stimulation, required for their identification, based on cytokine production capacity, causes down-modulation of most chemokine receptors (data not shown). We could only detect CCR6 on a large majority of Th17 cells (up to 88%), as previously reported, and CCR4 on a smaller fraction (up to 38%). Expression of CCR1 or CCR11 was undetectable in all T cell subsets (data not shown). No major differences in chemokine receptor profiles were observed between PBMCs of patients and healthy donors (online supplementary figure S2).
Defining chemokine signatures underlie CRC infiltration by individual T cell subsets

Chemokines significantly correlating with any T cell marker were assigned scores, calculated according to the formula (Spearman r value × percentage of corresponding chemokine receptor-positive cells) (online supplementary table S5). Thus, we identified putative chemokine signatures for each T cell subset (figure 3). In particular, for CTLs, we identified CCL3, CCL4, CCL5 and CCL8 (binding to CCR5), CXCL9 and CXCL10 (binding to CXCR3) and CXCL12 (binding to CXCR4). Th1-associated chemokine signature mainly included the CCR4 ligands CCL17 and CCL22, together with CCL3, CCL5, CXCL9, CXCL11 and CXCL12. CXCL12, CCL17 and CCL22, and to a lower extent CCL5 and CXCL9, also underlay tumour infiltration by Tregs. Infiltration by Tfh was exclusively associated with expression of CXCL13, whereas Th17-associated signature included CCL20 and CCL17 and to a lower extent CCL25, CCL27 and CCL28.

Identified signatures were validated in a larger cohort, including 311 CRC samples from The Cancer Genome Atlas (cancergenome.nih.gov) (online supplementary figure S3). Unsupervised hierarchical analysis identified different clusters. One (indicated in red) included samples overexpressing CD8, IRF-1/T-bet, FoxP3 and CXCR5, and all corresponding chemokines; a second one (blue) grouped samples showing low expression of all markers and related chemokines. Consistent with observations in our cohort, expression of IL-17-related and Th17-related chemokines clustered separately from other T cell markers and chemokines (cluster orange). Two remaining clusters (indicated in green) showed heterogeneous expression of all markers and chemokines (online supplementary figure S3A). Importantly, samples overexpressing all T cell markers (except IL-17) and their chemokine signatures showed improved survival as compared with those from other clusters (online supplementary figure S3B). Thus, overexpression of the identified chemokines associates with infiltration by beneficial T cell populations and improved prognosis.

T cell-recruiting chemokines are expressed by tumour cells

To identify cellular sources of T cell-recruiting chemokines, we evaluated their potential production by tumour cells, the major component of CRC microenvironment. Gene expression analysis of chemokines in primary CRC cells, isolated from freshly excised CRC specimens based on EpCAM expression, revealed that tumour cells express most relevant chemokine
For some chemokines, including CCL3, CCL4 and CCL20, gene expression levels were significantly higher in purified CRC cells than in total tumour tissues, suggesting that tumour cells are likely major contributors of these chemokines within CRC microenvironment. In contrast, purified tumour cells did not express CCL7, CCL8, CCL11, CCL13, CCL17 and CCL27 genes (data not shown).

Remarkably, in vitro cultured CRC cell lines expressed fewer chemokine genes and to lower extents as compared with primary tumour cells, suggesting that chemokine expression in tumour cells may be stimulated by microenvironmental factors absent in vitro.

Chemokine expression is induced in CRC cells by gut flora-derived stimuli

Translocation of commensal bacteria or derived stimuli across altered epithelia was described in CRC. We hypothesised that chemokine production in tumour cells may be triggered by gut flora-derived microbial stimuli. Indeed, CRC cells from primary tumours and established cell lines express TLRs potentially sensing them (online supplementary figure S4). Stimulation of CRC cells from cell lines (figure 5A and online supplementary figure S5) and CRC organoids (online supplementary figure S6) with TLR agonists induced 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. In contrast, no CXCL12 expression was observed.

Figure 2: CRC-infiltrating T cells express receptors binding overexpressed chemokines. Single cell suspensions obtained from freshly excised clinical CRC specimens were surface stained with fluorochrome-labelled antibodies specific for CD8, or CD4, in combination with the indicated chemokine receptors. Intracellular staining for Foxp3 was then performed. For detection of IL-17-producing cells, cell suspensions were stimulated with PMA/Ionomycin, and after 4 hours they were fixed, permeabilised and intracellularly stained with antibodies specific for IL-17 and the indicated chemokine receptors. Percentages of positive cells within gated CD8+, CD4+ Foxp3−, CD4+ Foxp3+, CD4+ CXCR5+ and CD4+ IL-17+ cells are shown. Means and SD are indicated by bars. CRC, colorectal cancer; IL, interleukin; n.d., not detected; n.t., not tested.

Figure 3: Defined chemokine signatures are associated with CRC infiltration by individual immune cell subsets. Chemokines significantly associated with expression of immune cells markers were assigned a score calculated as detailed in online supplementary table S5. A heat map of score values is shown. CRC, colorectal cancer; IL, interleukin.
Gut microbiota in tumours correlate with extent of T cell infiltration

To assess actual impact of bacteria-induced chemokines on T cell recruitment into tumour tissues, we adoptively transferred CRC-derived CD4+ and CD8+ TILs into tumour-bearing NSG mice and evaluated their homing to i.p. or i.c. tumours. Strikingly, TILs migrated into i.c. xenografts to significantly higher extents than in i.p. tumours (figure 6B, C), indicating that presence of gut microbiota enhances T cell recruitment into xenografts.

Consistently, in human CRC samples displaying high densities of CD3+ cells, gut bacteria, as detected by FISH analysis, appeared more abundant than in poorly infiltrated tumours (online supplementary figure S8A). However, only weak-to-moderate correlations were observed between 16S and IRF-1 (r=0.267; p=0.034), CCL3 (r=0.457; p=0.0019) and CXCL12 (r=0.348; p=0.005), suggesting that specific bacteria families, rather than total bacterial loads, influence T cell recruitment into CRC tissues. We therefore analysed gut flora composition of CRC samples previously characterised for abundance of CD3+ infiltrates (German cohort, n=27) and corresponding tumour-free tissues. Overall, the majority of detectable bacteria were represented by Bacteroidetes, Proteobacteria and Firmicutes, as previously reported19 (online supplementary figure S8B). In line with previous studies,18,19 Bacteroidetes and Firmicutes were slightly reduced in tumour tissues as compared with corresponding tumour-free tissues, whereas Fusobacteria were significantly increased (p=0.014, see online supplementary figure S8B). Unsupervised hierarchical analysis of OTU abundances revealed a reduction of specific bacteria families within Bacteroidetes and Firmicutes in xenografts from antibiotic-treated versus untreated mice (online supplementary figure S7B,C and supplementary table S6). Moreover, significant correlations between abundance of Rikenellaceae, Ruminococcaceae and Lachnospiraceae and expression levels of CCL5, CCL20 and CXCL11 were detected (online supplementary figure S7D and supplementary table S7).

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Upregulation of most chemokine genes was also induced on exposure of CRC cells to bacterial species enriched in CRC tissues, including *Fusobacterium nucleatum, Bacteroides fragilis* and *Escherichia coli*18–20 (figure 5B and online supplementary figure S6). Thus, microbial stimulation appears to be sufficient to partially recapitulate in cell lines chemokine expression profiles of primary CRC.

We further investigated effects of gut commensal bacteria on chemokine expression in vivo. Levels of chemokine expression were evaluated in tumour xenografts generated in NSG mice on i.p. or i.c. injection of human CRC cells from established cell lines. Strikingly, intracecal tumours displayed significantly higher levels of CCL5 (70-fold increase), CCL20 (19-fold increase), CXCL10 (12-fold increase) and CXCL11 (threefold increase), as compared with i.p. xenografts (figure 6A), suggesting that chemokine expression is strongly induced by exposure to gut flora. Importantly, antibiotic treatment of tumour-bearing mice dramatically reduced tumour-derived chemokine expression in i.c. xenografts (figure 6A), indicating that commensal bacteria are main chemokine inducers in CRC cells. Indeed, expression levels of CCL20, CXCL10 and CXCL11 in xenografts significantly correlated with bacterial loads, as assessed by ribosomal subunit 16S expression (online supplementary figure S7A). Furthermore, gut flora composition analysis revealed a reduction of specific bacteria families within Bacteroidetes and Firmicutes in xenografts from antibiotic-treated versus untreated mice (online supplementary figure S7B,C and supplementary table S6). Moreover, significant correlations between abundance of Rikenellaceae, Ruminococcaceae and Lachnospiraceae and expression levels of CCL5, CCL20 and CXCL11 were detected (online supplementary figure S7D and supplementary table S7).

Figure 4 Tumour cells express genes encoding T cell recruiting chemokines. Expression of the indicated chemokine genes was evaluated by qRT-PCR in total CRC tissues and corresponding purified tumour cells (n=10), or in the indicated established CRC cell lines. Means are indicated by bars. Statistical significance was assessed by Mann-Whitney test (*p<0.05). CRC, colorectal cancer; qRT, quantitative reverse transcription PCR.
strongly associated with each other and with previously identified chemokine signatures (figure 8A and online supplementary table S9). Unfortunately, correlations with IL-17 were not evaluable since this cytokine was poorly expressed in these samples. Most importantly, significant correlations between abundance of specific bacteria and expression of individual T cell markers and chemokines genes were observed. In particular, abundance of different families of Firmicutes, mainly including Lachnospiraceae and Ruminococcaceae, significantly correlated with expression of CCR5 and CXCR3 binding chemokines. Abundance of Proteobacteria and, in particular, Methylobacteriaceae, was also associated with expression of all prognostically favourable T cell markers and most corresponding recruiting chemokines (figure 8B and online supplementary table S10). Thus, expression of chemokine genes in human CRC tissues is associated with abundance of specific bacteria.

DISCUSSION
Aims of this study were to elucidate the nature of chemotactic factors promoting infiltration of human CRC by T cell populations associated with favourable prognosis and to gain insights on cellular sources and stimuli eliciting their production within CRC microenvironment.

Based on correlations between expression of T cell markers and chemokines in freshly excised CRC tissues, and on chemokine receptor profiles of tumour infiltrating immune cells, we identified prominent chemokine signatures associated with recruitment of individual immune cell populations into CRC tissues. In particular, expression of CCR3-binding and CCR5-binding chemokines, including CCL3, CCL4, CCL5, CCL7, CCL8 and CCL13, and of CXCR3 ligands, including CXCL9 and CXCL10, correlated with presence of both CTLs and Th1 cells. This may suggest that these subsets, expressing similar chemokine receptor patterns, may be concomitantly recruited. Indeed, in our sample cohort, a significant positive correlation between expression of CD8 and IRF-1 was detected. More surprisingly, Th1-related and, to a lower extent, CTL-related signatures also partially overlapped with that underlying Tregs infiltration. Remarkably, both Th1 and Tregs markers were associated with expression of CXCL12, and of CCR4 ligands CCL17 and CCL22. Also, a weak but significant correlation was detected between Tregs infiltration and expression of CCL5 and CXCL9. Although unexpected, these findings are consistent with chemokine receptor profiles displayed by Tregs, largely resembling those of Th1 and, to lower extents, of CTLs. Thus, within CRC tissues, recruitment of Tregs may parallel that of effector T cells. Accordingly,
Foxp3 expression in CRC samples significantly correlated with that of both CD8 and IRF-1 genes.

In contrast, chemokine signatures associated with infiltration by Th17 were clearly distinct, the first being exclusively represented by CXCL13, and the second by CCL20 and CCL17, and to lower extents CCL25, CCL27 and CCL28. Unexpectedly, however, in both cohorts analysed, expression of CXCR5 and CXCL13 clustered with that of CD8, IRF-1, Foxp3 and correlating chemokines, suggesting that infiltration by Tfh cells may also parallel that of CTLs, Th1 and Tregs. Thus, expression of CXCL13, by primary CRC cells, could be evoked by stimuli also inducing secretion of CTL, Th1 and Tregs recruiting chemokines (see below).

Overexpression of defined chemokines in CRC tissues, particularly CCL5, CXCL9 and CXCL10, was previously reported to be associated with improved patient survival.14 However, specific cell populations recruited by these chemokines were not investigated. Our study demonstrates that receptors binding these chemokines, that is, CCR5 and CXCR3, are widely expressed by different subsets of TILs, including CTLs Th1 and Tregs, predictive of favourable clinical outcome. Therefore, positive prognostic significance of these chemokines might rely on their capacity to
attract beneficial T cell populations within tumours. Due to the prospective nature of our study, we could not assess in our cohort the impact on identified chemokines on patient's survival, usually evaluated after 5 years. However, analysis of a public database including transcriptomic and clinical data confirmed the associations between expression of identified T cell markers and chemokines, and their link with improved clinical outcome.

Our work identifies tumour cells as important chemokine sources. CRC cells per se express a spectrum of chemokines relevant for the recruitment of favourable immune cells, including CCL3, CCL4, CCL5, CCL20 and CXCL10, binding receptors expressed by CTLs and Th1 cells. In contrast, genes encoding chemokines involved in recruitment of Tregs, including CCL17 and CCL22, although detected in CRC tissues, were rarely expressed by purified CRC cells, suggesting that they may be mainly produced by other cell types within tumour microenvironment. Notably, in a melanoma model, Tregs-recruiting chemokines were secreted by tumour-infiltrating CTLs.21 In our sample cohort, a significant correlation between expression of CD8 and CCL17 and CCL22 genes was observed, possibly suggesting that also in CRC they might derive from CTLs.

Remarkably, CRC cells isolated from different samples displayed heterogeneous chemokine gene expression levels, possibly reflecting distinct molecular characteristics and/or exposure to different microenvironmental conditions. Genomic
alterations occurring in CRC cells were reported to result in loss or amplification of chemokine genes. More recently, epigenetic silencing of Th1-type chemokines in CRC was also described. Thus, differential genomic and epigenomic instability may at least partially explain heterogeneity of chemokine gene expression across different samples. Accordingly, different CRC cell lines, although maintained under comparable culture conditions, displayed variable degrees of chemokine production capacity. Importantly, in vitro cultured cell lines generally display significantly lower chemokine gene expression levels than primary CRC cells, indicating that microenvironmental stimuli also play relevant roles in modulating chemokine gene expression. Previous studies showed that gut commensal bacteria translocated across the neoplastic epithelium may interact with tumour cells and induce direct protumourigenic effects or release of protumourigenic cytokines. However, their potential modulation of chemokine production by tumour cells was not evaluated so far. Strikingly, we found that stimulation by gut commensal bacteria in vitro and in vivo induces in tumour cell lines upregulation or de novo expression of multiple chemokine genes, recapitulating profiles and levels of chemokine gene expression of primary CRC cells. Most interestingly, we demonstrated that tumour cell exposure to gut bacteria ultimately results in higher T cell recruitment into tumour xenografts, revealing a role of gut commensal bacteria in controlling extent of tumour infiltration by beneficial immune cells.

Consistent with in vivo findings, we found that extent of T cell infiltration in primary human CRCs is significantly associated with presence of specific bacteria families and genera. Furthermore, we observed significant correlations between abundance of defined bacteria families and expression levels of specific chemokine genes, indicating that gut commensal bacteria trigger production of immune cell-recruiting chemokines within tumour tissues. Importantly, composition of gut flora also predicted improved survival. Notably, the cluster associated to more favourable prognosis also included three samples displaying low T cell infiltration, possibly suggesting that certain bacteria may favour recruitment of immune cells other than T cells, also beneficially impacting on clinical outcome, including neutrophils and NK cells. Although additional studies are warranted to elucidate potential interactions between gut microbiota and additional immune cell populations, it is tempting to speculate that gut flora composition in patients with CRC may concur with tumour genetic characteristics to determine extent and quality of immune cell infiltration, determining clinical outcome.

Specific bacterial species or strains mostly contributing to high chemokine expression and immune cell infiltration in human CRC samples remain to be identified. In in vitro experiments, we found that different species of CRC-associated bacteria, including E. coli and B. fragilis, may promote, although to different extents, expression of T cell recruiting chemokine genes. Ex vivo analysis of human samples showed that Firmicutes, and in particular Lachnospiraceae and Ruminococcaceae, although represented in gut flora to lower extents than other phyla, are mostly associated with expression of T cell-recruiting chemokines. Furthermore, abundance of Bacteroides and Proteobacteria also correlated with expression of most T cell recruiting chemokines and with tumour infiltration by all T cell subsets predictive of good prognosis. Notably, defined bacteria types were associated with expression of multiple chemokine encoding genes, possibly indicating their capacity to promote simultaneous recruitment of different T cell populations. This is consistent with the clustering of the expression of CD8, Th1, Foxp3 and CXCR5 observed in clinical samples, characterised by favourable prognosis.

Surprisingly, also bacterial species, such as Fusobacteria, reportedly associated with severe clinical outcome and that we found enriched in poorly infiltrated tumours, were capable to evoke expression of T cell recruiting chemokine genes by CRC cells in vitro. Further studies are warranted to clarify the final impact of individual bacterial species, and Fusobacteria in particular, on T cell function. It is conceivable that some bacteria might attract immune cells into tissues while inhibiting their functions. For instance, F. nucleatum has been reported to inhibit T and NK cell functions via TIGIT. Also, defined bacterial strains may play multifaceted functions differentially impacting on clinical outcome. In this line, a recent study has described an association between abundance of Fusobacteria species and high expression of IL-12 and TGF-β, ultimately promoting differentiation of a T cell subset characterised by low Foxp3 expression and predictive of favourable survival.

Molecular mechanisms mediating the cross-talk between CRC cells and gut bacteria also remain to be elucidated. Colon epithelial cells are capable of sensing gut micro-organism through...
pattern recognition receptors (PRRs), including TLRs.28 Our data suggest that bacteria-induced chemokine gene expression may be initiated on TLR triggering on tumour cells. Indeed, we observed TLR expression on primary CRC cells. Furthermore, stimulation with purified TLR agonists resulted in marked induction of chemokine gene expression in CRC cells. However, further studies are warranted to precisely identify which TLRs and, possibly, other PRRs, are engaged by individual CRC-associated bacterial species.

In conclusion, our study identifies tumour cells as a major chemokine source in CRC and reveals the key role played by gut microbiota in triggering chemokine production ultimately leading to T cell recruitment in tumour tissues and improved prognosis. This knowledge might eventually pave the way towards development of innovative treatments aimed at modifying gut flora to promote CRC infiltration by immune cell populations of favourable prognostic significance.

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