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Gut microbiota modulate T cell trafficking into human colorectal cancer

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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.

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Gut microbiota modulate T cell trafficking into human colorectal cancer

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Abbreviations: CRC, colorectal cancer; Th1, T-helper type 1; CTLs, cytotoxic T lymphocytes; Tregs, regulatory T cells; Th17, IL-17 producing T-helper cells ; Tfh, follicular T-helper cells ; NK, natural killer cells; HDs, healthy donors ; i.p., intra peritoneum; i.c., intra cecum; TLR, Toll-like receptor; PPR, pattern recognition receptor ; OTU, operational taxonomic units.

Abstract (Word count: 250)

Objective: Tumor infiltrating lymphocytes (TILs) favor 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 micro-environmental triggers.

Design: Expression of genes encoding immune cell markers, chemokines, and bacterial 16S ribosomal RNA (16SrRNA) was assessed by quantitative RT-PCR in fresh CRC samples and corresponding tumor-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*, upon generation of intraperitoneal or intracecal tumor xenografts in immune-deficient mice. T cell trafficking was assessed upon adoptive transfer of human TILs into tumor-bearing mice. Gut flora composition was analyzed 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 IL-17-producing Th cells. These chemokines were expressed by tumor cells upon 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 tumor-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 favoring recruitment of beneficial T cells into tumor tissues.

Significance of this study

What is already known about this subject?

- ➢ Infiltration of human colorectal cancers (CRC) by cytotoxic T lymphocytes (CTLs), T-helper 1(Th1), Foxp3+ regulatory T cells (Tregs), and CXCR5+ follicular T-helper cells (Tfh) predicts prolonged survival. In contrast, the prognostic relevance of IL-17-producing T-helper cells (Th17) is still controversial. Chemotactic factors recruiting these immune cell types into tumor tissues have not been fully investigated yet.

- ➢ High expression of CXCL9, CXCL10, CXCL16, and CX3CL1 genes, correlates with more favorable clinical outcome, but mechanisms underlying their positive prognostic significance remain to be elucidated.

- ➢ 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 favorable immune cell populations is associated with expression of defined chemokine genes, i.e. mainly CCL5, CXCL9 and CXCL10 for CTLs and Th1, CCL17, CCL22, and CXCL12 for Th1 and Tregs, CXCL13 for Tfh, and CCL20 and CCL17 for Th17.

- Most chemokine genes identified may be expressed by CRC cells in response to stimulation with gut microbiota-derived stimuli.

- In tumor 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 favorable prognostic significance. This knowledge might pave the way toward the development of innovative treatments aimed at modifying the gut flora to promote CRC infiltration by beneficial immune cells.

Introduction

Infiltration by immune cells heavily impacts on clinical outcome in human colorectal cancer (CRC) (1). High densities of cytotoxic CD8+ T cells (2;3), IFN-g expressing T-helper 1 cells (Th1)(4), CXCR5+ follicular T-helper cells (Tfh) (5) and, surprisingly, Foxp3+ regulatory T cells (Tregs) (6;7) 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 favorable clinical course (8). In contrast, prognostic significance of IL-17 producing T-helper cells (Th17) is controversial: their presence within CRC tissues was reported to correlate either with poor (4) or improved prognosis (9). In addition, innate immune cells were also shown to predict clinical outcome. Tumor infiltration by CD16+ myeloperoxidase (MPO)+ myeloid cells, mostly consisting of activated neutrophils, is an independent predictor of favorable prognosis (10-12). Notably, infiltration by neutrophils or natural killer (NK) cells was also found to increase favorable prognostic significance of cytotoxic CD8+ T cells (11;13).

Chemotactic factors driving these cell populations into CRC tissues remain largely undefined. Expression of defined chemokines, including CXCL9 (14), CXCL10 (14), CXCL16 (15), and CX3CL1 (5), was reported to correlate with high densities of tumor infiltrating lymphocytes (TILs), and to predict favorable clinical outcome. However, putative responding cell subsets within immune infiltrating cells have not been carefully characterized. Furthermore, chemokine sources and micro-environmental stimuli leading to chemokine production within CRC tissues were not addressed so far.

Upon intestinal tumorigenesis gut commensal bacteria translocate across altered epithelia and stimulate immune cells infiltrating lamina propria to release pro-inflammatory cytokines (16). 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 cohort, n=62), and at Klinikum Rechts Der Isar, Münich, Germany (German cohort, n=31). Use of human samples was approved by local ethical authorities (Ethikkommission Nordwest und Zentralschweiz, Comitato etico cantonale Ticino, and Ethics committee of the Faculty of Medicine of the TUM). Clinical-pathological characteristics of patients included in these cohorts are reported in Table S1. For details on sample processing and analysis by flow cytometry see "Supplementary Methods" and Table S2.

CRC cell stimulation with Toll-like receptor (TLR) agonists and bacteria

Cells from established cell lines or CRC-derived organoids were incubated with indicated concentrations of TLR agonists or heat-inactivated bacteria (bacteria: tumor cell ratio = 30:1) at 37 C. After 4 hours, chemokine expression was assessed by qRT-PCR. Details regarding cell lines, organoids, bacteria cultures, TLR agonists, and qRT-PCR are provided in Supplementary Methods and Table S3.

In vivo experiments

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

Tumor xenografts were generated in eight-week old NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ,) upon intra peritoneum (i.p.) and intra cecum (i.c.) injection (17) of LS180 cells.

(see "Supplementary Methods" for full details). From day 10, a randomized 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. Tumors were harvested on day 31 and assessed for chemokine gene expression.

To evaluate T cell migration, at day 29 tumor bearing mice were adoptively transferred with CFSE-labeled 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 tumors by flow-cytometry.

Details of mice, tumor cell injection protocols, and TIL isolation are provided in "Supplementary Methods".

16SrRNA gene sequencing and analysis

Bacterial flora was analyzed based on 16S rRNA sequencing. Total RNA was purified from 27 CRC tissue samples (German cohort) and reverse transcribed. V4 region of the bacterial 16SrRNA gene was amplified by PCR. Amplicons were sequenced on the Illumina MiSeq platform. Sequences were analyzed by USEARCH software (v8.1.1861). Differential operational taxonomic unit (OTU) analysis of normalized abundance counts was performed by DESeq2 software (v1.12.4). Libraries, sequencing and data analysis were performed by Microsynth AG (Balgach, Switzerland) as detailed in "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 analyzed expression of genes encoding immune cell markers, including CD3 for total T cells, CD4 for T-helper cells, CD8 for CTLs, T-bet and IRF-1 for Th1, IL-4, IL-5 and IL-13 for Th2, IL-17 for Th17, CXCR5 for Tfh, and Foxp3 for Tregs, in 62 CRC and corresponding tumor-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, 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 tumors as compared to 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 (Table S4).

Normalization of immune cell marker expression in tumor samples versus corresponding tumor-free colonic tissues revealed a consistent subgroup of samples displaying upregulation of one or more T cell markers (Figure S1). Upon unsupervised hierarchical analysis, samples clustered in three main groups: one characterized by overexpression of most T cell markers (cluster “high”), a second displaying heterogeneous expression (cluster “het”), and a third characterized 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 tumors (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 tumor tissues.

TILs express receptors binding overexpressed chemokines

We then analyzed chemokine receptor profiles of CD8+, CD4+ (Foxp3-) effector T cells, and CD4+ Foxp3+ Tregs, in freshly excised CRC specimens as compared to control tissues or PBMCs from patients or HDs (Figure 2 and Figure S2). All three subsets in tumors 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 to PBMCs (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 tumor 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 (9), 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 (Figure S2).

Table 1. Correlations between expression of genes encoding chemokines and immune cell markers in CRC specimens (n=62) *

	CD8		T-bet		IRF1		IL-17		CXCR5		Foxp3	
	Spearman r	P value	Spearman r	P value	Spearman r	P value	Spearman r	P value	Spearman r	P value	Spearman r	P value
CCL2	**0.3960**	**0.0020**	**0.4100**	**0.0010**	**0.4250**	**0.0010**	0.0060	0.9660	0.1880	0.1470	**0.4620**	**<0.0001**
CCL3	**0.4036**	**0.0015**	0.1247	0.3468	**0.3205**	**0.0133**	-0.2230	0.0890	-0.0560	0.6730	0.1519	0.2509
CCL4	**0.3454**	**0.0069**	0.0162	0.9021	0.0687	0.6017	-0.0700	0.5960	-0.0440	0.7370	-0.0533	0.6857
CCL5	**0.6544**	**<0.0001**	**0.4252**	**0.0007**	**0.5578**	**<0.0001**	-0.0300	0.8180	0.2750	0.0330	0.3399	0.0079
CCL7	**0.3258**	**0.0104**	0.0736	0.5730	0.2613	0.0420	-0.0010	0.9930	-0.0230	0.8600	0.1244	0.3396
CCL8	**0.4632**	**0.0002**	0.1333	0.3056	0.2969	0.0202	-0.0720	0.5820	0.0880	0.5000	0.2038	0.1152
CCL11	**0.3410**	**0.0080**	**0.3620**	**0.0040**	**0.4680**	**<0.0001**	0.0620	0.6370	0.1560	0.2330	**0.5540**	**<0.0001**
CCL13	**0.4097**	**0.0010**	0.1364	0.2944	**0.3265**	**0.0102**	-0.0240	0.8520	0.2040	0.1150	0.1764	0.1739
CCL14-15	0.0230	0.8610	0.1020	0.5340	-0.0310	0.8110	**0.3460**	**0.0060**	-0.1950	0.1330	-0.0820	0.5280
CCL17	**0.3234**	**0.0110**	0.1834	0.1571	**0.4450**	**0.0003**	**0.3000**	**0.0190**	0.2310	0.0740	0.2646	0.0393
CCL18	0.2420	0.0610	**0.3500**	**0.0060**	**0.3110**	**0.0150**	-0.0710	0.5870	0.2090	0.1060	0.2310	0.0730
CCL19	**0.5270**	**0.0010**	**0.5900**	**<0.0001**	**0.5270**	**<0.0001**	0.0020	0.9880	**0.5380**	**<0.0001**	**0.4890**	**0.0001**
CCL20	-0.2528	0.0493	-0.2051	0.1128	-0.0933	0.4743	**0.4440**	**0.0003**	-0.1510	0.2450	-0.1297	0.3192
CCL21	**0.4070**	**0.0010**	**0.5560**	**<0.0001**	**0.4770**	**<0.0001**	-0.1890	0.1450	0.1970	0.1270	**0.5900**	**<0.0001**
CCL22	**0.3754**	**0.0029**	**0.4432**	**0.0003**	**0.6803**	**<0.0001**	0.1570	0.2280	**0.3550**	**0.0050**	**0.7033**	**<0.0001**
CCL23	**0.3510**	**0.0050**	0.1270	0.3280	**0.3410**	**0.0070**	-0.0940	0.4720	0.1120	0.3920	0.1640	0.2070
CCL24	-0.1050	0.1230	0.1350	0.3010	0.2620	0.0410	0.0540	0.6780	-0.0560	0.6660	0.0660	0.6160
CCL25	0.2174	0.0923	0.2541	0.0482	0.1778	0.1703	**0.3830**	**0.0020**	0.1580	0.2240	0.2226	0.0847
CCL26	0.1350	0.3010	0.2190	0.0890	**0.3010**	**0.0180**	0.0450	0.7280	0.2170	0.0930	0.1780	0.1700
CCL27	0.0110	0.2460	0.0410	0.7530	0.0110	0.9310	**0.4180**	**0.0010**	0.1630	0.2110	0.1120	0.3900
CCL28	-0.0320	0.3930	0.0110	0.9310	-0.0320	0.8070	**0.3570**	**0.0050**	-0.0450	0.7280	-0.1290	0.3200
CXCL3	-0.1850	0.1250	-0.0330	0.8000	-0.1850	0.1540	**0.3790**	**0.0030**	0.0420	0.7490	-0.2030	0.1170
CXCL9	**0.5509**	**<0.0001**	**0.4138**	**0.0010**	**0.5125**	**<0.0001**	0.1590	0.2260	**0.3910**	**0.0020**	**0.3458**	**0.0068**
CXCL10	**0.4268**	**0.0006**	0.2867	0.0251	0.1835	0.1569	0.1670	0.1970	0.2560	0.0460	0.1431	0.2711
CXCL11	0.2075	0.1085	0.2361	0.0669	**0.3708**	**0.0033**	0.1200	0.3570	0.2060	0.1110	0.1436	0.2697
CXCL12	**0.5330**	**<0.0001**	**0.3592**	**0.0045**	**0.5795**	**<0.0001**	-0.2650	0.0390	0.2060	0.1120	**0.4887**	**0.0001**
CXCL13	**0.4810**	**0.0001**	**0.6110**	**<0.0001**	**0.4270**	**0.0010**	0.1110	0.3920	**0.5760**	**<0.0001**	**0.5090**	**<0.0001**
CXCL14	0.0800	0.5610	0.0810	0.5550	**0.4150**	**0.0020**	0.2130	0.1180	0.1360	0.3240	0.2740	0.0430
CXCL16	0.2864	0.0252	0.1562	0.2293	**0.4231**	**0.0007**	0.0040	0.9740	0.0860	0.5110	0.2046	0.1138
CX3CL1	0.2703	0.0351	**0.3134**	**0.0139**	**0.5272**	**<0.0001**	-0.2010	0.1200	0.0190	0.8870	**0.4085**	**0.0011**

*Significant correlation coefficients ≥0.3 are indicated in bold

Defined 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 x percentage of corresponding chemokine receptor-positive cells) (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 CCR4-ligands CCL17 and CCL22, together with CCL3, CCL5, CXCL9, CXCL11, CXCL12, CCL17 and CCL22, and to a lower extent CCL5 and CXCL9, also underlay tumor 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 (TCGA, cancergenome.nih.gov) (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 and Th-17 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 (Figure S3A). Importantly, samples overexpressing all T cell markers (except IL-17) and their chemokine signatures showed improved survival as compared to those from other clusters (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 tumor cells

To identify cellular sources of T cell recruiting chemokines, we evaluated their potential production by tumor 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 tumor cells express most relevant chemokine genes (Figure 4). For some chemokines, including CCL3, CCL4, and CCL20, gene expression levels were significantly higher in purified CRC cells than in total tumor tissues, suggesting that tumor cells are likely major contributors of these chemokines within CRC microenvironment. In contrast, purified tumor 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 to primary tumor cells, suggesting that chemokine expression in tumor 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 (16). We hypothesized that chemokine production in tumor cells may be triggered by gut flora-derived microbial stimuli. Indeed, CRC cells from primary tumors and established cell lines express TLRs potentially sensing them (Figure S4). Stimulation of CRC cells from cell lines (Figure 5A and Figure S5) and CRC organoids (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.

Upregulation of most chemokine genes was also induced upon exposure of CRC cells to bacterial species enriched in CRC tissues, including *Fusobacterium nucleatum, Bacteroides fragilis, and Escherichia coli* (18-20) (Figure 5B and 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 tumor xenografts generated in NSG mice upon i.p. or i.c. injection of human CRC cells from established cell lines. Strikingly, intracecal tumors displayed significantly higher levels of CCL5 (70-fold increase), CCL20 (19-fold increase), CXCL10 (12-fold increase) and CXCL11 (3-fold increase), as compared to i.p. xenografts (Figure 6A), suggesting that chemokine expression is strongly induced by exposure to gut flora. Importantly, antibiotic treatment of tumor bearing mice dramatically reduced tumor-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 (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 (Figure S7B, C and Table S6). Moreover, significant correlations between abundance of Rikenellaceae, Ruminococcace, and Lachnospiracee and expression levels of CCL5, CCL20, and CXCL11 were detected (Figure S7D and Table S7).

Gut microbiota in tumors correlate with extent of T cell infiltration

To assess actual impact of bacteria-induced chemokines on T cell recruitment into tumor tissues, we adoptively transferred CRC-derived CD4+ and CD8+ TILs into tumor bearing NSG mice and evaluated their homing to i.p. or i.c. tumors. Strikingly, TILs migrated into i.c. xenografts to significantly higher extents than in i.p. tumors (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 tumors (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 analyzed gut flora composition of CRC samples previously characterized for abundance of CD3+ infiltrates (German cohort, n=27) and corresponding tumor-free tissues. Overall, the majority of detectable bacteria were represented by Bacteroidetes, Proteobacteria, and Firmicutes, as previously reported (19) (Figure S8B). In line with previous studies (18;19), Bacteroidetes and Firmicutes were slightly reduced in tumor tissues as compared to corresponding tumor-free tissues, whereas Fusobacteria were significantly increased (p=0.014, Figure S8B). Unsupervised hierarchical analysis of operational taxonomic unit (OTU) abundances in tumor tissues, identified two main clusters one (indicated in orange) including 10/13 samples displaying low CD3 densities (CD3low), and a second (green) including all 14 tumors displaying high CD3 densities (CD3high) plus 3 CD3low tumors (Figure 7A). Importantly, samples from the green cluster displayed a significantly prolonged survival as compared to orange cluster samples (Figure 7B). Specific bacteria genera, including Alloprevotella, Treponema, and Desulfovibrio, were significantly enriched in CD3high tumors, whereas, Prevotella, Bacteroides, and Fretibacterium, among

others, were overrepresented in CD3low tumors (Figure 7B and Table S8). Expression of T cells markers and chemokine genes was also assessed in these samples. Consistent with results from previous cohort, CD8, IRF-1, Foxp3 and CXCR5 were strongly associated with each other and with previously identified chemokine signatures (Figure 8A and 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 favorable T cell markers and most corresponding recruiting chemokines (Figure 8B and Table S10). Thus, expression of chemokine genes in human CRC tissues is associated with abundance of specific bacteria.

Discussion

Aim of this study was to elucidate the nature of chemotactic factors promoting infiltration of human CRC by T cell populations associated with favorable 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 tumor infiltrating immune cells, we identified prominent chemokine signatures associated with recruitment of individual immune cell populations into CRC tissues. In particular, expression of CCR3- 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- 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 Tfh and 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 analyzed, 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, i.e. CCR5 and CXCR3, are widely expressed by different subsets of TILs, including CTLs Th1, and Tregs, predictive of favorable clinical outcome. Therefore, positive prognostic significance of these chemokines might rely on their capacity to attract beneficial T cell populations within tumors. 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 tumor cells as important chemokine sources. CRC cells *per se* express a spectrum of chemokines relevant for the recruitment of favorable 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 tumor

microenvironment. Notably, in a melanoma model, Tregs recruiting chemokines were secreted by tumor 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 (5). More recently, epigenetic silencing of Th1-type chemokines in CRC was also described (22). 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 micro-environmental 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 tumor cells and induce direct pro-tumorigenic effects (23;24) or release of pro-tumorigenic cytokines (16). However, their potential modulation of chemokine production by tumor cells was not evaluated so far. Strikingly, we found that stimulation by gut commensal bacteria *in vitro* and *in vivo* induces in tumor 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 tumor cell exposure to gut bacteria

ultimately results in higher T cell recruitment into tumor xenografts, revealing a role of gut commensal bacteria in controlling extent of tumor 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 tumor tissues. Importantly, composition of gut flora also predicted improved survival. Notably, the cluster associated to more favorable prognosis also included three samples displaying low T cell infiltration, possibly suggesting that certain bacteria may favor recruitment of immune cells other than T cells, also beneficially impacting on clinical outcome, including neutrophils (10;11) and NK cells (13). 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 CRC patients may concur with tumor 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 *Escherichia coli* and *Bacteroides 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 tumor 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, characterized by favorable prognosis.

Surprisingly, also bacterial species, such as Fusobacteria, reportedly associated with severe clinical outcome (25), and that we found enriched in poorly infiltrated tumors, 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, *Fusobacterium nucleatum* has been reported to inhibit T and NK cell functions via TIGIT (26). 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 characterized by low Foxp3 expression and predictive of favorable survival (27).

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 microorganism through pattern recognition receptors (PRRs), including TLRs (28). Our data suggest that bacteria-induced chemokine gene expression may be initiated upon TLR triggering on tumor cells. Indeed, we observed TLRs 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 tumor 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 tumor tissues and improved prognosis. This knowledge might eventually pave the way toward development of innovative treatments aimed at modifying gut flora to promote CRC infiltration by immune cell populations of favorable prognostic significance.

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Patient consent: Obtained

Ethics approval: EKBB/EKNZ (study protocol n. 2014-388), Ethics Committee of the Faculty of Medicine of the TUM (#1926/2007), Cantonal Veterinary Office Basel-Stadt (license n. 2266), Cantonal Veterinary Office Zürich (license n. 35/2014).

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Figure Legend

Figure 1: CRC infiltration by T cells is associated with overexpression of defined chemokines. Expression of the genes encoding the indicated immune cell markers and chemokines was analyzed in 62 freshly excised CRC tissues and corresponding tumor-free colonic mucosa samples by qRT-PCR. **A.** Gene expression levels in CRC tissues (bold) and tumor-free colonic mucosa samples (gray) relative to GAPDH. Statistical significance was assessed by Wilcoxon signed rank test (*$p<0.05$; ***$p<0.0001$). **B.** Unsupervised hierarchical clustering of the expression of the indicated genes in individual CRC specimens as related to corresponding tumor-free colonic tissues **C.** Expression of 38 chemokine genes (see Table S3) in tumors as related to corresponding tumor-free colonic tissues, according to their distribution within the different clusters.

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 labeled 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, permeabilized 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 standard deviations are indicated by bars; 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 Table S5. A heat map of score values is shown.

Figure 4. Tumor 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 tumor cells (n=10), or in the indicated established CRC cell lines. Means are indicated by bars. Statistical significance was assessed by Mann Whitney test (* $p<0.05$).

Figure 5. Gut bacteria-derived stimuli induce chemokine gene expression in CRC cells. CRC cell from LS180 cell line were treated with (**A**) LPS (1 μg/ml), Poly (I:C) (10 μg/ml), Flagellin (100 ng/ml), or FSL-1 (1 μg/ml), or (**B**) with the indicated heat-inactivated bacteria (bacteria: CRC cell ratio = 30:1). After four hours, expression levels of the indicated chemokine genes were analyzed by qRT-PCR, using GAPDH as reference gene. Cumulative data from three independent experiments are shown. Statistical significance was assessed by two-way ANOVA test (*=$p<0.05$).

Figure 6. Exposure of tumor cells to gut flora promotes chemokine expression and T cell recruitment into CRC. A NSG mice were inoculated i.p. (n=17) or i.c. (n=66) with LS180 cells (10^5 cells/mouse). Starting from day 10, a randomized group of mice inoculated i.p., (n=7) or i.c. (n=28), were treated with Ampicillin and Vancomycin for three weeks. On day 31 xenografts were removed and expression levels of the indicated chemokine genes and of

16S were analyzed by qRT-PCR, using GAPDH gene as reference. Cumulative data from three independent experiments are shown. Statistical significance was assessed by two-way ANOVA test (*= $p<0.05$, **$p<0.01$, ***$p<0.0001$). **B, C.** NSG mice were inoculated with LS180 i.p. (n=11) or i.c. (n=14). On day 29, mice were adoptively transferred with equal numbers (5x10^6 cells/mouse) of human CFSE-labelled CRC-derived CD4+ and CD8+ T cells. After 48 hours, extent of human TIL migration into tumors was evaluated by flow cytometry. Cumulative data from two independent experiments performed with TILs derived from two different samples (P312, red, and P326, black) are shown. Statistical significance was assessed by one-way ANOVA test (***$p<0.0001$).

Figure 7. Abundance of defined bacteria families correlates with T cell infiltration and improved clinical outcome in human CRCs. The gut flora composition of 27 primary CRC samples pre-assessed for density of CD3+ infiltrate (CD3high n=14, CD3low n=13) was analyzed by 16SrRNA sequencing. **A.** Unsupervised hierarchical clustering of OTU abundances was performed using the hclust algoritm based on the Ward D2 method. **B.** Kaplan-Meier curves illustrating overall survival (OS) probability of patients included in clusters identified by hierarchical analysis. Statistical significance was assessed by log-rank test. **C.** Fold change (log2) of bacteria genera and families enriched in CD3high as compared to CD3low tumors are shown. Differential OTU analysis on normalized abundance counts was performed with DESeq2 software (v1.12.4).Staistical significance ($p<0.05$) was assessed using a Wald test.

Figure 8. Abundance of defined bacteria families correlates with expression of individual chemokines and T cell markers. Expression of genes encoding for the indicated

markers or chemokines was assessed in 27 CRC samples (see above) and Spearman correlations between all markers were calculated. Correlation matrixes illustrating major correlations found between expression of T cell markers and chemokine genes (**A**) to each other and (**B**) versus OUT abundances. Color and diameter of the spots are proportional to the calculated Spearman correlation coefficients as indicated in the blue to red bar. All r and corresponding p values are listed in Tables S9 and S10.

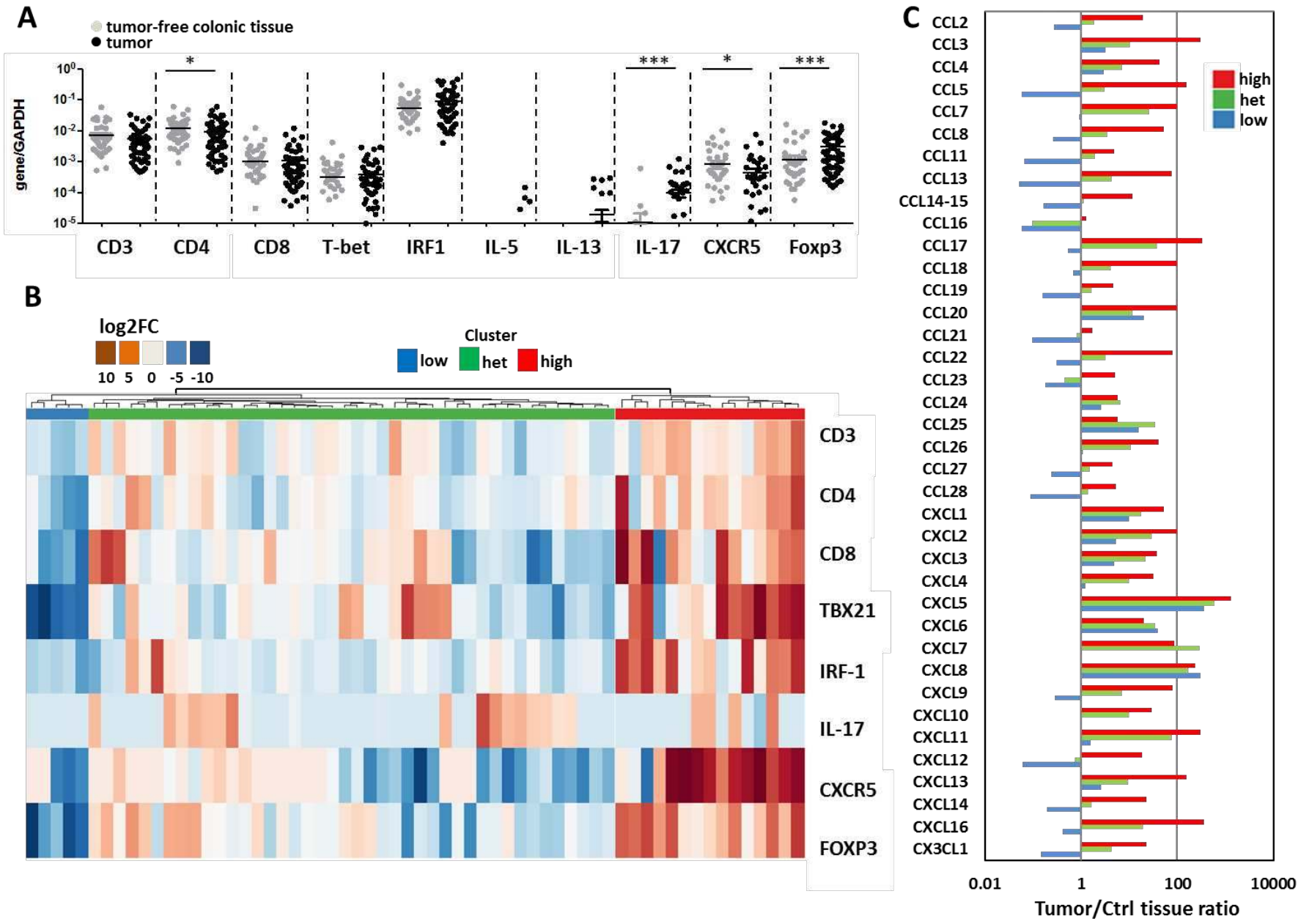
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Figure 1

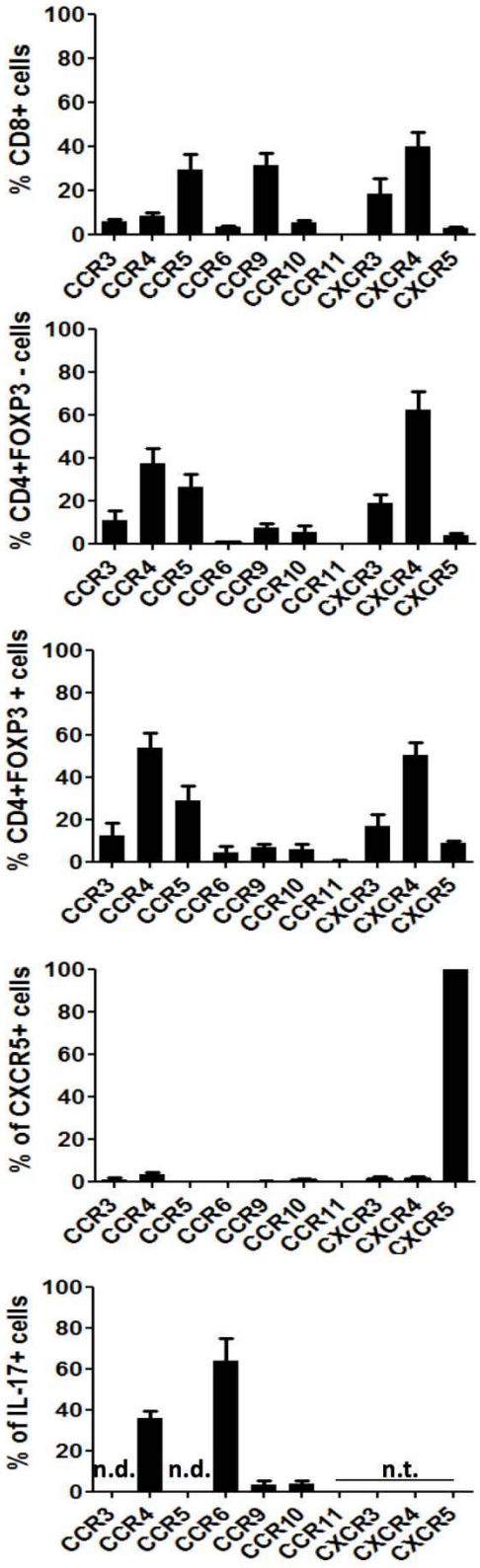
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Figure 2

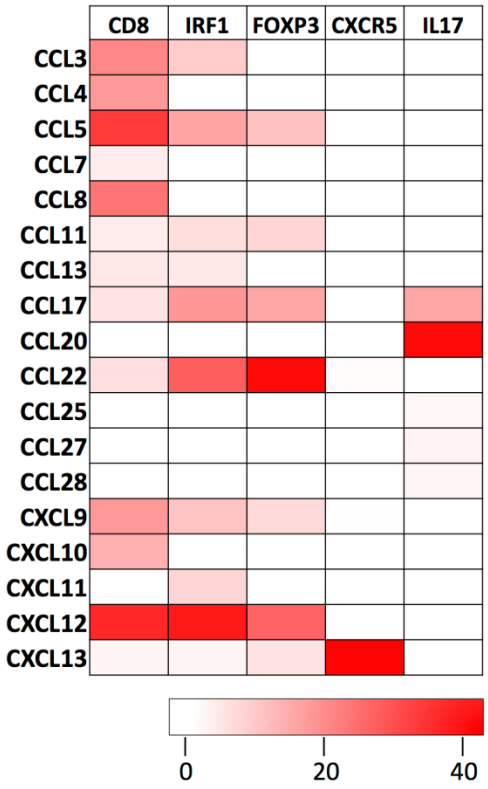
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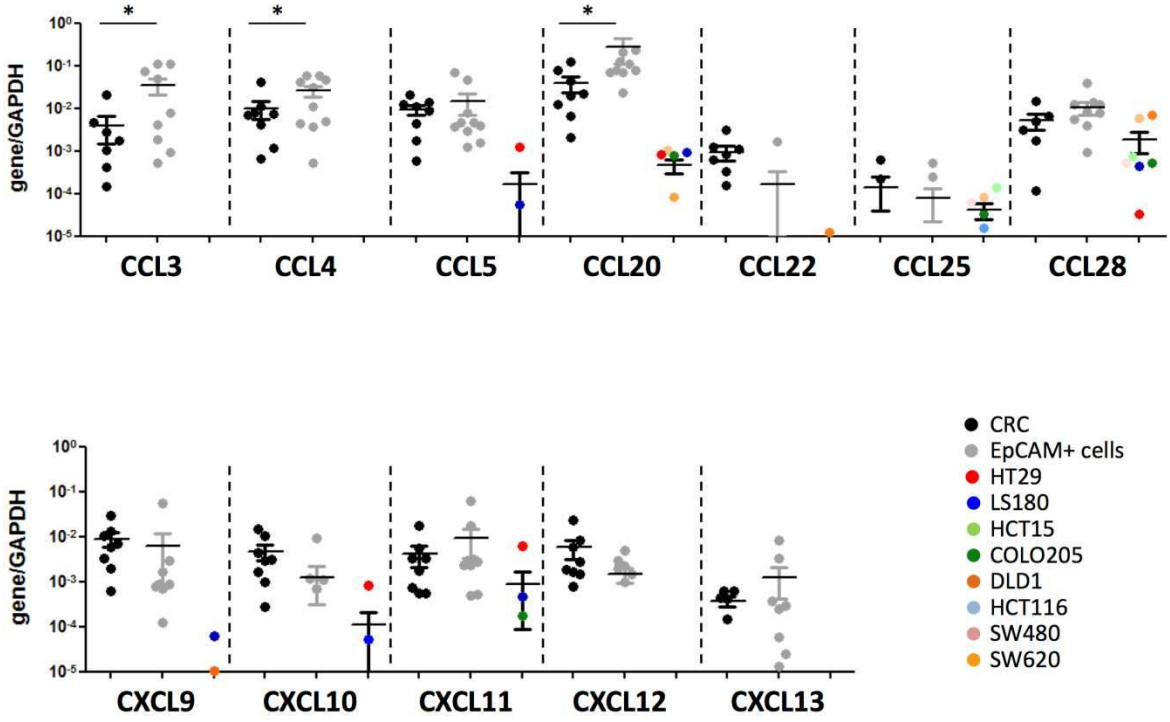
Figure 3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SW480
SW620

Figure 4

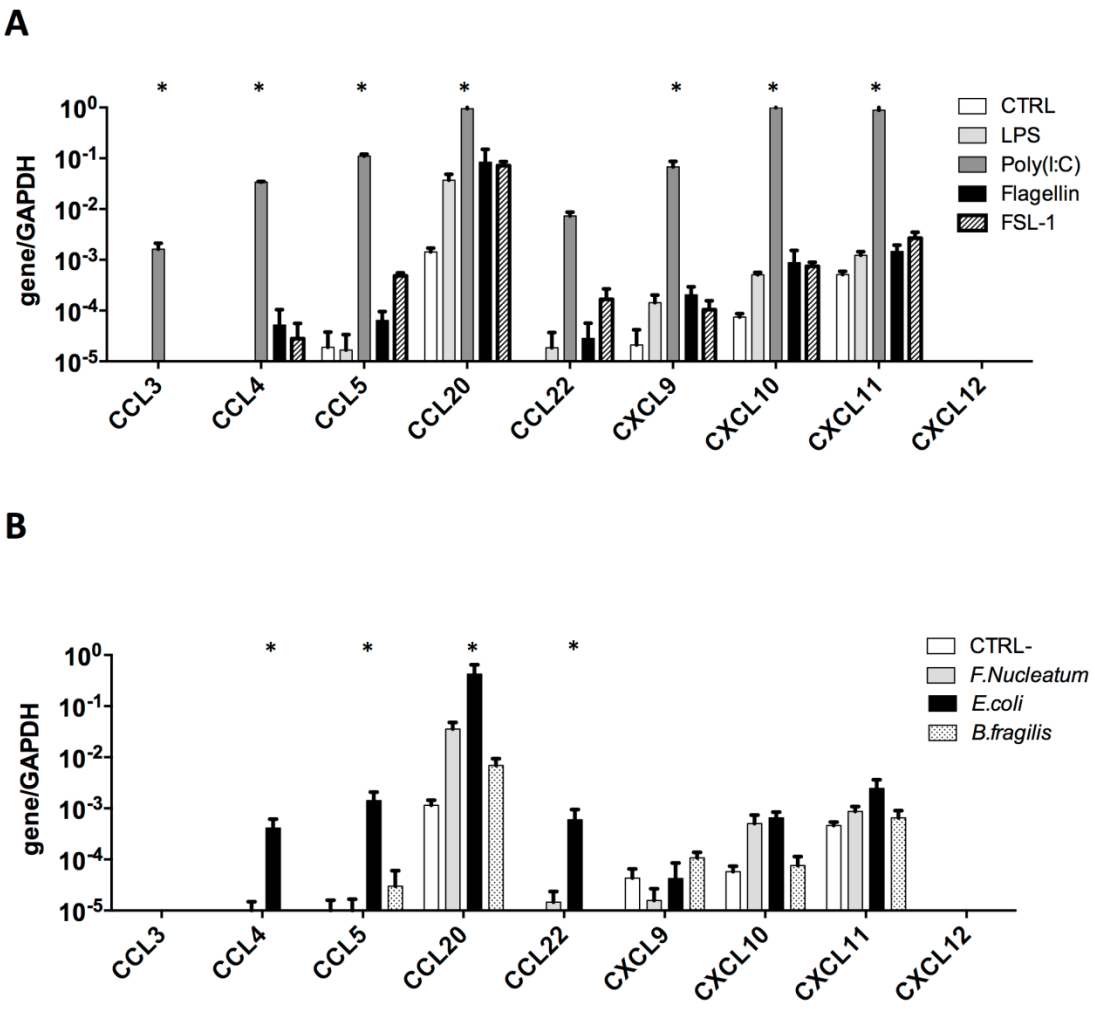
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Figure 5

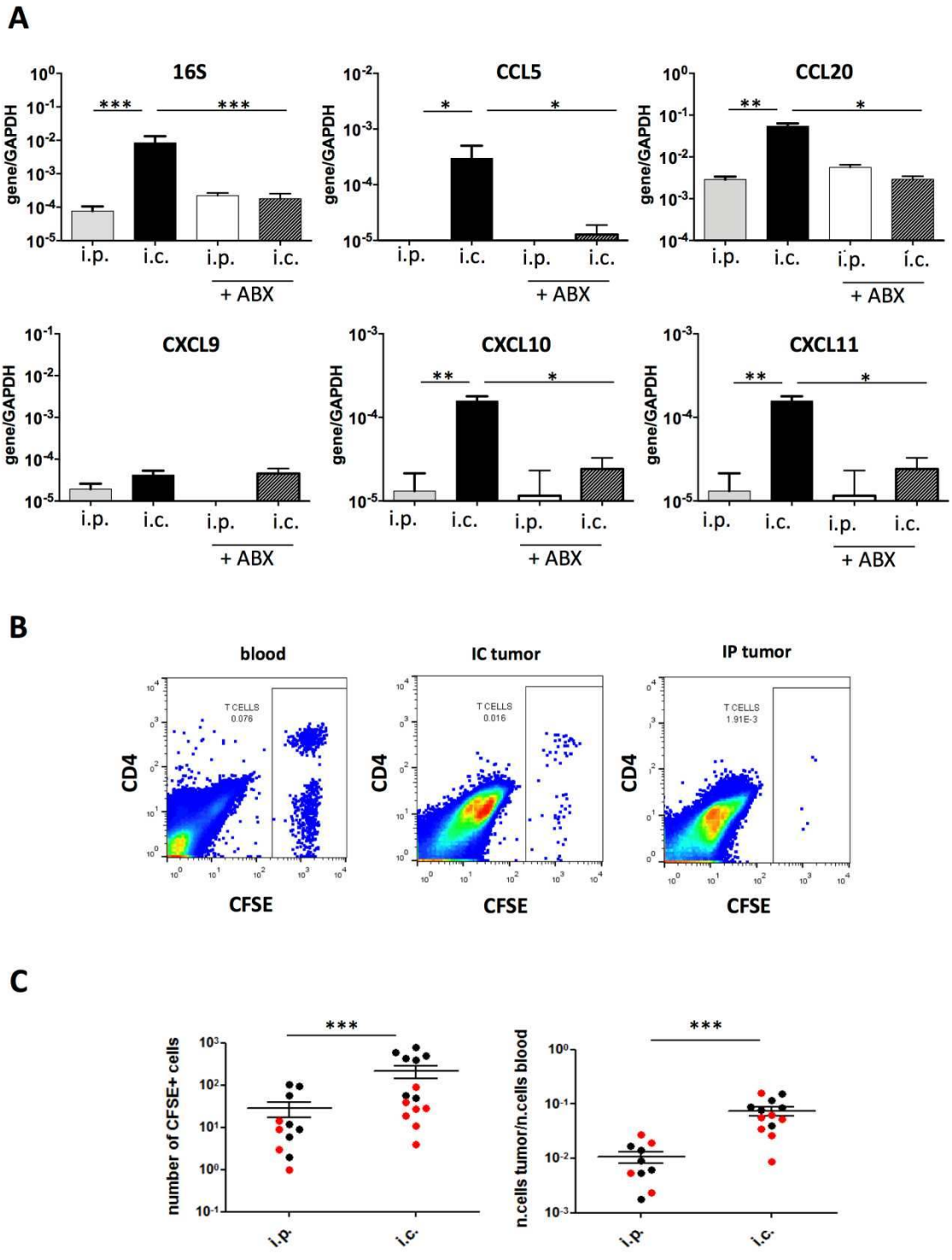
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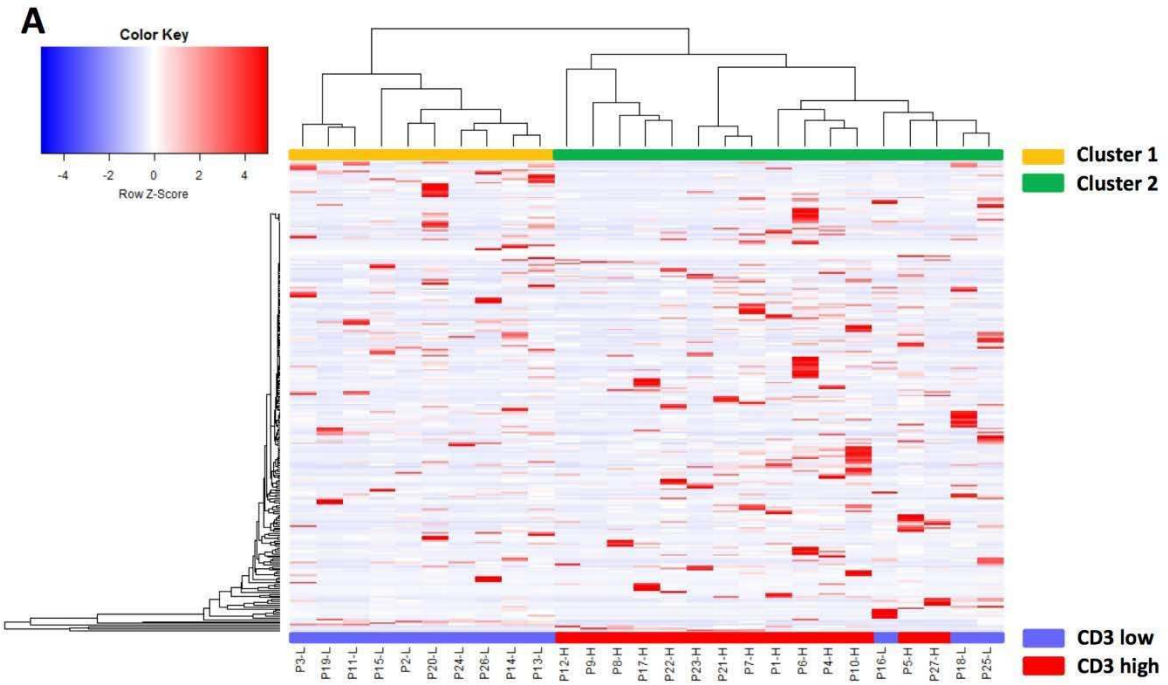
Figure 6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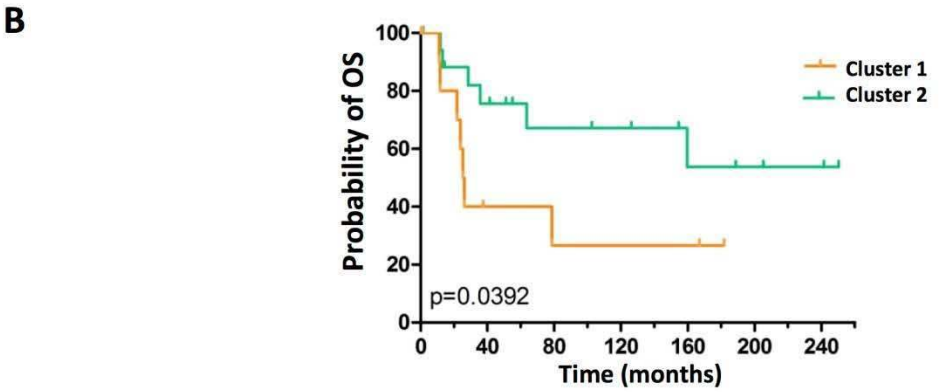
P2-L
P20-L
P24-L
P26-L
P14-L
P13-L
P12-H
P9-H
P8-H
P17-H
P22-H
P23-H
P21-H
P7-H
P1-H
P6-H
P4-H
P10-H
P16-L
P5-H
P27-H
P18-L
P25-L


B
Probability of OS
100
80
60
40
20
0
p=0.0392
0
40
80
120
160
200
240
Time (months)
Cluster 1
Cluster 2

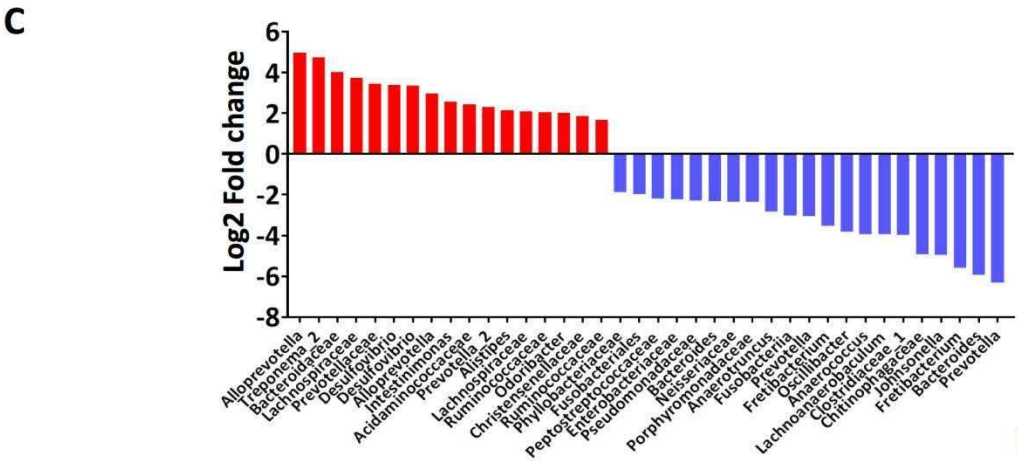

C
Log2 Fold change
6
4
2
0
-2
-4
-6
-8
Alloprevotella
Treponema 2
Bacteroidaceae
Lachnospiraceae
Prevotellaceae
Desulfovibrio
Alloprevotella
Intestinimonas
Acidaminococcaceae
Prevotella 2
Alistipes
Lachnospiraceae
Ruminococcaceae
Odoribacter
Christensenellaceae
Ruminococcaceae
Phylobacteriaceae
Fusobacteriales
Peptostreptococcaceae
Enterobacteriaceae
Pseudomonadaceae
Bacteroides
Neisseriaceae
Porphyromonadaceae
Anaerotruncus
Fusobacteria
Prevotella
Fretibacterium
Oscillibacter
Anaerococcus
Lachnoanaerobaculum
Clostridiaceae 1
Chitinophagaceae
Johnsonella
Fretibacterium
Bacteroides
Prevotella

Figure 7

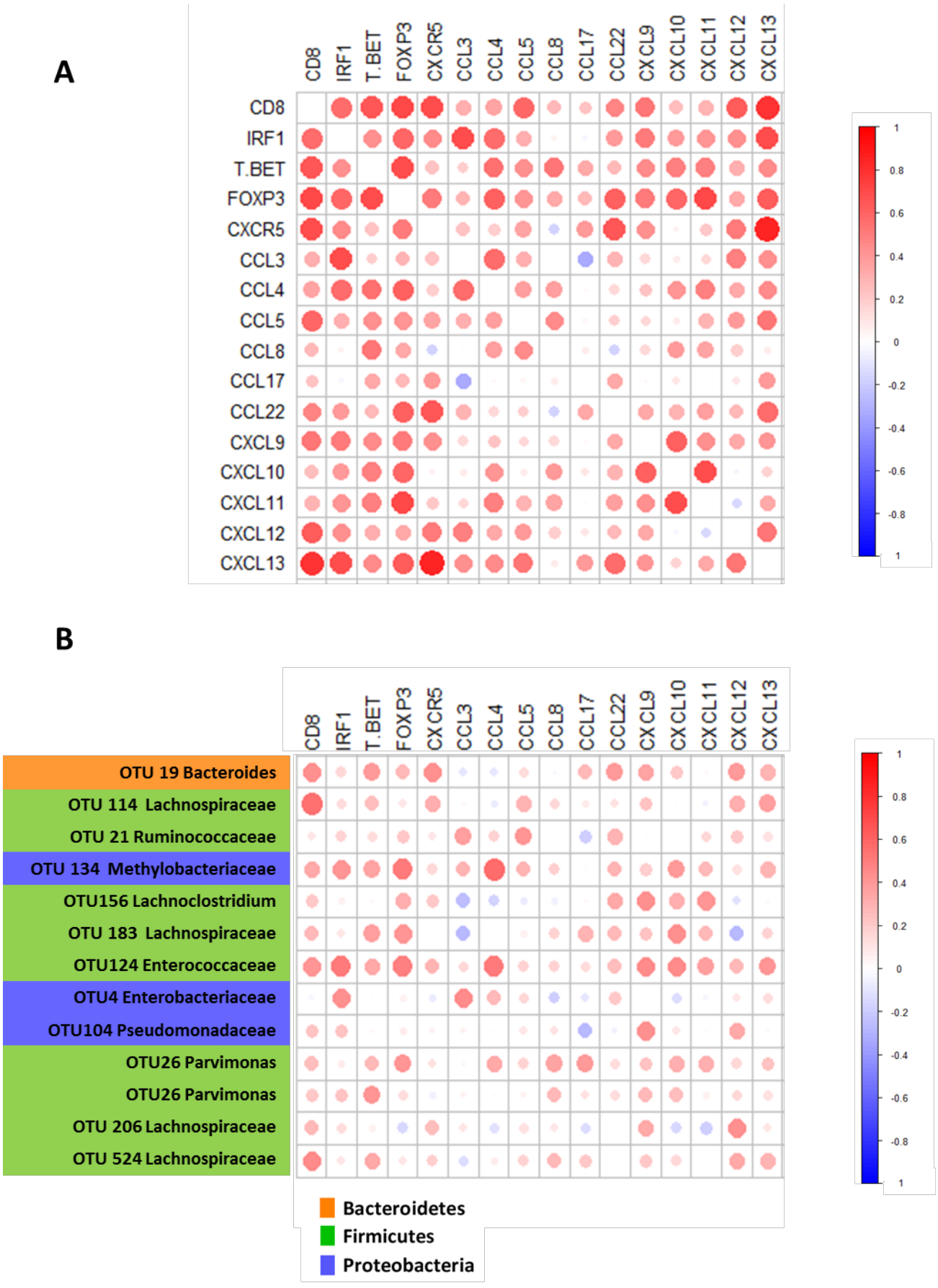

A
CD8
IRF1
T.BET
FOXP3
CXCR5
CCL3
CCL4
CCL5
CCL8
CCL17
CCL22
CXCL9
CXCL10
CXCL11
CXCL12
CXCL13
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
1
B
OTU 19 Bacteroides
OTU 114 Lachnospiraceae
OTU 21 Ruminococcaceae
OTU 134 Methylobacteriaceae
OTU156 Lachnoclostridium
OTU 183 Lachnospiraceae
OTU124 Enterococcaceae
OTU4 Enterobacteriaceae
OTU104 Pseudomonadaceae
OTU26 Parvimonas
OTU26 Parvimonas
OTU 206 Lachnospiraceae
OTU 524 Lachnospiraceae
Bacteroidetes
Firmicutes
Proteobacteria

Figure 8