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FRACTALKINE-FRACTALKINE RECEPTOR AXIS IN HUMAN COLORECTAL CANCER

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SUMMARY

Although the presence of genetic and epigenetic abnormalities is important for tumor occurrence, the formation of an inflammatory microenvironment plays a pivotal role in tumor progression. Even if the link between cancer and inflammation has been widely accepted in the last decade, no inflammation-associated markers or potential targets have yet been translated into clinical practice. Human colorectal cancer (CRC), the second cause of cancer-related death in Western countries, represents a paradigm of the cancer-related inflammation.

The aim of this study is to provide an overview of the inflammatory mediators expressed in tumor microenvironment of CRC, in order to select and characterize new candidates for clinical or therapeutic strategies.

In this study, the RNA profile of a large panel of inflammatory genes, in particular cytokines, chemokines and receptors, has been analysed in eight surgical tumor samples and corresponding normal mucosa, from CRC patients. Several chemokines and receptors were found to be strongly up regulated in tumor tissues. The expression of selected genes, such as CCL3, CCL4, CXCL8 and CX_3CL1, was further investigated and confirmed in a larger number of samples.

Further studies focus on a specific chemokine-receptor pair: CX_3CL1 and CX_3CR1. CX_3CL1 is a transmembrane protein acting both as an adhesion molecule as well as a chemokine when cleaved by specific protease. It has a single receptor, CX_3CR1, which has been primarily studied on immune cells. A number of reports indicate that the CX_3CL1-CX_3CR1 axis is involved in the pathogenesis of different chronic inflammatory conditions, but its role in cancer has not been elucidated. By immunohistochemistry, we analysed the expression of CX_3CL1 and CX_3CR1 in a series of 144 paraffin-embedded CRC tissues. We found that CX_3CL1 and CX_3CR1 were already faintly expressed by healthy epithelial cells, and their expression progressively increased in pre-cancerous lesions and in tumor tissues, with a heterogeneous staining. Based on the median score value of CX_3CL1 and CX_3CR1 staining, we divided our case list in high and low expressing tumors. Correlating these results with patients' clinical and pathological features, we found that patients with high expression of CX_3CL1 and CX_3CR1 showed a trend to better prognosis. Of great interest, the concomitant high expression of both CX_3CL1 and CX_3CR1 (positive-axis tumors) resulted in significantly increased disease-specific survival and disease-free survival.

Given the role of CX_3CL1 in the recruitment of immune cells at sites of inflammation, we investigated whether high expression of CX_3CL1 correlated with an increased amount of tumor infiltrating lymphocytes ($CD3^+$ cells, known to be protective in CRC) or macrophages ($CD68^+$ cells). To our surprise, CX_3CL1 expression by tumor cells did not correlate either with the density of $CD3^+$ nor $CD68^+$ cells at tumor invasive front, indicating that the CX_3CL1-associated favourable prognosis is not mediated via the recruitment of these immune cells.

Since CX_3CL1 is able to act as an adhesion molecule for CX_3CR1^+ cells, we hypothesized that the concomitant expression of CX_3CL1 and CX_3CR1 by tumor epithelial cells could function as an adhesive loop, increasing the adherence among cells and preventing tumor spreading. This hypothesis is supported by our findings that CRC metastatic tumor cells in lymph nodes and liver showed a reduced expression of CX_3CL1 and CX_3CR1 in comparison to the corresponding primary tumors. More interesting, patients with positive-axis tumor showed a reduced risk of developing metachronous metastasis.

To have insights into the functional interaction of the two molecules, we ectopically overexpressed CX_3CL1-cherry fusion protein and CX_3CR1-GFP fusion protein in two different human colorectal cancer cell lines. *In vitro* studies demonstrated that the expression of CX_3CR1 and CX_3CL1 strongly increased tumor cell adhesion but not cell proliferation. Using a multicellular tumor spheroid (MTS) model obtained by mixing CX_3CL1-$cherry^+$ cells with CX_3CR1-GFP^+ cells, we observed by immunofluorescence the presence of several spots of colocalization, confirmed by FRET analysis, suggesting that tumor cells indeed interact in a CX_3CL1-CX_3CR1-mediated mechanism. To gain support on our hypothesis regarding the putative protective role of CX_3CL1-CX_3CR1 in preventing tumor metastasis, *in vivo* experiments have been undertaken. Of note, the presence or absence of the CX_3CL1-CX_3CR1 axis did not influence tumor growth (subcutaneous) in nude mice. A syngenic model of spleen-liver metastasis is in progress.

Taken together, these data indicate that the presence of CX_3CL1-CX_3CR1 axis is a good prognostic marker in CRC patients. The lack of this axis is associated with an increased risk of tumor spreading and metastasis. The mechanistic explanation of these results still needs to be elucidated.

Our observation on the role of CX_3CL1-CX_3CR1 axis in CRC suggests a new biological significance of the involvement of chemokines in tumors. While the presence of chemokines and their receptors in cancer has been usually associated with tumor progression, we demonstrate in this study that the expression of the intact CX_3CL1-CX_3CR1 loop by CRC epithelial cells is associated with lower degree of tumor recurrence and metastasis.

These results suggest a different point of view in the paradigm of chemokines and cancer-related inflammation.

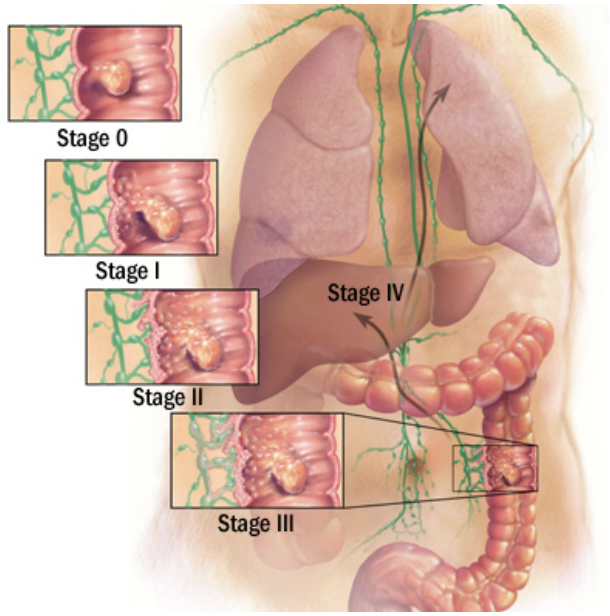
INTRODUCTION

Chapter 1

COLORECTAL CANCER

Colorectal cancer (CRC) is one of the most frequent human neoplasia and the third cause of cancer death in industrialized countries [1]. The pathogenesis of CRC is a multi-step process of genetic alterations that confer a selective growth advantage to the colonic epithelial cells and drive the transformation from normal epithelium to aberrant crypt foci, polyps, adenomas and finally carcinomas. Most cases of CRCs are sporadic, and genetic or environmental factors, such as diet, sedentary lifestyle, obesity and smoking, are important [1]. About 20% of all patients with CRC are estimated to have some component of familial risk without fulfilling the strict criteria for hereditary colorectal cancer. 5-10% of all CRCs develop in the settings of well-defined hereditary cancer syndromes, such as hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) [2].

Although broad generalization can be made regarding the diagnosis and treatment approach of CRCs based on Tumor Node Metastasis (TNM; T: depth of tumor invasion, N: regional lymph node metastasis, M: evidence of distant metastasis) staging, there is a considerable heterogeneity in the natural history of individual tumors (fig 1). Careful pathologic reporting of colorectal cancer resection specimen is of primary importance to determine the diagnosis and to plan the treatment of individual patients. According to the WHO (World Health Organization) classification, essential pathologic parameters are: histologic type, grade of differentiation, depth of tumor invasion, margins of resection, regional lymph node, response to neoadjuvant therapy and vascular invasion. Several other histopathologic variables, including perineural invasion, pattern of growth, tumor infiltrating lymphocytes (TILs) at tumor margin and tumor budding have been proposed as prognostic factors in colorectal cancer. Nowadays, these parameters are not commonly employed in the clinical setting. As a matter of fact, tumor budding and TILs represent the most promising prognostic factors to be introduced in the pathologic evaluation of these tumors [3,4]



T—primary tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through the muscularis propria into subserosa or into non-peritonealised pericolic or perirectal tissues
T4	Tumour directly invades other organs or structures and/or perforates visceral peritoneum
N—regional lymph nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1 to 3 regional lymph nodes
N2	Metastasis in 4 or more regional lymph nodes
M—distant metastasis	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage	T	N	M	5-year overall survival[62–66]
Stage I	T1, T2	N0	M0	80–95%
Stage IIA	T3	N0	M0	72–75%
Stage IIB	T4	N0	M0	65–66%
Stage IIIA	T1, T2	N1	M0	55–60%
Stage IIIB	T3, T4	N1	M0	35–42%
Stage IIIC	Any T	N2	M0	25–27%
Stage IV	Any T	Any N	M1	0–7%

Adapted from Weitz J et al, Lancet 2005

Fig. 1 Colorectal staging based on TNM classification

TNM staging system for colorectal cancer and published survival rate for different stages are indicated.

Molecular Basis of Colorectal Cancer

It is generally accepted that colorectal cancer develops by genetic alterations. The analysis of the molecular basis of the disease allows developing a more targeted approach to prevention and treatment of this cancer. There are two major pathways involved in CRCs: the chromosomal instability pathway (CIN) and the microsatellite instability pathway (MSI). Recent studies have suggested that there are other routes, including the serrated and epigenetic pathways. Moreover, a cross talk between these pathways has been reported, indicating that many genes are involved in the pathogenesis of colorectal cancer (fig.2) [5,6].

Chromosomal Instability

This is the most common genetic aberration to occur in colorectal carcinomas and has been identified in almost 85% of all cases [7]. While it is widely recognized that colorectal cancers with this abnormality are associated with a poorer prognosis,

the molecular mechanisms involved in this abnormality are actually poorly understood [8].

This pathway is characterized by a multi-step process in which mutations in the adenomatous polyposis coli (*APC*) gene occur early in the development of polyps; *K-ras* mutations arise during the adenomatous stage and mutations of *p53* and deletions on chromosome 18q occur concurrently with the transition to malignancy [5,9]. The long arm of chromosome 18 contains the genes Deleted in Colorectal Cancer (DCC) and two genes involved in TGF-β signalling pathway, *SMAD2* and *SMAD4*.

APC: *APC* is a tumor suppressor gene involved in the Wnt signaling pathway. Normally, *APC* binds to β-catenin to form a complex with AXIN and glycogen synthase kinase (GSK) 3β which is degraded through ubiquitylation: when it is inactivated, accumulated β-catenin translocates from the lateral cell membrane to the nucleus, where it drives the transcription of multiple genes implicated in tumor growth and invasion. Mutations in *APC* gene usually cause defective binding of APC to β-catenin [10], increasing the levels of β-catenin and thus promoting its translocation into the nucleus [11]. About 50% of sporadic tumors with wild-type *APC* are reported to show mutations of β-catenin itself, resulting in the accumulation of β-catenin [12]. Germ-line *APC* mutations give rise to familial adenomatous polyposis (FAP), an inherited cancer-predisposing syndrome in which more than 100 adenomatous polyps can develop: in carriers of the mutant gene, the risk of colorectal cancer by age of 40 years is almost 100% [13].

K-Ras: *K-Ras* is a proto-oncogene which plays a major role in the transduction of intracellular signals mostly related to cellular proliferation. The majority of *K-Ras* mutations occur as activating point mutation in codons 12,13 and 64 [14-16]. Mutated K-Ras protein activates different effector pathways, including RAF/MAPK, JNK and phosphatidylinositol 3-kinase (PI3-K), leading to constitutive growth promotion. Some of the downstream gene targets of K-Ras include the cyclin D1, DNA methyltransferase and vascular endothelial growth factor (VEGF) [17].

K-Ras mutations have been detected in various types of carcinoma, particularly in gastroenterological cancer, such as colon, pancreatic and bile duct

cancer, but also in lung cancer. They have been found in 15%-68% of sporadic colorectal adenomas and in 40%-65% of other cancers [5,18]. Increased expression is seen in dysplastic polyps and is also associated with the progression of the adenoma-carcinoma sequence [19].

p53: *p53* is the most commonly mutated tumor suppressor gene in various malignant tumors. p53 protein plays a variety of crucial roles in the cell including cell cycle regulation, apoptosis, DNA repair and differentiation. *p53* mutations [9] have been identified in 40%-50% of sporadic colorectal cancers [20]. The frequency of *p53* mutations is higher in distal colon and rectal cancer than in proximal colon cancers [21]. Patients with cancers involving a *p53* mutation have a worse outcome and shorter survival time than patients whose cancers do not have a mutation in this gene [22].

DCC/SMAD: *DCC* and *SMAD* genes are both located on 18q. Allelic losses on chromosome 18q have been identified in approximately 70% of primary colorectal cancers, in particular in advanced tumors with hepatic metastasis [9]. Point mutations of the *DCC* tumor suppressor gene have been identified in approximately 6% of sporadic colorectal cancers [23]. Inactivation of *SMAD* genes, in particular *SMAD4,* has been causally associated with tumor progression [5].

SMAD2 and SMAD4 proteins are involved in TGF-β signalling. The TGF-β/SMAD signalling pathway is composed of TGF-β receptor type I (TGF-βRI) and type II (TGF-βRII) and SMAD proteins. When TGF-β binds to TGF-βRII, which then complexes with TGF-βRI, SMAD2 is phosphorylated and binds to SMAD4. This complex translocates into the nucleus inducing cell growth arrest. Mutations leading to the inactivation of the *SMAD4* gene have been found in 16%-25% of colorectal cancer cases, while alterations of *SMAD2* gene have been identified in 6% of cases [24-26]. Both *TGF-βRII* and *SMAD* mutations are reported to occur with the same timing during carcinogenesis, at the transition from adenoma to carcinoma.

COX-2: aberrant overexpression of cyclooxygenase-2 (COX-2) is thought to have an important role in development of CRC. *COX-2*, an early-response gene induced by growth factors, cytokines, inflammatory mediators and tumor promoters, is overexpressed in as many as 43% of adenomas and 86% of carcinomas [27]. The

tumorigenic effects of COX-2 can be attributed to the production of prostaglandin E2, whose increased levels have been reported in colorectal cancer [28,29]. COX-2 and prostaglandin E2 regulate proliferation, survival, migration and invasion in colorectal tumors. COX-2 also regulates angiogenesis, through the induction of proangiogenic factors such as VEGF and basic fibroblast growth factor [30].

Microsatellite instability

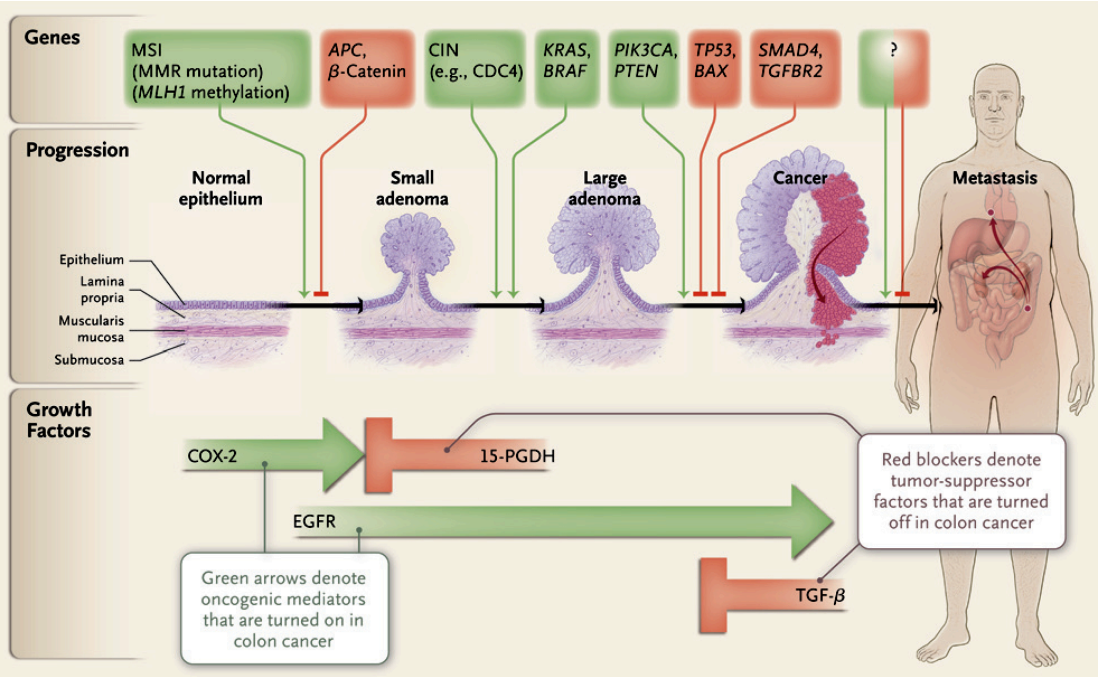
Microsatellite instability (MSI) is characterized by expansion or contraction in the number of tandem repeats of simple DNA sequences, called microsatellites. In contrast to the CIN pathway, the molecular mechanisms that result in MSI are relatively well known. This pathway is closely related to the Mismatch Repair (MMR) system, which corrects any error made by DNA polymerase during the replication of DNA [31]. The microsatellite sequences are particular susceptible to replication errors and therefore any mutation impairing the efficacy of MMR system would predispose to the development of MSI [32].

The MMR system comprises at least seven different proteins (MLH1, MLH3, MSH2, MSH3, MSH6, PMS1 and PMS2) associated in different ways to form heterodimeric proteins that subsequently recognise and repair mismatch errors in DNA replication [33]. An alternative route leading to MMR system deficiency is the silencing of MMR genes by the hypermethylation of their promoters. This appears to be the mechanism occurring in sporadic MSI tumors, involving in particular the MLH1 promoter [34]. MSI tumors can be identified also by the presence of at least 30% unstable loci present among a panel of five mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) [35]. Moreover, mutations in microsatellite of target genes such as TGF-β have been identified in MSI positive tumors [5]. Depending on the degree of instability, MSI tumors can be divided into two sub-classes, MSI-high (MSI-H) and MSI-Low (MSI-L): tumors with 10-29% unstable loci are designated as MSI-L [36,37]. In sporadic colorectal cancers MSI-H is present in 10%-20% and MSI-L in 5%-50% of such cancers.

Sporadic MSI-H colorectal cancers display distinct clinicopathological features compared to microsatellite stable (MSS) or MSI-L tumors, occurring

predominantly in the proximal colon and more frequently in women than in men [38]. Moreover, these cancers are characterized by distinct histopathological features (mucinous or signet-ring cell differentiation), medullary features and high lymphocyte infiltration.

Patients with MSI colorectal tumors show a longer survival times than patients with MSS colorectal tumors [39], indicating the microsatellite instability as a putative prognostic marker for colorectal cancer outcome. In addition, test for MSI can be used to predict patient response to adjuvant chemotherapy, although this practice is controversial: it has been suggested that fluorouracil-based adjuvant chemotherapy is beneficial in patients with stage II or stage III colon cancer exhibiting MSS or MSI-L tumors, but not in those exhibiting MSI-H tumors [40].



Adapted from Markowitz and Bertagnolli, New England Journal of Medicine 2008

Fig. 2: Genes and Growth Factors Pathways Driving the Progression of Colorectal Cancer

In the progression of colon cancer, genetic alterations target the genes that are identified at the top of the diagram. The question mark indicates that genetic or epigenetic changes specific to metastatic progression have not been identified. Key growth factor pathways that are altered during colon neoplasia are shown at the bottom of the diagram. CIN: chromosomal instability; EGFR: epidermal growth factor receptor; 15-PGDH: 15-prostaglandin dehydrogenase; TGF-β: transforming growth factor β.

The serrated and the epigenetic pathways

The serrated pathway has only recently been considered as a distinct pathway to colorectal carcinogenesis. Previously, colorectal polyps were divided into two subgroups: adenomas, normally considered precursors to colorectal cancer, and hyperplastic polyps, normally indicated to be benign with no metastatic potential. Recently, several studies identified hyperplastic polyps as a heterogeneous group of polyps, indicated as traditional serrated adenomas (TSA), sessile serrated adenomas (SSA) and true hyperplastic polyps. There is now compelling evidence to suggest that both TSA and SSA have the potential to develop into malignancy, progressing via different pathways. TSA are usually associated to MSS or MSI-L tumors, while SSA is usually related to MSI-H colorectal cancer. In addition *K-Ras* mutations have been found in almost 80% of TSA but are very rare in SSA. On the contrary, SSA lesions tend to be characterized by mutations in *BRAF* gene, a member of the RAF family of serine/threonine kinase involved in the RAS/RAF/MAPK pathway.

Recent molecular biology studies have revealed that epigenetic mechanisms play an important role in colorectal carcinogenesis. CpG-rich islands located at the 5' end of coding sequences can undergo hypermethylation, leading to the silencing of the associated gene. Cancers showing methylation and silencing of multiple genes are indicated as CIMP positive. The *MLH1* gene involved in MSI-H phenotype is a frequent target of hypermethylation. Many other genes, including $p16^{INK4A}$, estrogen receptor (*ER*), *APC* and cycloxigenase-2 (*COX-2*) have been reported to undergo hypermethylation and silencing in human colorectal cancers.

Predictive/prognostic markers and therapeutic treatments

Studies that aid in the understanding of colorectal cancer on a molecular level have provided important tools for genetic testing for high-risk familial forms of the disease, predictive markers for selecting patients for certain classes of drug therapies, and molecular diagnosis for the non-invasive detection of early cancers. In addition, biological pathways that could form the basis of new therapeutic agents have been identified [6].

Up to date, anti-EGFR therapy is the only application of colorectal cancer genomics used in clinical therapies [41-44]. On the contrary, several genomics markers resulted to be useful for prognosis. Germ line mutations in tumor-suppressor genes, such as *APC, MHL1* and *MSH2* indicate a very high risk of colorectal cancer and guide the frequency of colorectal cancer surveillance and recommendations for surgery (fig. 3) [39,45-47].

Poor survival in stage II and III colon cancer is associated with the loss of heterozygosity at chromosomal location 18q [46]. Sporadic colorectal cancers with mismatch-repair deficiency generally are less likely to be associated with distant metastasis and have a favourable prognosis [48].

Screening is effective in reducing mortality from colorectal cancer. Screening procedures include faecal occult-blood tests, flexible sigmoidoscopy, double-contrast barium enema and colonoscopy [49]. Colonoscopy is the gold standard for diagnosis of colorectal cancer, together with abdominal ultrasound and chest radiography [1]. For patients with a personal or familial history of colorectal neoplasia, FAP, HNPCC or inflammatory bowel disease, special surveillance guidelines exist taking into consideration the higher risk [50].

DNA Marker	Comments
Prognostic	
APC	A germ-line mutation defines the colorectal-cancer predisposition syndrome, familial adenomatous polyposis, with an 80 to 100% lifetime risk of colorectal cancer. Patients with germ-line *APC* mutations undergo prophylactic colectomy or proctocolectomy.
MLH1, MSH2, MSH6	A germ-line mutation in these and, less commonly, in other mismatch-repair genes defines hereditary non-polyposis colon cancer, with a 40 to 80% lifetime risk of colorectal cancer, as well as an increased risk of endometrial cancer. Patients with germ-line mismatch-repair gene mutations undergo frequent colonoscopic surveillance and may be considered for prophylactic colectomy and hysterectomy.
MLH1 methylation–associated silencing	The somatic inactivation of *MLH1* in primary colorectal cancers is evidenced by either detection of DNA microsatellite instability or loss of tumor MLH1 protein expression on immunohistochemical analysis, and is more frequent in early-stage colorectal cancers than in advanced disease. Such inactivation may be a marker of more indolent disease or a better prognosis in the absence of adjuvant chemotherapy.
18q Loss of heterozygosity	The somatic loss of heterozygosity at chromosomal location 18q, a site containing genes associated with colorectal cancer (e.g., *SMAD4* and *SMAD2*), is associated with a poorer outcome in patients with stage II or stage III colon cancer than that in patients with tumors retaining both parental alleles at 18q.
Predictive	
KRAS	The somatic mutation produces unrestricted activity of signaling through the MAPK and PI3K cascades. Patients with stage IV colorectal cancer and activating mutations in *KRAS* do not have a response to EGFR-inhibitor therapy.
BRAF V600E	The somatic mutation activating this kinase causes unrestricted MAPK pathway signaling. Patients with stage IV colorectal cancer and the activating *BRAF* V600E mutation do not have a response to EGFR-inhibitor therapy.
MLH1 methylation-associated silencing	The loss of the mismatch-repair function contributes to the loss of other tumor suppressors (e.g., TGFBR2 and BAX). Patients with mismatch-repair–deficient tumors may not have a response to fluorouracil and may have an improved response to irinotecan-containing regimens.

Adapted from Markowitz and Bertagnolli, New England Journal of Medicine 2008

Fig. 3: Prognostic and Predictive DNA markers in Colorectal Cancer

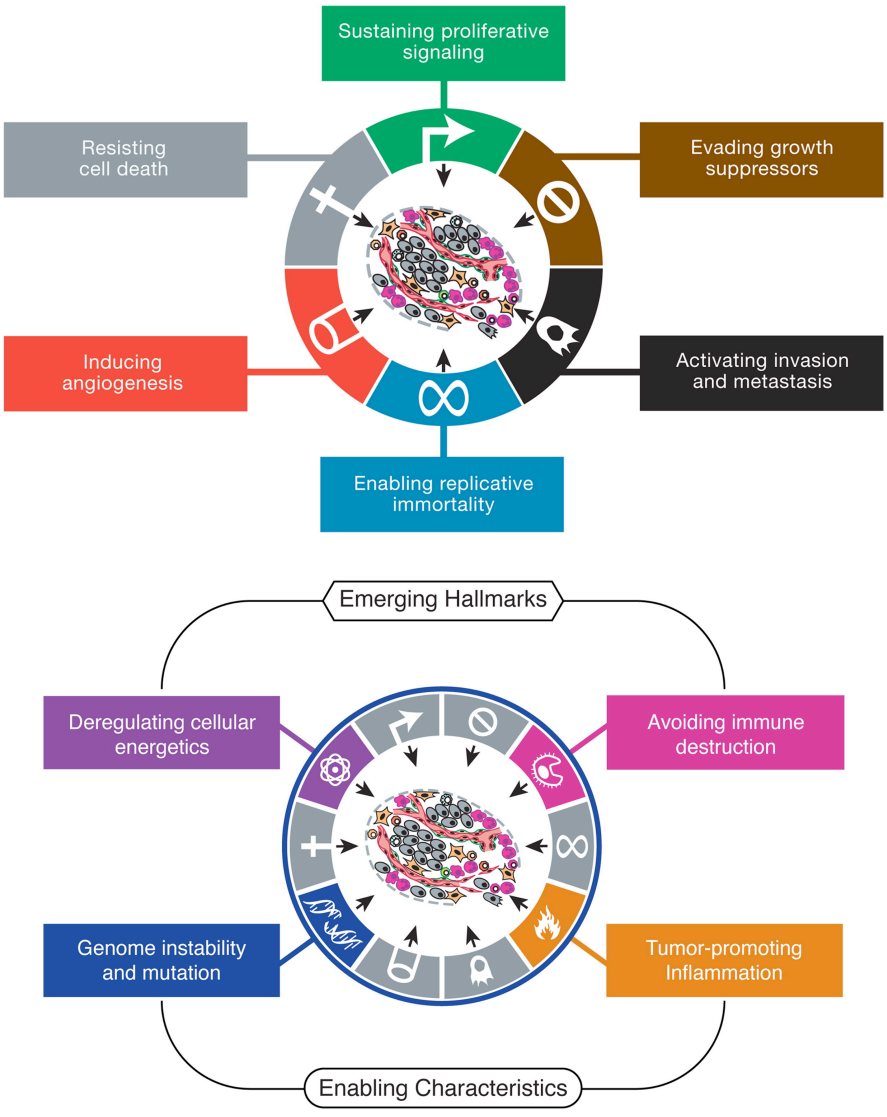
BAX denotes BCL-1 associated X protein, EGFR epidermal growth factor receptor, MAPK mitogen-activated protein kinase, PI3K phosphatidylinositol 3-kinase, TGFβR2 transforming growth factor receptor β type II.

Surgery remains the basis of therapy for colorectal cancer and important developments have occurred. With good surgical technique and selection of patients, excellent results can be obtained by surgery alone without radiotherapy, even in lymph node positive patients [51]. Radiotherapy aims to reduce local recurrence and improve survival in patients with rectal cancer. Traditionally, adjuvant radiochemotherapy was considered standard care for patients with stage II and III rectal cancer. Neo-adjuvant radiochemotherapy was reserved for advanced rectal cancer. Irinotecan, oxaliplatin, capecitabine and 5-fluorouracil are the four most commonly used drugs, while monoclonal antibodies, including cetuximab (anti-EGFR), panitumumab (anti-EGFR), bevacizumab (anti-VEGF-A) and other drugs have been used alone or in combination with chemotherapy. Fluorouracil-based adjuvant chemotherapy is beneficial in patients with stage II or stage III colon cancer exhibiting MSS or MSI-L tumors, but not in those exhibiting MSI-H tumors [40].

Chapter 2

CANCER AND INFLAMMATION

In addition to genetic and epigenetic abnormalities, the formation of an inflammatory microenvironment plays a pivotal role in carcinogenesis and disease progression (fig.4) [52,53]. Links between tumor and inflammation were first made in the nineteenth century, on the basis of observations that tumors often arose at sites of chronic inflammation and that inflammatory cells were present in biopsied samples from tumors [52,54,55].



Adapted from Hanahan and Weinberg, Cancer Cell 2011

Fig. 4: Inflammation as the major hallmark of cancer.

In 2000, Hanahan and Weinberg proposed a model to define the six properties that a tumor acquires. This model has now been revised to include cancer-related inflammation as an additional hallmark.

Epidemiological studies have shown that chronic inflammation predisposes to various types of cancer. There are many triggers of chronic inflammation that increase the risk of developing cancer. Such triggers include microbial infection (for example, infection with *Helicobacter pylori* is associated with gastric cancer and gastric mucosal lymphoma), autoimmune disease (inflammatory bowel disease is associated with colon cancer) and inflammatory conditions of unknown origin (for example, prostatitis is associated with prostate cancer). Accordingly, treatment with non-steroidal anti-inflammatory agents decreases the incidence of several tumor types [56-58].

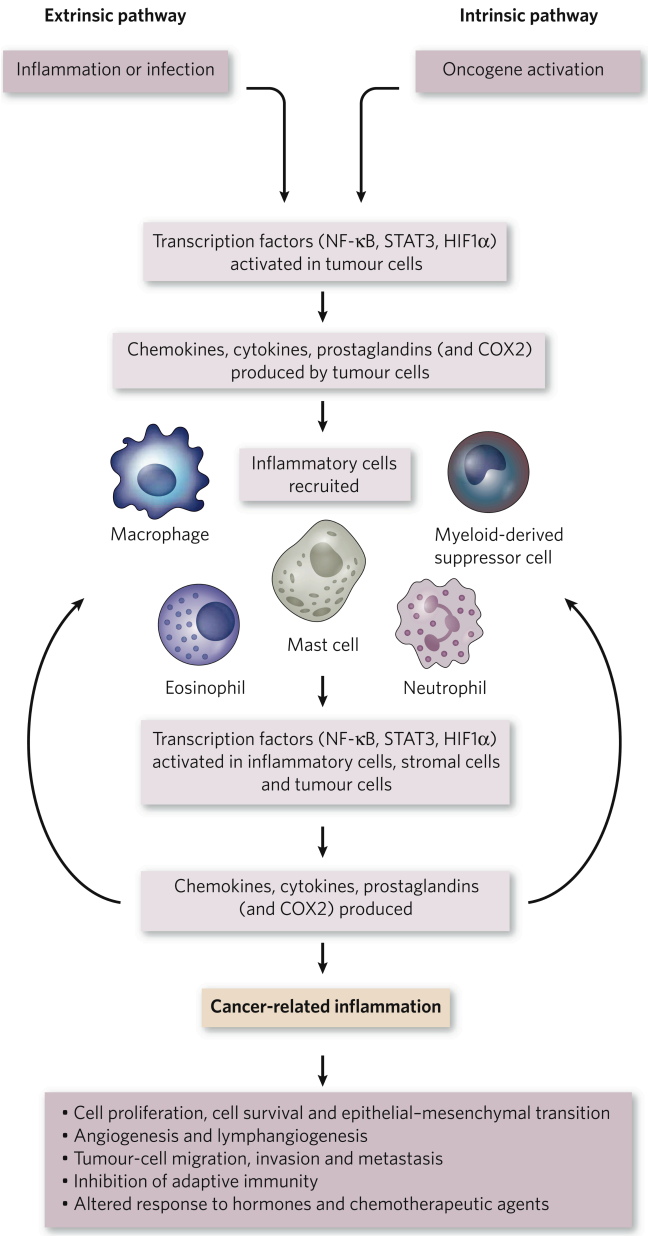
The hallmarks of cancer-related inflammation include the presence of inflammatory cells (mainly macrophages and lymphocytes), the presence of specific inflammatory signalling molecules (such as cytokines, chemokines and growth factors), tissue remodelling and the formation of new blood vessels (angiogenesis) [52,59].

The intrinsic and the extrinsic pathways

The connection between inflammation and cancer can be viewed as consisting of two pathways: an extrinsic pathway, driven by inflammatory conditions that increase cancer risk, and an intrinsic pathway, driven by genetic events that cause neoplasia (fig. 5). These events include the activation of various types of oncogenes by mutation, chromosomal rearrangement or amplification and the inactivation of tumor suppressor genes. In addition to promote cell autonomous proliferation, activated oncogenes are able to induce down stream a signalling cascade leading to the production of inflammatory mediators, thereby generating an inflammatory tumor microenvironment. For example, activated components of the RAS-RAF signalling pathway induce the activation of the nuclear transcription factor κB (NF-κB) and the production of several inflammatory cytokines and chemokines [60,61]. Another oncogene, MYC, encodes a transcription factor that is overexpressed in many human tumors. In addition to promoting cell-autonomous proliferation, MYC regulates the remodelling of the extracellular microenvironment, with inflammatory cells and mediators having important roles in this process [62,63]. A

further example is offered by the tyrosine kinase RET, a prototypic transforming oncogene in human papillary carcinoma of the thyroid (PTC). RET activates an inflammatory programme leading to the production of several mediators, including colony-stimulating factors (CSFs), interleukin-1 (IL-1), COX-2 and chemokines attracting monocytes and dendritic cells (CCL2 and CCL20) [64].

While these results unequivocally demonstrate that dominant oncogenes (the intrinsic pathway) promote the formation of an inflammatory microenvironment, they do not address the issue how persistent inflammatory conditions (the extrinsic pathway) increase cancer risk. Indeed, cancer-associated inflammation affects many aspect of malignancy, including cell proliferation and survival, angiogenesis and tumor metastasis [52,53,59,65,66]. This interplay is likely to occur in pancreatic carcinoma, in which both pancreatitis and mutations in the gene encoding *K-Ras* are frequently found. Both mild chronic pancreatitis and mutated *K-Ras* are required to induce pancreatic cancer, indicating that, although the RAS-RAF pathway can drive tumor-promoting inflammation, an extrinsic inflammatory condition (pancreatitis) is needed to drive full-blown carcinogenesis [67]. Tumor-suppressor proteins are also able to regulate the production of inflammatory mediators: examples of such proteins are the von Hippel-Lindau tumor suppressor (VHL), TGF-β and phosphatase and tensin homologue (PTEN) [68-70]. VHL is a component of the molecular complex that targets the transcription factor hypoxia-inducible factor 1 α (HIF-1α). HIF-1α promotes the cellular and tissue responses to hypoxia, including the angiogenesis process. It also interacts with NF-κB resulting in the production of the inflammatory cytokine tumor-necrosis factor α (TNF-α) and the chemokine receptor CXCR4 [65,68,70,71]. The overexpression of CXCR4 is particularly relevant, since it is frequently overexpressed in cancer and is involved in metastasis [68].



Mantovani A. et al, Nature 2008

Fig. 5: Pathways that connect cancer and inflammation: extrinsic and intrinsic pathways

Two pathways connect Cancer and inflammation: the intrinsic pathway is activated by genetic events that cause neoplasia; by contrast, in the extrinsic pathway, inflammatory or infectious conditions augment the risk of developing cancer at certain anatomical site.

Chapter 3

INFLAMMATION AND COLORECTAL CANCER

Colorectal cancer represents a paradigm of the cancer-related inflammation. Patients affected by inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), are at increased risk of developing neoplasia, with an extended incidence rate of 2.75 and 2.64 of CRC in patients with UC and CD, respectively [72-74]. Interestingly, in *Helicobacter Pylori*-induced gastric tumors, pro-inflammatory signalling by TNF-α can induce β-catenin nuclear accumulation even in the absence of mutations in the *APC* gene.

During colorectal carcinogenesis, colonic epithelial cells accumulate genetic mutations that confer a selective growth advantage to the neoplastic epithelial cells, leading to the transformation from normal epithelium to adenomatous polyps and finally to invasive colorectal cancer. Transformed epithelial cells are also able to secrete several pro-inflammatory mediators that act on various types of pro-inflammatory leukocytes, endothelial cells and fibroblasts to establish a tumor promoting reactive microenvironment: among these, are cytokines such as TNF-α, IL-1, IL-6, chemokines (see chapter **5**) cyclooxygenase-2 (COX-2), innate immunity receptors and signalling molecules, such as Toll-like receptors (TLR)-4 MyD88 and the transcription factor NF-κB.

- ***TNF-α:*** TNF-α is a member of the TNF cytokine superfamily and is a key molecule regulating inflammation and host defence. Activation of TNF receptors (TNFRs) can trigger NF-κB and downstream survival pathways, or can activate caspase 8 and the associated apoptotic signal [75]. This cytokine has controversial roles in cancer, serving as a tumor-promoting or tumor-destructive factor. The contribution of TNF-α in the development of CRC has been recently investigated in a genetic mouse model lacking the type 1 TNFR-p55 [76]. In this study, the abrogation of TNF-α signalling in mice resulted in a significantly reduced colitis after treatment with azoxymethane (AOM) and dextran sodium sulphate (DSS), with decreased tissue damage, inflammatory cell infiltrates and cytokine production in the mucosa.

- ***NF-κB:*** NF-κB is a key regulator of innate immunity and inflammation. While in normal condition NF-κB is kept in an inactive form, in various types of cancer, including CRC, is constitutively activated. It has been demonstrated that the abrogation of NF-κB in intestinal epithelial cells in mice did not affect the extent of

inflammation, but resulted in a dramatic reduction in tumor number as a consequence of enhanced epithelial cell apoptosis during early tumor development [77]. In contrast, the conditional inactivation of NF-κB in myeloid cells strongly reduced the expression of many genes involved in the inflammatory responses (such as IL-1β, TNF-α, MIP-2, COX-2 and intercellular adhesion molecule ICAM), resulting in a reduction of tumor size. Thus, NF-κB activation controls both the survival of transformed epithelial cells as well as the leukocyte-driven inflammation that provides signalling molecules that sustain tumor growth.

- ***Toll-like receptors (TLRs):*** TLRs play a major role in sensing gut microbiota and activation of these receptors is required to maintain intestinal homeostasis. Genetic or functional dis-regulation of TLRs may be linked to chronic inflammation and tumor development. For example, polymorphisms in TLR-4, a receptor required for innate immunity against gram-negative bacteria, have been associated with UC and CD [78]. In addition to their involvement in IBD, aberrant TLR signalling may contribute to the tumor-promoting activity of NF-κB. Recently, it was shown that a deficiency in MyD88, the TLR adaptor protein, significantly reduces the number and size in the APC^{min} mouse model of intestinal tumorigenesis [79]. Moreover, mice inoculated with colon cancer cells in which *TLR4* was silenced showed an increased survival and a reduction in tumor size in comparison to control mice [80].

- ***COX-2:*** COX-2, the enzyme involved in the synthesis of prostaglandins and prostacyclins from arachidonic acid, is strongly related to colorectal carcinogenesis. COX-2 is not constitutively expressed in the colon mucosa, but its expression increased in adenomas and colon carcinoma [81]. Indeed, overexpression of COX-2 in mouse tumor xenografts enhanced tumor growth due to a proangiogenic effect. On the other hand, its absence inhibits the development of colorectal polyps in mice [82,83].

  Several polymorphisms in the flanking regions of COX-2 have been described, but their association with the risk of CRC remains unclear. However, a COX-2 variant (c.3618A/G), possibly affecting RNA stability, was associated with the presence of clinical features of good prognosis and higher survival rate of patients [81].

- ***IL-6:*** IL-6 is considered a major key player in the transition between acute and chronic inflammation as well as innate and acquired immunity [84]. IL-6 modulates the expression of chemokines and adhesion molecules, suppressing neutrophil infiltration, while promoting the accumulation of mononuclear leukocytes, leading to the resolution of acute inflammation and the activation of acquired immunity [85,86]. Evidence of the role of IL-6 in intestinal inflammation comes from the demonstration that the inhibition of IL-6 signalling affects chemotaxis and apoptosis of lamina propria mononuclear cells, improving disease outcome in animal model of colitis [87]. In the last years, the involvement of IL-6 in CRC has been deeply investigated. Several studies indicate that IL-6 stimulates the growth of colon cancer cells *in vivo* [88], increases invasiveness of colon cancer cells and likely promotes secondary tumor formation [89,90]. Moreover, serum levels of IL-6 in colorectal cancer patients are higher than in healthy controls and significantly correlate with tumor staging and poorer survival rate [91]. In addition, IL-6 production by immune cells within the lamina propria in response to intestinal injury is NF-κB-dependent and regulates the proliferation of intestinal epithelial cells during acute inflammation and colitis-associated cancer (CAC) [92]. Therefore, myeloid cell-derived IL-6 is considered an important player in the leukocyte-driven inflammation that promotes colon carcinogenesis.

  Formal evidence has been provided that IL-6-activated JAK-STAT3 pathway is also of major importance. STAT3 induces the expression of genes involved in cell cycle progression and suppression of apoptosis, eventually promoting cell survival and proliferation during colitis-associated carcinogenesis. The capacity of STAT3 to support intestinal cell proliferation not only facilitates healing after colitis-induced tissue injury, but also promotes mutagen-triggered transformation [92,93].

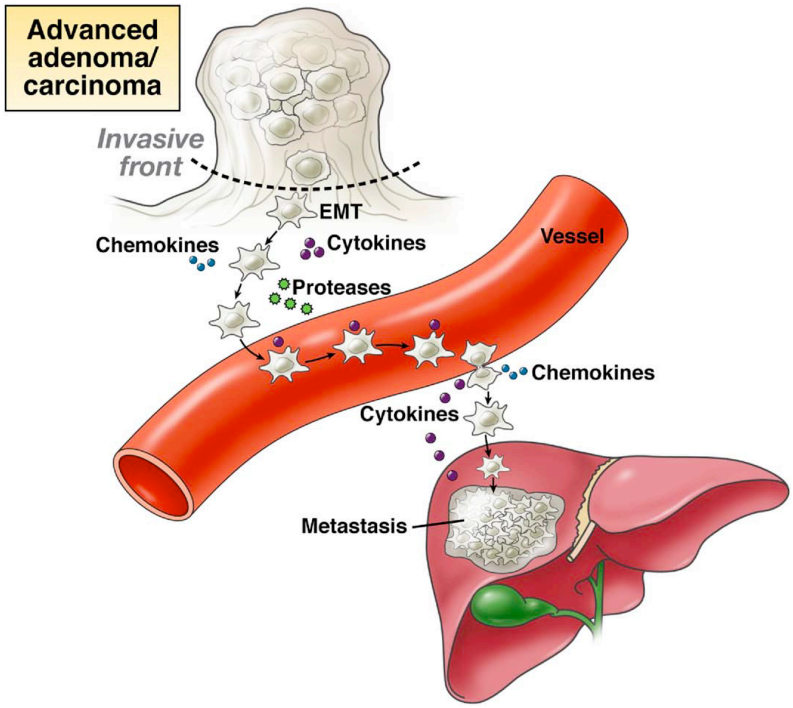
Inflammation in invasive and metastatic CRC

The role of immune cells and their products in metastasis of colorectal and colitis-induced tumors has not been examined in details because of the lack of suitable mouse models. Nevertheless, immune and inflammatory cells are present in advanced and metastasizing tumors, especially at the leading edge of the tumor,

where invasion takes place [94,95]. The inflammatory microenvironment can influence several key stages of metastasis [96]. The first step is represented by the "epithelial to mesenchymal transition" (EMT), in which cancer cells acquired fibroblastoid characteristics that increase their motility and allow them to invade epithelial lining/basal membranes to reach efferent blood vessels or lymphatics. The process is largely regulated by TGF-β and is characterized by loss of E-cadherin expression by malignant epithelial cells (fig. 6) [97].

EMT can be facilitated by the activation of NF-κB and/or STAT3 signalling [98,99]. In addition to TGF-β, EMT can be regulated by several proinflammatory cytokines, including IL-1, TNF-α, HIF-1α and IL-6: these molecules activate transcription factors that regulate EMT, such as ZEB1, ZEB2, Twist, Kiss and Snail [96,100,101]. Activated Snail represses E-cadherin transcription in epithelial cells. TNF-α signalling stabilizes Snail by preventing its degradation, thus promoting metastasis of colon cancer cells [96].

Inflammatory signals also regulate the production and activity of proteases that degrade the extracellular matrix and facilitate invasion and extravasation of cancer cells [102]. Human colorectal cancer overexpress MMP 1, 2, 3, 7, 9 and 13 [103]. Pro-inflammatory cytokines (e.g. TNFα and TGFβ) and chemokines can increase the expression of various MMPs, providing an independent stimulus for increased cell migration [103,104]. In addition, TNF family cytokines regulate the expression of MMP inhibitors such as TIMP3 or maspin [105,106].



Terzić J, Gastroenterology 2010

Fig. 6. The role of inflammation in angiogenesis, invasion and metastasis

Cytokines, chemokines and proteases (MMP) produced by inflammatory cells influence the processes of angiogenesis and metastasis. They alter the migratory properties and behaviour of cancer cells, degrade the ECM, and increase the tumor blood supply and access to vasculature. Immune cells also create chemokine gradients that guide the migration of metastatic cells through the tissue to distant organs, and facilitate other aspects of metastasis.

Chapter 4

CROSSTALK BETWEEN CANCER AND IMMUNE SYSTEM

Tumor immunosurveillance in human cancer

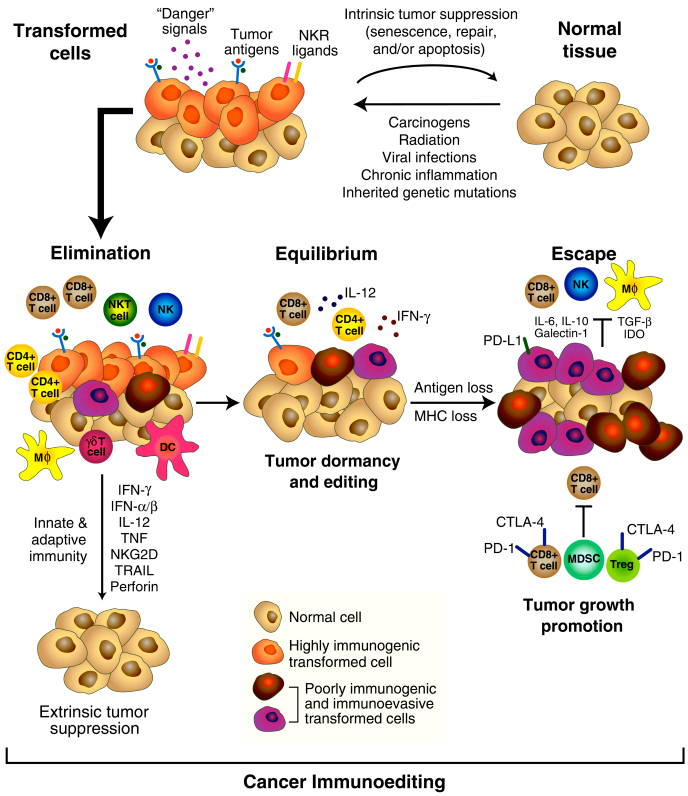
The complex relationship between tumours and the immune system has long been studied. Cancer cells express tumour-associated antigens able to trigger the host immune response [107,108,55,109] Indeed, in the last decades, there has been growing evidence that cells of the adaptive immunity are present at tumour sites and are usually associated with more favourable prognosis. The best evidence is in human colorectal cancer, where $CD3^{+}CD8RO^{+}$ lymphocytes are clearly associated with longer disease free and specific survival [110,111]. Also in other human tumours, such as ovary, melanoma and breast, the density of T cells is usually correlated with a more favourable outcome. In marked contrast, cells of the innate immunity, especially macrophages, most frequently favour tumour progression, in the majority of cancers. Tumour-Associated Macrophages (TAMs) are recruited early at tumour sites where they most frequently display pro-tumour functions, such as the activation of the neo-angiogenic switch, the secretion of soluble factors that support tumour cell resistance to apoptotic stimuli and stimulate the proliferation and invasion of malignant cells. TAMs have been also associated with the suppression of adaptive immunity [112-117].
Thus, in a simplified scheme, adaptive immunity is usually protective and limits tumour progression, while innate immunity favours disease development. However, biological mechanisms are more complex: recent evidence has been provided that components of adaptive immunity (e.g. IL-4-producing $CD4^{+}$ T cells and antibody-producing B cells) may activate innate immune cells in a pro-tumour manner [116,118]. Therefore, the dynamic interplay between innate and adaptive immunity is of paramount importance in the outcome of tumour progression or regression.

The "immunoediting" process

In the recent years, data collected from large cohorts of human cancers have demonstrated that the immune contexture of the primary tumors is an essential prognostic factor for patients' disease-free and overall survival. Global analysis of tumor microenvironments showed that the nature, the functional orientation, the

density and the location of innate and adaptive immune cells could influence the tumor progression and the risk of relapse events [119].

The notion that the immune system not only protects the host against tumor formation but also shape tumor immunogenicity is the basis of the cancer immunoediting hypothesis, which stresses the dual host-protective and tumor-promoting actions of immunity on developing tumors. The cancer immunoediting process proceeds sequentially through three distinct phases termed "elimination", "equilibrium" and "escape" (fig. 7) [109]. In the elimination phase, innate and adaptive immune system work together to detect the presence of a developing tumor and destroy it before it becomes clinically apparent. Rare tumor variants may survive the elimination phase and enter the equilibrium phase, in which adaptive immune system prevents tumor cell outgrowth and also sculpts the immunogenicity of the tumor cells [120]. In the escape phase, tumor cells that have acquired the ability to circumvent immune recognition and/or destruction emerge as progressively growing, visible tumors [121,122].



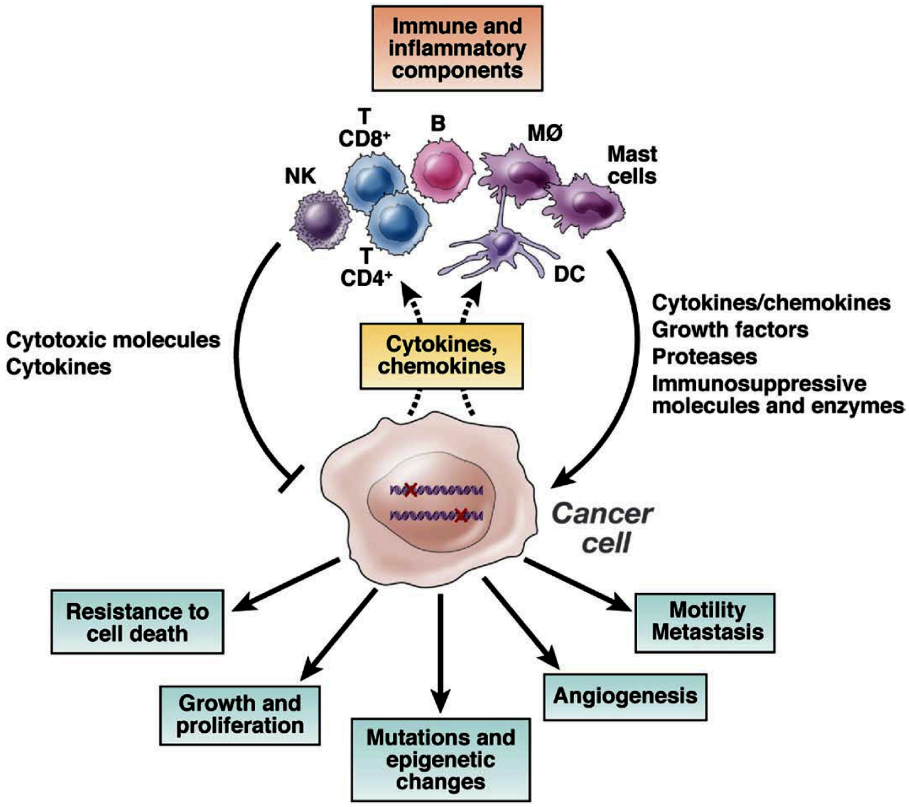
Schreiber RD, Science 2011

Fig. 7: The cancer immunoediting concept

Cancer immunoediting is an extrinsic tumor suppressor mechanism that engages only after cellular transformation has occurred and intrinsic tumor suppressor mechanism has failed. In its most complex form, cancer immunoediting consists of three sequential phases: elimination, equilibrium and escape.

Tumor infiltrating leukocytes in CRC

A leukocyte infiltrate is already present in benign adenoma and is markedly increased in CRC tissues. Immune cells are localized both at the periphery and in tumor stroma, occasionally invading cancer cell nests. Most represented leukocytes are T lymphocytes and macrophages, although some eosinophils, mast cells, NK cells and rare DC can be found (fig. 8)[123-126].



Terzić J, Gastroenterology 2010

Fig. 8: Immunosurveillance and inflammation in CRC

Immunosurveillance is typically mediated by cytotoxic and helper T lymphocytes as well as natural killer (NK) cells. Innate and adaptive immune cells can also promote tumor development. In the pathogenesis of CRC, inflammation can promote colitis-induced tumorigenesis.

Tumor infiltrating Lymphocytes (TILs) in CRC

As for other neoplastic tissues [114], an abundance of $CD3^+$ T cells in CRC is usually associated with more favourable prognosis. Indeed, the most convincing results of a protective anti-tumor effect of $CD3^+$ lymphocytes have been provided in human CRC.

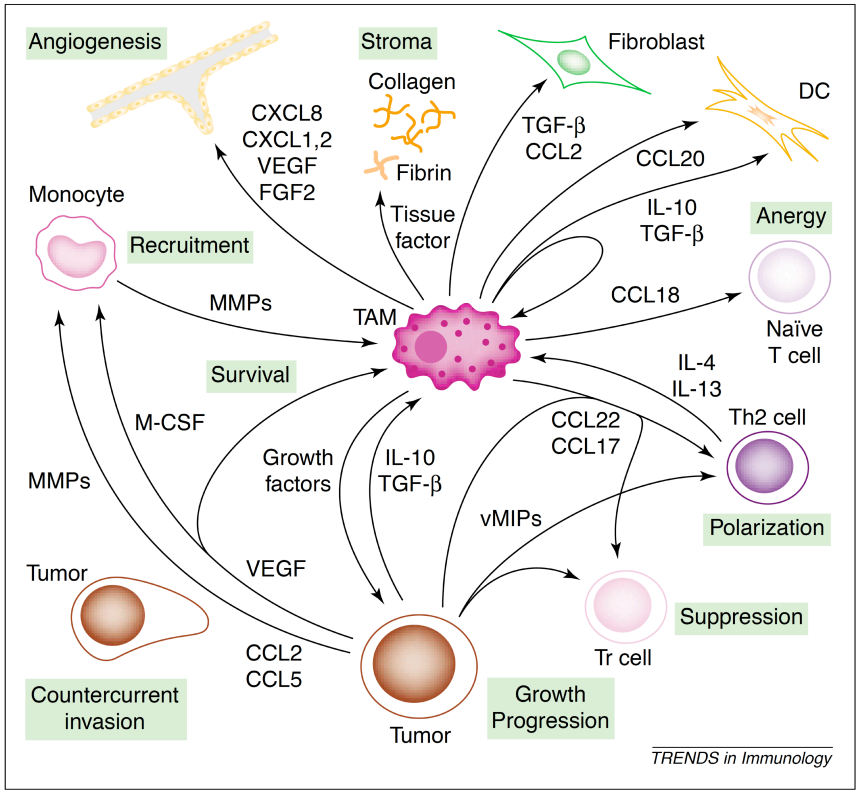
Several studies confirmed that high rate of tumor-infiltrating lymphocytes (TILs), in particular located intra-epithelially, is beneficial for patient outcome, being associated with earlier tumor stage, decreased local recurrence rate after surgery and improved overall and disease-free survival both in metastatic and non-metastatic patients [127-129]. Furthermore, it has been demonstrated that genes involved in Th1 immune responses are inversely correlated with tumor recurrence, indicating that adaptive immunity may promote patient survival [130]. The increased levels of mRNA for products and markers of Th1 effector T cells (CD8, T-BET, IFNγ, granulosin and Granzyme-B), are associated with prolonged survival and the absence of pathological signs of early metastatic invasion, such as vascular emboli, lymphatic invasion and perineural invasion (VELIPI) [110]. Moreover, the presence of effector memory T cells within tumors, defined by the presence of CD3, CD8, CD45RO, CCR7, CD28 and CD27 markers was associated with VELIPI-negative tumors. More recently, it ha been demonstrated that $CD3^+$ cells at the invasive front are associated with a lower risk of metachronous metastasis and a consequently survival advantage. However, this holds true only in node-negative patients (stage II) and loses statistical significance in stage III patients [111].

Tumor-associated Macrophages (TAMs) in CRC

In spite of an abundant literature on many pro-tumor functions of TAM in several tumor types (fig. 9), [113, 131-134], their role in colorectal cancer is quite controversial.

Some studies indicated that macrophages in CRC appear to have anti-tumor activity and are associated with improved survival [135,136]. On the contrary, in other studies

there is evidence that a massive macrophage infiltration is correlated with tumor progression, growth and disease aggressiveness.



Mantovani A. et al, TRENDS in Immunology 2002

Fig. 9: TAMs as M2-polarized macrophages

TAMs are source and target for cytokines and chemokines in the tumor microenvironment. These molecules and other local mediators regulate tumor growth, progression and invasion, interaction with other components of the stroma and the activation and orientation of adaptive immunity.

- ***Pro-tumoral activity of TAMs:*** TAMs of colorectal cancer have been shown to secrete VEGF, thereby promoting angiogenesis and tumor growth [137]. As discussed above, IL-6 has a crucial role in colon tumorigenesis and TAMs are the major producers of this cytokine. In addition, TAM-derived IL-6 induces STAT3-mediated IL-10 production in tumor cells, which has been correlated with poor prognosis [138]. TGF-β is produced by both tumor cells and macrophages, and plays a key role in the epithelial to mesenchymal transition (EMT), thus promoting tumor progression and metastasis [139,140]. Colon cancer cells can also stimulate the production by macrophages of MMP2 and MMP9, promoting cell invasion by disrupting extra-cellular matrix and cleaving cell-adhesion molecules such as E-cadherin [141-143].

Recently, it has been reported that TAMs, through IL-1β, promote Wnt signalling in colon cancer, supporting tumor growth. The reduction or loss of β-catenin and peroxisome proliferator-activated receptor-γ (PPAR-γ) expression was strongly correlated with high TAM infiltration, increased COX-2 and tumor aggressiveness [144-146].

- ***TAMs anti-tumoral activity in CRCs:*** a number of studies have correlated the presence of high macrophage infiltration with good prognosis. Considering the effect of TAMs in colon cancer, their localization appears of primary importance. The expression of co-stimulatory molecules, such as CD80 and CD86, and ICAM-1 were increased in peritumoral macrophages while TAM from cancer stroma had significantly reduced expression [147]. In addition, macrophages along the tumor margin were able to induce apoptosis in cancer cells through a Fas Ligand-dependent mechanism. The number of macrophages correlated with the number of apoptotic cancer cells; further, the degree of cancer cell apoptosis was inversely correlated with haematogenous metastasis, underlining the protective role of TAMs [148]. The anti-tumor effect of TAMs was confirmed by other studies correlating macrophage infiltration and prognosis. The presence of VEGF-expressing TAMs was associated with a favourable outcome [149], and high levels of macrophage infiltration at the invasive margin correlated with an increased overall survival rate. Patients with low levels of macrophages infiltration had more advanced disease, higher rate of vascular invasion and lymph node metastasis [150]. More recently, it has been demonstrated that a dense macrophage infiltration at the tumor front positively influenced prognosis in colon cancer and that direct macrophage-to-tumor cell contact was required to manifest their anti-tumorigenic activity [135]. In agreement with these data, high density of TAMs at the invasive front was associated with lower occurrence of hepatic metastasis and improved prognosis of colorectal cancer patients [151].

Overall, these data highlight an ambiguous role of macrophages in the progression of colorectal cancer, and suggest that anti-tumor or pro-tumor activity of TAMs may depend on their localization within cancer tissue. Peritumoral macrophages are likely to have less exposure to tumor-derived cytokines and are located in less hypoxic area: thereby they may differentiate into a tumoricidal rather

than pro-tumoral activity. The divergent biological activities of TAM in CRC underscore the concept of functionally distinct TAM subsets, whose polarization is ultimately dictated by microenvironmental cues.

TAMs and the modulation of immune response in CRC

Activation of innate immunity is indispensable for the stimulation and orientation of adaptive immune responses, and macrophages are key players in this crosstalk. In the tumor context, TAMs and related myeloid cells have been mainly characterized as inhibitors of T-cell activation. In the last years, several studies focused on the interaction between macrophages and two $CD4^+$ T cells subsets in CRCs: Th17 and regulatory T cells (T_{reg}).

- ***Th17 lymphocytes:*** TAMs produce IL-23, a crucial cytokine for the polarization of Th17 lymphocytes, in concert with IL-1β, IL-6 and TGF-β [152-154]. Th17 lymphocytes produce IL-17 and related cytokines IL-21 and IL-22, which are important mediators to maintain mucosal homeostasis [155]. Human CRC express higher levels of IL-23 mRNA compared to the corresponding cancer-free mucosa [156]. The role of Th17 cells in tumor pathogenesis is still not well defined. Some studies suggested that IL-17 and/or Th17 cells may promote protective tumor immunity [157]; other studies, instead, showed that IL-17 inhibits the expression of Th1- chemokines, such as CXCL10, CXCL11 and CCL5, thus inhibiting the recruitment of Th1 effector cells [158].

- ***Regulatory T Cells (T_{reg}):*** Th17 cells are reciprocally related to T_{reg} cells. The early differentiation of T_{reg} and Th17 cells from naïve $CD4^+$ T cells shares a requirement for TGF-β, indicating a substantial plasticity in the development of these lymphocyte subsets [159]. TAMs are a major source of TGF-β, together with cancer cells, and can directly induce T_{reg} by cell-cell interaction via membrane-bound TGF-β [160]. In human CRC, T_{reg} infiltration is significantly higher in tumor tissues compared to normal mucosa [161]. T_{reg} can suppress the cytotoxicity of $CD8^+$ anti-tumor effectors and are considered to importantly contribute to tumor immune evasion [162]. On the

other hand, other studied showed that the presence of T_{reg} was associated with better patient survival and that they represent an even stronger prognostic factor than tumor infiltrating $CD8^+$ and $CD45RO^+$ T cells [163]. As a confirmation of their potential protective effect, adoptive transfer of T_{reg} into *APC*^{min} mice prevented the formation of intestinal adenoma, and promoted the regression of some established tumors [164].

Very recently, a new regulatory T cell population characterized by the expression of $CD8^+$ $CD25^+$ and $FoxP3^+$ ($T8_{reg}$) was identified in blood and tissues of CRC patients [165]. Interestingly, $T8_{reg}$ cells are significantly elevated and are able to suppress the proliferation and cytokine production of conventional $CD4^+CD25^-$ T cells *ex vivo*. $T8_{reg}$ numbers correlate with levels of IL-6 and TGF-β, as well as with tumor stage and micro-invasive status [165].

Taken together, despite the evidence that T_{reg} inhibit adaptive immunity and promote tumor progression in several neoplasia [166], distinct results are found in CRC. By contrasting the production of inflammatory cytokines, T_{reg} may limit inflammation-dependent cancers, as are most CRC neoplasia. On the other hand, in spontaneous and inflammation-independent cancer, the predominant effects of tumor-induced T_{reg} may result in suppression of protective adaptive immunity.

Chapter 5

CHEMOKINES AND CHEMOKINE RECEPTORS

The eponymous function of chemokines and their receptors is to mobilize cells in a gradient direction. This biological function is particularly important and has been mostly studied for the leukocyte recruitment to sites of inflammation. Over the last two decades, however, it has become increasingly clean that chemokines regulate other important biological function, such as angiogenesis, cell proliferation and survival, stem cell mobilization from hematopoietic niches and extracellular matrix remodeling [167,168].

The chemokine family: structure and nomenclature

Chemokines are a group of small (~8-14 kDa), mostly basic, structurally related molecules that regulate trafficking of various cell types, especially leukocytes, through interaction with a subset of seven-transmembrane, G protein-coupled receptors. About 40 chemokines have now been identified in humans [169].

Gene products are assigned to the chemokine gene superfamily according to the organization of a characteristic cysteine signature in their predicted primary amino acid structure. Based on this cysteine motif, the various members can be divided into four families, indicated as CXC, CC, C and CX_3C (or α, β, γ and δ, respectively). CXC and CX_3C chemokines are distinguished by the presence of one (CXC) or three (CX_3C) amino acids between the first and the second cysteines, whereas the first two cysteines of CC chemokines are adjacent [170]. CXC chemokines can be further sub-divided into ELR (glutamic acid/leucine/arginine)-containing and non-ELR containing chemokines depending on the presence of an ELR motif just prior to the CXC signature (fig. 10).

The ELR-containing CXC chemokines mostly attract neutrophils whereas the non-ELR-containing CXC chemokines attract lymphocytes.

The CC subfamily contains the largest number of chemokines. CC chemokines are chemoattractants for a wide range of cells, including monocytes, granulocytes, neutrophils, lymphocytes and dendritic cells [171]. The only C chemokine cloned to date, XCL1/lymphotactin, has been reported to selectively attract $CD8^+$ T lymphocytes and NK cells. This chemokine has only two cysteines in its primary amino acid sequence [172]. Finally, CX_3CL1/Fractalkine, the only member of the CX_3C

sub-family, has a unique primary amino acid sequence with three amino acids between its first two cysteines, and is chemotactic for monocytes, T and NK cells.

McColl SR, Immunology and Cell Biology 2002

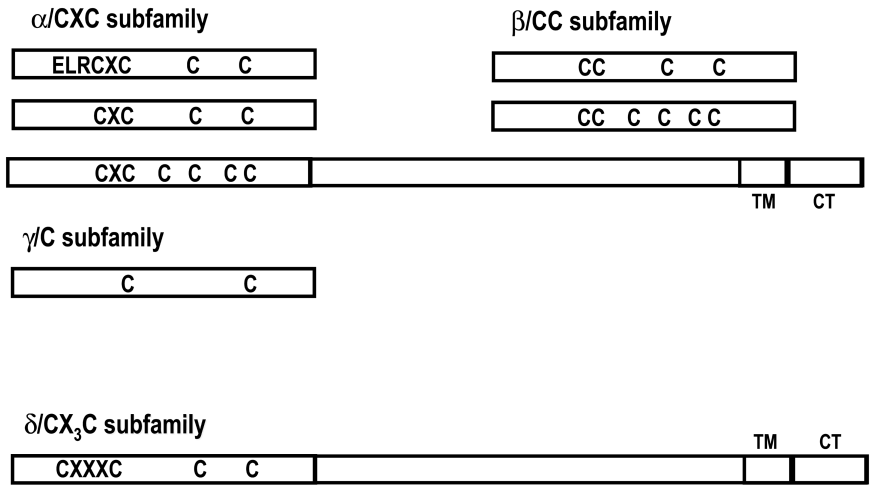
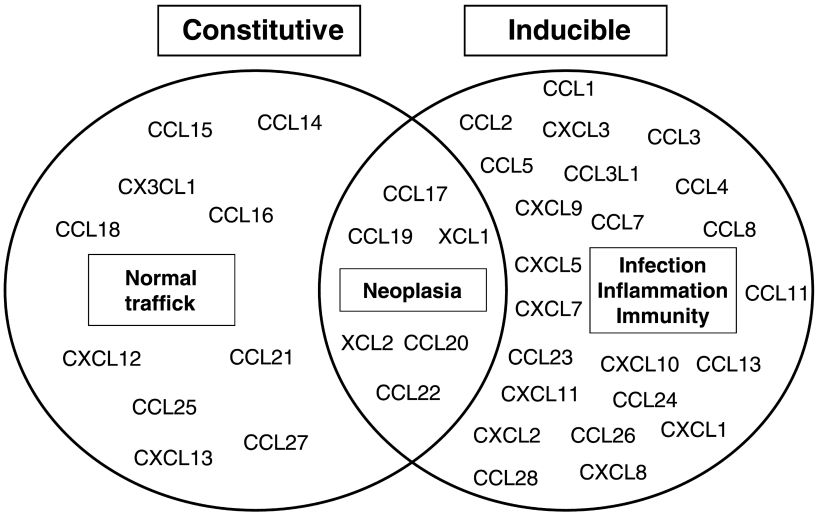


Fig. 10: General structure of chemokine gene superfamily members

Schematic representation of the cysteine signature motifs present in the predicted primary amino acid structure that distinguish members of the chemokine gene superfamily into four basic sub-family. Further subdivision can be made within CXC and CC families according to the presence or absence of an ELR motif immediately preceding the CXC motif, or the presence of either four or six cysteines.

This structural classification of chemokines based on the cysteine motif did not provide any indication of the functional role of the various chemokines. An alternative system that relates expression to function has been developed [173,174]. According to this system, the chemokines can be classified based on their functional expression as homeostatic/constitutive or inflammatory/inducible (fig. 11). Homeostatic chemokines are expressed constitutively within lymphoid tissues and appear to be responsible for the movement of thymocytes through the thymus during selection and for the physiological trafficking of cells including lymphocytes and dendritic cells into secondary lymphoid organs, under normal condition or during immune responses [169,175,176]. Inflammatory chemokines are up regulated at site of inflammation, and play a key role in the recruitment of effector leukocytes to peripheral tissues in response to immunological challenge [177-179]. There is also evidence that the maintenance of resident immune surveillance cells in peripheral

tissues is regulated by low-level basal expression of some homeostatic and some inducible chemokines.



Locati M. et al, Allergy 2002

Fig. 11: Functional classification of chemokines

According to regulation of their production, chemokines can be classified as constitutive chemokines, regulating normal leukocyte trafficking, or inducible chemokines, associated to inflammatory and immune responses.

Redundancy, promiscuity and "polyspeirism" of chemokines

Chemokines are redundant in their action on target cells. No chemokine is uniquely active on one leukocyte population and usually a given leukocyte population has receptors that respond to different molecules. Interestingly, mononuclear phagocytes, the most evolutionary ancient cell type involved in innate immunity, respond to the widest range of chemokines. These include most CC chemokine, the CX_3C chemokine and certain CXC molecules, such as stromal cell-derived factor-1 (SDF-1) and, under certain conditions, interleukin-8 (IL-8) [180].

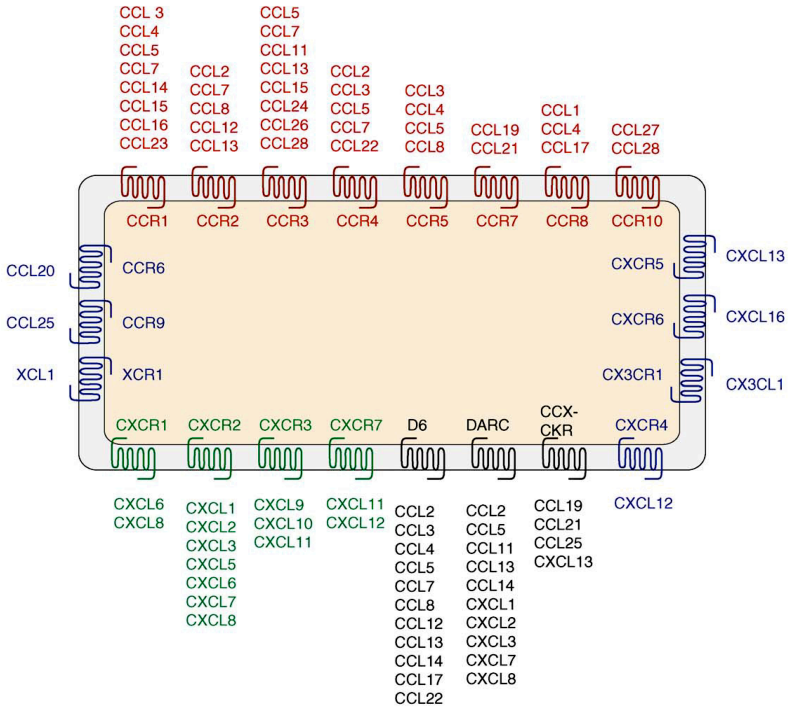
Moreover, the interaction of chemokines with their receptors is characterized by considerable promiscuity. Most known receptors have been reported to interact with multiple ligands and most ligands interact with more than one receptor. For instance, all four monocyte chemotactic proteins (MCPs) interact with CCR2, and at least MCP-2, MCP-3 and MCP-4 also recognize other receptors (CCR1 and CCR3).

Probably, all cell types under appropriate conditions are able to produce chemokines. Interestingly, multiple chemokines are produced in a redundant way by a single cell (polyspeirism). Usually, a cell produces many chemokines concomitantly in response to the same stimulus. Again, polyspeirism is particularly striking for mononuclear phagocytes and endothelial cells exposed to bacterial products or primary inflammatory cytokines. In these cells, bacterial lipopolysaccharide, IL-1 and TNF elicit production of MCPs, macrophage inflammatory proteins (MIPs), RANTES (CC), Fractalkine (CX_3C) and various CXC chemokines [181-185].

The chemokine receptors: structure and nomenclature

Chemokines induce cell migration and activation by binding to specific G-protein-coupled cell-surface receptors on target cells [186,187]. Seven human CXC chemokine receptors, ten human CC chemokine receptors and one human CX_3C chemokine receptor have been identified. Chemokine receptors are expressed on different types of leukocytes. Some receptors are restricted to certain cells, whereas others are more widely expressed. In addition, chemokine receptors are constitutively expressed on some cells, whereas they are inducible on others. For instance, CCR1 and CCR2 are constitutively expressed on monocytes but they are expressed on lymphocytes only after stimulation by interleukin-2 [188]. Again, the expression of other chemokine receptors is restricted to a cell state of activation and differentiation. Some chemokine receptors are also expressed on non-hematopoietic cells, including neurons, astrocytes, epithelial cells and endothelial cells. This suggests that the chemokine system has other roles in addition to leukocytes chemotaxis.

Although most chemokine receptors bind more than one chemokine, CC receptors bind only CC chemokine and CXC receptors bind only CXC chemokines (fig. 12). This ligand-receptor restriction may be related to structural differences between CC and CXC chemokines, which have similar primary, secondary and tertiary structures but different quaternary structures [189]



Lazennec and Richmond, Trends in Molecular Medicine 2010

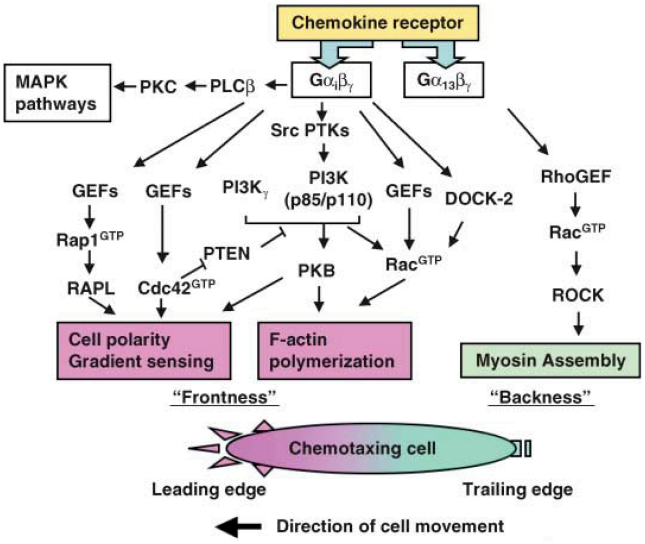
Fig. 12: Chemokines and chemokine receptors family

Most chemokines can interact with multiple receptors, and a single receptor can interact with multiple chemokines. This is the case for most CC (red) and CXC (green) chemokines. Decoy receptors (black) can bind multiple chemokines. By contrast, a minority of receptors (blue) have only one ligand.

Signal transduction of chemokine receptors

The G-protein-coupled chemokine receptors bind chemokine ligands with high affinity to activate the heterotrimeric $G\alpha_i\beta\gamma$. Many chemokine-induced signalling events are inhibited by *Bordetella Pertuxin* toxin, confirming that chemokine receptors are linked to G protein of the class G_i [189]. The exchange of GDP for GTP induces separation of $G\alpha$ (usually $G\alpha_i$) and $G\beta\gamma$ subunits followed by the activation of a series of downstream effectors, such as PLC and PI3Kγ [190]. PLC cleaves PIP2 yielding to two second messengers: IP3, which induces the release of calcium ions from intracellular stores leading to a transient rise of free calcium concentrations, and DAG, which activates several isoforms of PKC.

A number of adaptor proteins dynamically interact with chemokine GPCRs to facilitate internalization and signal transduction and this dynamic interaction continues during vesicular trafficking where the interactions exhibit both spatial and temporal plasticities. This network of proteins associating with chemokine GPCRs forms a dynamic "chemosynapse" that is vital for chemotaxis [191,192]. Overall, active $G\alpha_i$, $G\beta\gamma$ and "chemosynapse" facilitate directional sensing, polarization of the cell with the leading edge (pseudopodium) in the front and the formation of a trailing tail (uropod) at the back. Small GTPases Rac, CdC42 and phosphatidylinositol-3-kinase (PI3K) accumulate at the leading edge with resultant actin polymerization and F-actin formation, leading to stabilization of the lamellipodium. Phosphatase and tensin homolog (PTEN) tyrosine/PIP3 phosphatase resides along the sides and at the posterior end of the migrating cells, Rho GTPase and its effectors accumulate at the trailing edge facilitating actomyosin contraction and tail retraction, resulting in the migration of the cell (fig. 13). Like cytokine receptors, chemokine receptors are also able to signal through the JAK-STAT pathway. Chemokine GPCRs undergo constitutive homo- or hetero-dimerization and the functional impact of such dimerization in the pharmacological properties of the given chemokine receptor appears to be of great significance [193].



Tanaka T. et al, Cancer Science 2005

Fig. 13: Chemokine receptors signalling pathways.

Chemokine receptors can bind and activate heterodimeric G proteins. In a chemotaxing cell, signalling components responsible for the formation of cell polarity, directional sensing and F-actin polymerization, such as PI3K, Rac and CdC42, are preferentially recruited to the leading edge, whereas the mediators of actomyosin contraction, which are downstream of Rho, are recruited to the trailing edge.

Regulation of the chemokine system

As already discussed, chemokines can be distinguished in homeostatic/constitutive or inflammatory/inducible, depending on their regulation and production.

Post-translational modifications naturally occur for both CC and CXC chemokines and strongly affect their biological activities [194]. Several proteases, including metalloproteinases, thrombin and plasmin, act on different chemokines, leading to the production of distinct variants that differ by the extension of the amino-terminal or, less frequently, carboxi-terminal, region. Several lines of evidence indicate that extracellular processing of chemokines by proteases is an important way to regulate the activity and function of the chemokine system in different physiological and pathological conditions.

Regulation of chemokine activity can occur also at the level of chemokine receptors. Chemokine receptor signalling is transient, and the rapid termination of its

activity is achieved by three different mechanisms: inactivation, desensitization and internalization. Receptor inactivation is mediated by the intrinsic GTPase activity of the Gα subunit, which hydrolyses GTP and reunites with Gβγ to return to the initial conformation of inactive heterotrimers. Desensitization is caused by receptor phosphorylation through G-protein-coupled receptor kinase, such as PKC. Finally, internalization is caused by β-arrestin or adaptin-2 mediated receptor sequestration and internalization, through clathrin-coated pits or caveolae.

In addition, in certain conditions, chemokine receptors are uncoupled to the signalling machinery and are converted into functional decoy receptor. These "atypical" receptors appear to be structurally uncoupled to G-proteins, and do not induce any detectable intracellular signal after chemokine binding. Examples of such *decoy* receptors are DARC (Duffy Antigen Receptor for Chemokines) and D6, a decoy receptor selective for CC inflammatory chemokines [195].

The role of the chemokine system in homeostasis and in pathology

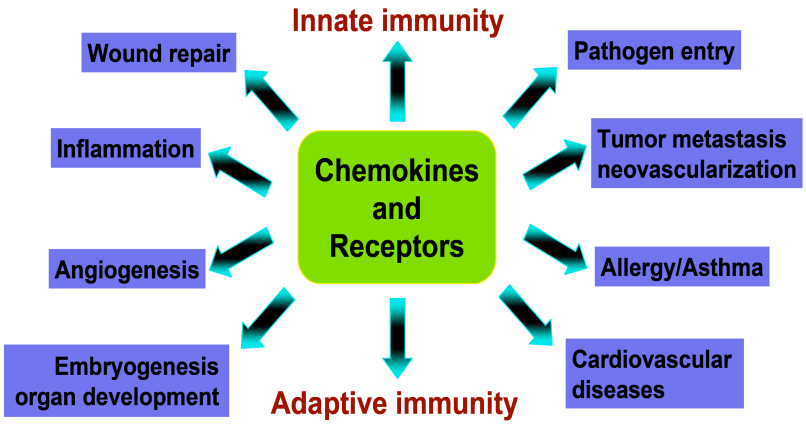
Chemokines play a pivotal role in innate and adaptive immunity. Chemokines are thought to provide the directional cues for the movement of leukocytes in development, homeostasis and inflammation. The chemokines CCL19 and CCL21 activate the chemokine receptor CCR7 present on näive T cells, B cells, mature DC/Langerhans cells (LC) and $CD56^{bright}$NK cells and induce their migration towards the T cell zone of the secondary lymphoid organs (SLO) [196,197]. The B cells express the chemokine receptor CXCR5 that directs their homing to follicles of the lymph node (LN) that express CXCL13 [198]. Immature DCs express CXCR1, CCR1, CCR2 and CCR6 and the ligands for these receptors are inflammatory chemokines that recruit these immature DCs to the site of inflammation [198].

Chemokines have been shown to participate in and control the process of a number of acute and chronic inflammatory conditions by promoting the infiltration and activation of inflammatory cells into injured or infected tissues [199,200]. Several of the CC chemokines, including CCL3 and CCL5, are expressed in sepsis and exert pro-inflammatory effects by mediating organ specific leukocyte influx and activation. Members of the CXC chemokines are implicated in the pathogenesis of systemic

inflammatory response. Indeed, the secretion of chemokines has been detected in a wide variety of inflammatory diseases, such as asthma, bacterial pneumonia, glomerulonephritis, rheumatoid arthritis, atherosclerosis and inflammatory bowel disease (IBD) [200] (fig. 14).

A central feature of inflammatory disease is the migration of leukocytes from the circulation, across the endothelium and the basement membrane and into the affected tissue. Leukocyte extravasation is a regulated multistep process involving a series of coordinated interactions between leukocytes and endothelial cells through the sequential engagement of adhesion molecules (e.g. integrins and selectins) [201,202]. Chemokines provide the signals that convert integrin-mediated interaction from low-affinity, to higher-affinity [203]. In addition, chemokines generate a chemoattractant gradient that drives leukocytes to migrate across the endothelium and through the extracellular matrix into the tissue [204].

One of the first recognized mechanisms of chemokines, different from cell chemotaxis, was their effect on angiogenesis. Chemokines have important implications in the regulation of the angiogenic switch in tumors, either directly or indirectly, by recruiting leukocytes that provide angiogenic factors [205]. Endothelial cells express CXCR4 and its triggering by CXCL12 induces endothelial cell migration and proliferation; moreover, CXCR4 act synergistically with VEGF to enhance neo-angiogenesis [206]. CXCL12 promotes tumor angiogenesis also by the local recruiting of circulating of bone marrow-derived endothelial precursors [207]. In addition, chemokines have been shown to induce gene expression and functional activation of various MMPs. Neo-angiogenesis and tissue remodelling through the activation of MMPs are crucial steps of the wound healing process. Chemokines and chemokine receptors, such as CXCL1, CXCL8, CXCR2, CX_3CL1 and CX_3CR1, are important and vital for proper wound healing.



Yingying Le, Cellular & Molecular Immunology, 2004

Fig. 14: The role of chemokines and chemokine receptors in patho-physiologic condition.

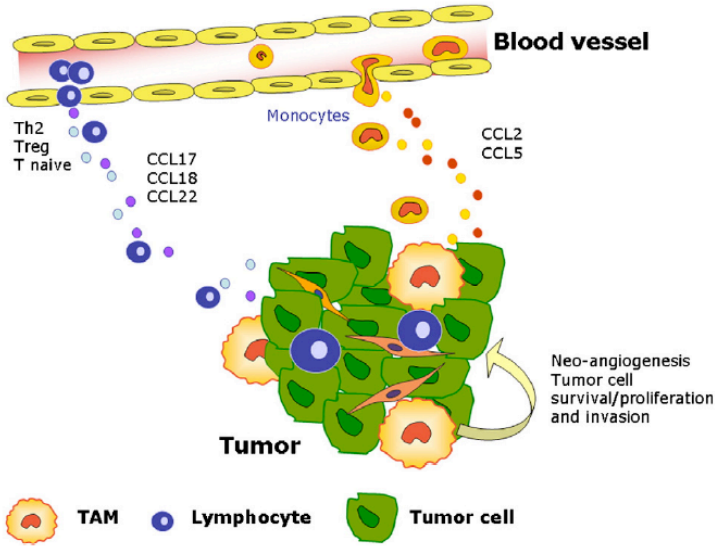
Chemokines and cancer-related inflammation

Chemokines are key player of the cancer-related inflammation. Components of the chemokine system affect multiple pathways of tumor progression, including leukocyte recruitment, neo-angiogenesis, tumor cell proliferation and survival, invasion and metastasis.

Chemokine ligands and receptors are downstream of genetic events that cause neoplastic transformation and are abundantly expressed in chronic inflammatory condition that predispose to cancer. For instance, components of the RAS-RAF signalling pathway induce the activation of NF-κB and the production of several chemokines (e.g.CXCL8), while the activation of the transcription factor c-Myc programme leads to the induction of several CC chemokine [60,61,63].

Chemokines have been historically associated to leukocyte recruitment to tumors. Major attractants of monocytic precursors are the CC-chemokines. In a variety of human cancers, CCL2 and CCL5 levels are correlated with high numbers of intra-tumor myeloid cells [68,113,208]. On the other hand, chemokines such as CCL17 and CCL22, abundantly produced by TAMs, preferentially attract T cell subsets devoid of cytotoxic functions (Th2 and T_{reg} cells)[68,209] (fig. 15). Therefore, the expression of a particular chemokine in the tumor microenvironment is a crucial determinant of the leukocyte composition within tumor tissue [185].

Chemokines can also regulate the recruitment and trafficking of cells of the adaptive immunity to site of tumor (fig. 15). TILs have been reported to express the CXCR3 receptor; the corresponding ligands CXCL9 and CXCL10 can elicit anti-tumoral responses that correlate with increased infiltration of CD4 and CD8 lymphocytes [210]. In gastric and colorectal carcinoma, significant levels of CXCL9 and CXCL10 are produced by stromal cells, and are correlated with CXCR3-positive TIL [211].



Allavena P. et al., Experimental Cell Research 2011

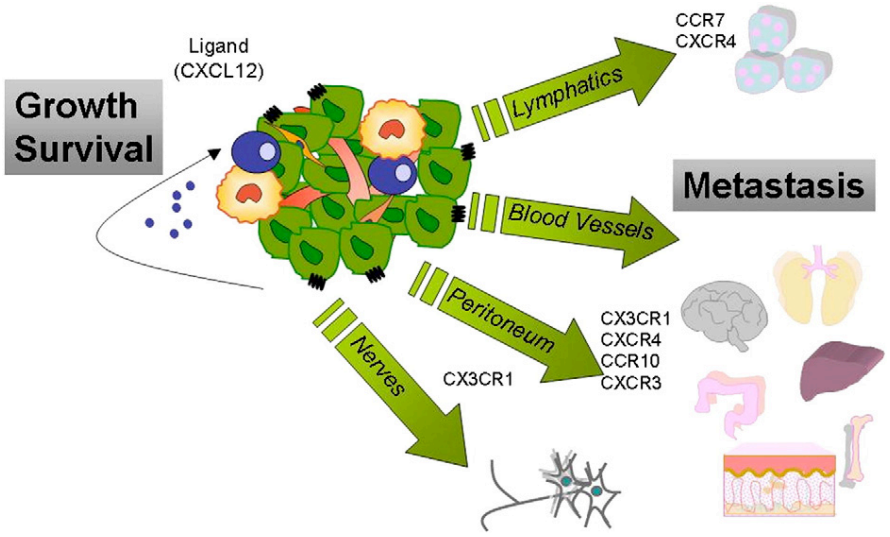
Fig. 15: Tumor-derived chemokines attract blood leukocytes ultimately having a pro-tumoral role
Chemokines secreted by tumor and stromal cells regulate the recruitment of circulating monocytes. Differentiated TAMs promote tumor progression regulating neo-angiogenesis, matrix remodelling, cancer cell survival, proliferation and invasion. By producing chemokines, TAMs recruit lymphocytes devoid of cytotoxic functions and suppress anti-tumor adaptive immune responses.

A major breakthrough, in the last decade, was the establishment that also tumor cells can express chemokine receptors and may functionally respond to ligands. In general, receptor engagement enhances cancer cell resistance to apoptotic stimuli and proliferation through the activation of the MAP/Erk and PI3K pathways [68,185]. Earlier studies already pointed out that some tumor cell lines were able to migrate in response to CXCL8, and that antibodies against CXCR2 inhibited tumor cell growth *in vitro*. Cellular senescence can have a dual function in cancer

progression, by favouring clearance of DNA damaged cells and progression of neighbouring cells via secreted factors. Chemokines, in particular CXCL8, have been associated with the phenomenon of senescence: CXCR2 and its ligand are secreted during senescence in a NF-κB and p53-dependent way to reinforce senescence [212,213].

There is now evidence that chemokines can serve as cues for the secondary localization of tumor cells. The most frequently overexpressed chemokine receptor on tumor cells is CXCR4. In general CXCR4 is associated with tumor progression and metastasis [68,185]. In a seminal paper, Muller and colleagues demonstrated that CXCR4 is involved in metastasis in a model of breast cancer [214]. Other CXC-receptors have been implicated in the malignant dissemination to distant organs. For instance, CXCR1, CXCR2 and CXCR3 in malignant melanoma [215,216]; CXCR3 in B-cell chronic lymphocytic leukemia cells [217]; CXCR5 in liver metastasis of colorectal carcinoma [218]; CX_3CR1 is implicated in the perineural invasion frequently occurring in pancreatic adenocarcinoma [219,220] (fig. 16).

Overall, the above studies indicate a strong involvement of chemokines and chemokine receptors in tumor progression and metastatic dissemination. Evidence in pre-clinical and clinical settings suggests that the chemokine system represents a valuable target for the development of innovative therapeutic strategies.



Allavena P. et al., Experimental Cell Research 2011

Fig. 16: Direct effects of chemokines on tumor cells

Cancer cells secrete chemokine ligands (e.g.CXCL12) that act in paracrine fashion on receptor-positive tumor cells (e.g. CXCR4) to sustain their resistance to apoptotic stimuli and active proliferation. Chemokine receptor also guide tumor cell dissemination to distant organs.

Chemokines and colorectal cancer

The role of chemokines and their receptors in CRC has been studied only in very recent years. Chemokines play an important role in enhancing growth and spread of malignancy by inducing angiogenesis and controlling tumor cell migration and invasion. Increased levels of pro-inflammatory chemokines and leukocyte infiltration were already observed in the intestinal mucosa of IBD patients and this elevation strongly correlates with the severity of the disease [221,222]. The levels of pro-inflammatory chemokines such as CCL2, CCL3, CCL4 and CCL5, and pro-angiogenic chemokines, such as CXCL1, CXCL5 and CXCL8 are elevated in human colon tumor tissues as compared to the matched normal tissues, indicating that these chemokines and their receptors play an important role in regulating colon tumor progression, angiogenesis and metastasis (fig. 17) [223]. The timing of expression of these chemokines during tumor progression may be very important. For example, one study reported that when CXCL5 levels are low in human CRC, there are reduced numbers of $CD8^+$ T cells and poor prognosis [224]; CXCL1 and CXCL5 have been identified to have a potential role in tumor progression from adenoma to cancer, being higher in CRC and liver metastasis compared to adenoma [225]. CXCL8 is considered a fundamental chemokine that comprises a network specific to colon cancer. Expression of both CXCL8 and IL-6 is elevated in oncogene-induced senescent colorectal cells that express the tumor suppressor $p16^{INK4A}$ [226]. In addition, CXCL8 can stimulate colon carcinoma cell migration/invasion, mediating TNFα/TGFβ-driven epithelial-to-mesenchymal transition.

Disruption of TGF-β signalling in cancer cells up-regulates CXCL12-CXCR4 and CXCL5-CXCR2 ligand-receptor pairs, promoting metastasis and dampening antitumor immune response [227]. CXCL12 and CXCR4 are up regulated in advanced-stage colorectal cancers and their overexpression correlates with poor prognosis [228]. CXCR4 signalling regulates metastasis by two different mechanisms, through the direct chemotactic attraction of migrating or invading metastatic seeds and through the stimulation of tumor neo-angiogenesis. Furthermore, CCR6 expression is significantly elevated in colorectal cancer cells that metastasize to the liver, and its specific ligand, CCL20, is highly expressed in the liver, suggesting that CCL20 may contribute to the selective recruitment of CCR6-positive cancer cells into the liver [222].

Chemokines and their receptors in IBD and CRC

Name	Class/expression	Receptor	Cell attracted	IBD	CRC
CC chemokines					
CCL1	Inducible	CCR8	Monocytes, T, B		
CCL2	Inducible	CCR2	Monocytes, T, NK, immature DC	+	+
CCL3	Inducible	CCR1, 5	Monocytes, macrophages T, NK, immature DC	+	+
CCL4	Inducible	CCR5, 8	Monocytes, macrophages T, Immature DC	+	+
CCL5	Inducible	CCR1, 3 and 5	Monocytes, macrophages T, NK, immature DC	+	+
CCL7	Inducible	CCR1, 2 and 3	Monocytes, macrophages T, NK, immature DC	+	?
CCL8	Inducible	CCR2, 3 and 5	Monocytes, macrophages T, NK, immature DC	+	?
CCL9	Inducible	CCR1	DC	?	+
CCL11	Inducible	CCR3	Eosinophils, basophils Th2 T, mast cells	?	+
CCL13	Inducible	CCR1, 2 and 3	Monocytes, T, NK eosinophils, immature DC	+	?
CCL14		CCR1, 3	Monocytes, macrophages NK, T cells	?	?
CCL15		CCR1, 3	Monocytes, macrophages T, NK, eosinophils, mast cells	?	?
CCL16		CCR1	Monocytes, macrophages T, NK cells	?	?
CCL17		CCR4	Th2 T cells, macrophages T, NK cells	+	+
CCL18			T cells	?	?
CCL19		CCR7	T, B, mature DC	+	?
CCL20		CCR6	T, B, immature DC	+	+
CCL21		CCR7	T, B, mature DC	+	+
CCL22		CCR4	Th2 T cells, macrophages	+	?
CCL23		CCR1	Monocytes	?	?
CCL24	Inducible	CCR3	Eosinophils, basophils mast cells, Th2 T cells	+	?
CCL25		CCR9	Thymocytes, T cells	+	?
CCL26	Inducible	CCR3	Eosinophils, basophils mast cells, Th2 T cells	+	?
CCL27		CCR10, 2, 3	T, B, monocytes immature DC	?	?
CCL28		CCR10, 3	T cells	+	?
CXC chemokines					
CXCL1	ELR^+, inducible	CXCR2, 1	Neutrophils	+	+
CXCL2	ELR^+, inducible	CXCR2	Neutrophils	+	+
CXCL3	ELR^+, inducible	CXCR2	Neutrophils	?	?
CXCL4	ELR^-, inducible		Fibroblasts, endothelial cells	?	+
CXCL5	ELR^+, inducible	CXCR2	Neutrophils	+	+
CXCL6	ELR^+, inducible	CXCR2	Neutrophils, macrophages	+	?
CXCL7	ELR^+, inducible	CXCR2	Neutrophils	?	?
CXCL8	ELR^+, inducible	CXCR1, 2	Neutrophils	+	+
CXCL9	ELR^-, inducible	CXCR3	Th1 T cells, NK	+	+
CXCL10	ELR^-, inducible	CXCR3	Th1 T cells, NK	+	+
CXCL11	ELR^-, inducible	CXCR3	Th1 T cells, NK	+	+
CXCL12	ELR^-	CXCR4, 7	$CD34^+$ progenitor cells hematopoietic cells	+	+
CXCL13	ELR^-	CXCR5	Naïve B cells, $CD4^+$ T cells	?	+
CXCL14	ELR^-		Monocytes, immature DC	?	?
CXCL16	ELR^-	CXCR6	Naïve $CD8^+$ cells, $CD4^+$ T cells NK-T cells	+	?
C chemokines					
XCL1		XCR1	T cells, NK	?	+
XCL2		XCR1	T cells, NK	?	?
CX3C chemokine					
CX3CL1		CX3CR1	T cells, monocytes, neutrophils DC	?	+

Wang D. et al., Current Opinion in Pharmacology 2009

Fig 17: Chemokines and their receptor in IBD and CRC

Chemokines and chemokines receptors are strongly involved in the recruitment of inflammatory mediators at the site of inflammation in both intestinal bowel disease and colorectal cancer tissues.

In contrast to CXCR4, which is constitutively expressed, CXCR3 is not expressed by normal intestinal epithelial cells, while it is up regulated during carcinogenesis. Chemokines that activate CXCR3, such as CXCL9, CXCL10, CXCL11 and CCL21 are up regulated by signalling of oncogenic proteins such as Ras, or by proinflammatory cytokines, such as TNF-α, IL-17 or even IFN-γ [229,230,231].

In conclusion, chemokines are responsible for recruiting inflammatory cells into the mucosa of the inflamed intestine, leading to the formation of a tumor-promoting microenvironment. In addition to the regulation of the tumor

microenvironment, chemokines have a direct effect on epithelial cells expressing chemokine receptors, inducing migration and metastasis.

Understanding the molecular mechanisms by which chemokines orchestrate the progression from a physiologic inflammatory condition to pathologic inflammation and neoplasia in the human colon will lead to the development of novel therapeutic approaches for prevention and amelioration of CRC.

AIM

OF THE

PROJECT

Among human tumors, colorectal cancer (CRC) is one of the most frequent in Western countries, and still a leading cause of high mortality.

CRC represents a paradigm of the cancer-related inflammation promoting tumor initiation and disease progression. While this concept has been clearly recognized in the last decade, no inflammation-associated markers or targeted therapy have been translated into clinical practice.

Aim of this study is first to obtain a global overview of the inflammatory mediators expressed in the microenvironment of human colorectal cancer surgical specimens. Secondly, is to identify which inflammatory mediators worth of further investigation as potentially useful biological and clinical markers (e.g. diagnosis, disease indicator or therapeutic target).

To this aim, a strategy has been designed to evaluate a large panel of different inflammatory mediators (mostly cytokines, chemokines, receptors of innate immunity) initially in a restricted number of samples. Interesting candidate proteins will be confirmed in a larger number of CRC cases.

The results of gene and protein expression of the inflammatory mediators will be correlated with patients' clinical and pathological features, in order to gain information on the possible role of the selected molecules in the progression of the disease.

In the last part of the project, I will make the effort to understand the biological mechanism of the molecules under study and their implication in tumor development.

RESULTS

Expression of chemokines and chemokine receptors in human colorectal cancer

TaqMan Low Density Array analysis of eight human colon cancer samples

To investigate the role of inflammation in human colon cancer, we performed a large screening of inflammation-related genes in eight human colon cancer samples and corresponding normal tissues, using the TaqMan Low Density Array (LDA, Applied Biosystem), customized with 91 inflammation-related genes, selected by us, and 5 housekeeping genes. Among the 91 inflammation-related genes, 24 were chemokines and 17 were chemokine receptors. Considering CC chemokines, CCL1, CCL3, CCL4 CCL7, CCL20, CCL25 and CCL26 were more frequently transcribed compared to the normal autologous tissues. The corresponding receptors, CCR8 and CCR6, were also up regulated, but other, like CCR1 and CCR5, were less than 2 fold expressed. Among CXC chemokines, high levels of CXCL1 and CXCL8 and the corresponding receptor CXCR2 were found. The ligands CXCL9 and CXCL10 were also highly expressed, but not their receptor CXCR3. Surprisingly, the chemokine CCL2, frequently produced by several tumor types including CRC, or the constitutive chemokine CXCL12, were not up regulated compared to normal intestinal mucosa (tab.1).

To better appreciate the configuration of the chemokine system in the normal colonic mucosa, the data were also analysed relative to the mean of the five housekeeping genes customized in LDA. Two representative cases are presented in fig. 18. Panels A and B show the mRNA levels of chemokines and receptors in one early stage tumor, while Panel C and D show levels in an advanced stage tumor, respectively. The normal colonic mucosa (white bars) expresses several chemokines: CCL2, CCL5, CCL14/15, CCL20, CCL21 and CXCL12 are expressed more frequently. Some of these chemokines were further up regulated in tumor samples (black bars), but not always they reached the two-fold cut-off. Other chemokines such as CCL3, CCL4, CXCL9, CXCL10 and especially CXCL8 were more selectively overexpressed in the tumor tissues. Among chemokine receptors, CXCR4 and CXCR7, both binding CXCL12, were already expressed in the normal mucosa,

and CXCR4 further up regulated in tumors. Other receptors were up regulated, including CCR1 and CCR5, both binding CCL3 and CCL4, and CXCR2, recognizing CXCL8.

	Stage1	Stage2	Stage2	Stage3	Stage3	Stage3	Stage4	Stage4
CCL1	4	3	1	0	3	2	6	28
CCL11	0	1	2	0	0	0	0	0
CCL14/15	1	0	1	0	0	0	6	1
CCL17	1	0	0	1	30	0	0	1
CCL18	1	0	1	4	2	1	12	1
CCL2	0	1	0	0	2	0	3	0
CCL20	0	6	0	0	3	0	2	42
CCL21	1	0	1	0	0	0	0	0
CCL25	20	0	582	3	0	0	28	694
CCL26	4	0	2	3	761	134	3	143
CCL3	5	0	1	1	8	1	26	0
CCL4	4	1	0	1	3	0	22	1
CCL7	1	3	2	1	4	0	7	0
CCL8	0	0	0	0	0	0	6	0
CCR1	1	0	0	1	1	0	1	1
CCR3	0	3	1	0	0	0	0	0
CCR5	1	0	1	0	0	1	1	0
CCR6	1	3	2	0	0	0	0	2
CCR8	4	5	6	2	8	3	2	3
CMKLR1	0	0	0	1	0	0	3	1
CXCL1	2	3	2	1	10	1	28	9
CXCL10	7	2	0	1	1	1	410	9
CXCL9	6	1	0	1	0	1	10	6
CXCR3	1	4	0	1	1	0	0	1
CXCR4	1	0	1	1	1	0	3	3
CXCR7	0	1	1	1	1	0	0	1
CXCL8	4	24	13	11	447	4	1492	1
CXCR2	0	4	3	3	10	3	34	0
CXCL12	0	0	0	0	0	0	0	0
CX3CL1	10	1	0	3	1	1	3	1
CX3CR1	5	1	1	2	0	1	1	0

Tab. 1: Overview of the expression of the chemokine system in the microenvironment of human CRC samples.

mRNA of chemokines and receptors from CRC tumors were analysed by a customized TaqMan Low Density Array. Data are expressed relative to autologous normal tissues. Grey box depicts a fold equal or >2. Eight tumor samples representative of different clinical stages are shown

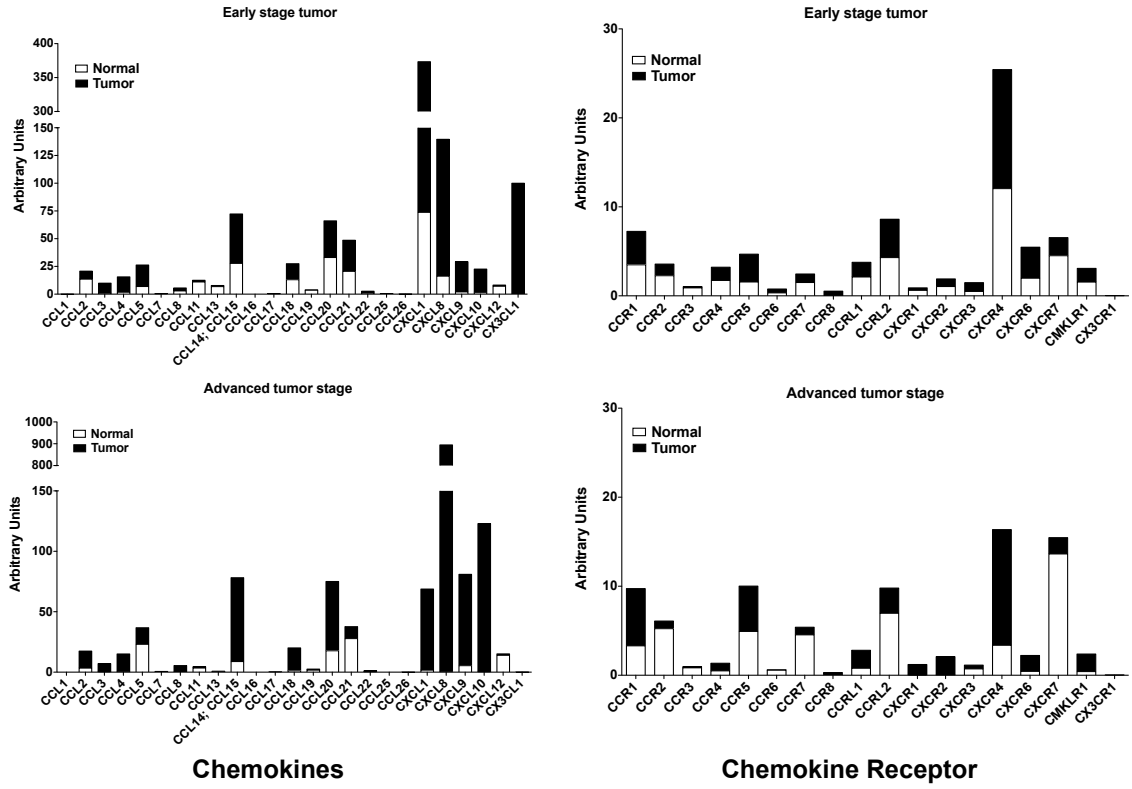


Fig. 18: Expression of mRNA for chemokines and chemokine receptors in CRCs

mRNA expression of chemokines and chemokine receptors in an early stage tumor (panel A and C) and in an advanced stage tumor (panel B and D). The analysis has been performed using a customized TaqMan Low Density Array. mRNA levels are expressed relative to the mean of five housekeeping genes. White bars: autologous normal colonic mucosa. Black bars: tumor tissue

CCL3, CCL4 and CXCL8 expression in CRC

To confirm data obtained by the LDA, we further investigated mRNA expression of CCL3 and CCL4 in other 20 colon cancer tissues and their corresponding normal mucosa. These two chemokines were picked up, as they are not thoroughly studied in CRC. Furthermore these ligands attract Th1 lymphocytes, important effectors in anti-tumor immunity. As shown in fig. 19, there is a significant increase in CCL3 expression in tumor samples compared to healthy tissue ($p=0.05$). CCL4 expression is higher in tumor tissues, but this difference is at the limit of significance ($p=0.07$).

Subsequently, we focused our attention on CXCL8, since it has been extensively demonstrated that this ligand is strongly overexpressed in various tumor types. We investigated CXCL8 mRNA expression in 30 colon cancer samples and corresponding normal mucosa. As shown in fig 19, there is a strong significant increase in CXCL8 expression in tumor tissues ($p<0.0001$). Both for CCL3, CCL4 and CXCL8 there is no significant correlation with tumor stage (not shown).

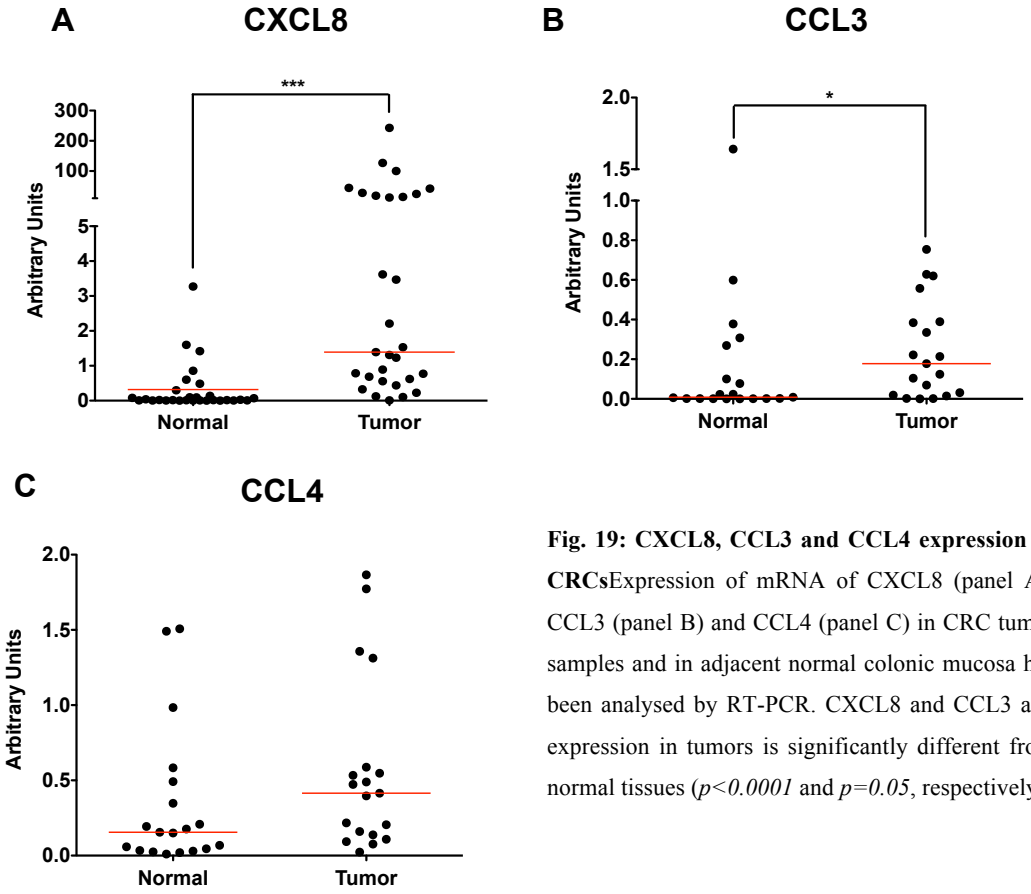


Fig. 19: CXCL8, CCL3 and CCL4 expression in CRCs Expression of mRNA of CXCL8 (panel A), CCL3 (panel B) and CCL4 (panel C) in CRC tumor samples and in adjacent normal colonic mucosa has been analysed by RT-PCR. CXCL8 and CCL3 and expression in tumors is significantly different from normal tissues ($p<0.0001$ and $p=0.05$, respectively)

CXCL8 correlation with OPN and SPARC

Among the most expressed non-chemokine genes in the LDA, we found osteopontin (OPN) and secreted protein acidic and rich in cysteine (SPARC). It has been demonstrated that OPN is not only a matrix protein, but has also a chemotactic activity for leukocytes and it is involved, together with CXCL8, in prostate cancer recurrence [232]. Both OPN and SPARC contribute to the deposition and remodelling of the extracellular matrix (ECM) and to the development of angiogenesis, a function shared by CXCL8 as well. We therefore investigated the expression of OPN and

SPARC in CRC samples. As shown in fig. 20, there is a significant increase in both mRNA OPN and SPARC in tumor tissues compared to normal mucosa ($p<0.0001$). Interestingly, we found a significant linear correlation between mRNA OPN and CXCL8 expression in tumor tissues ($p=0.001$), while there was no correlation between CXCL8 and SPARC ($p=0.9$). Interestingly, both CXCL8 and OPN are induced by hypoxia and are involved in neo-angiogenesis [233].

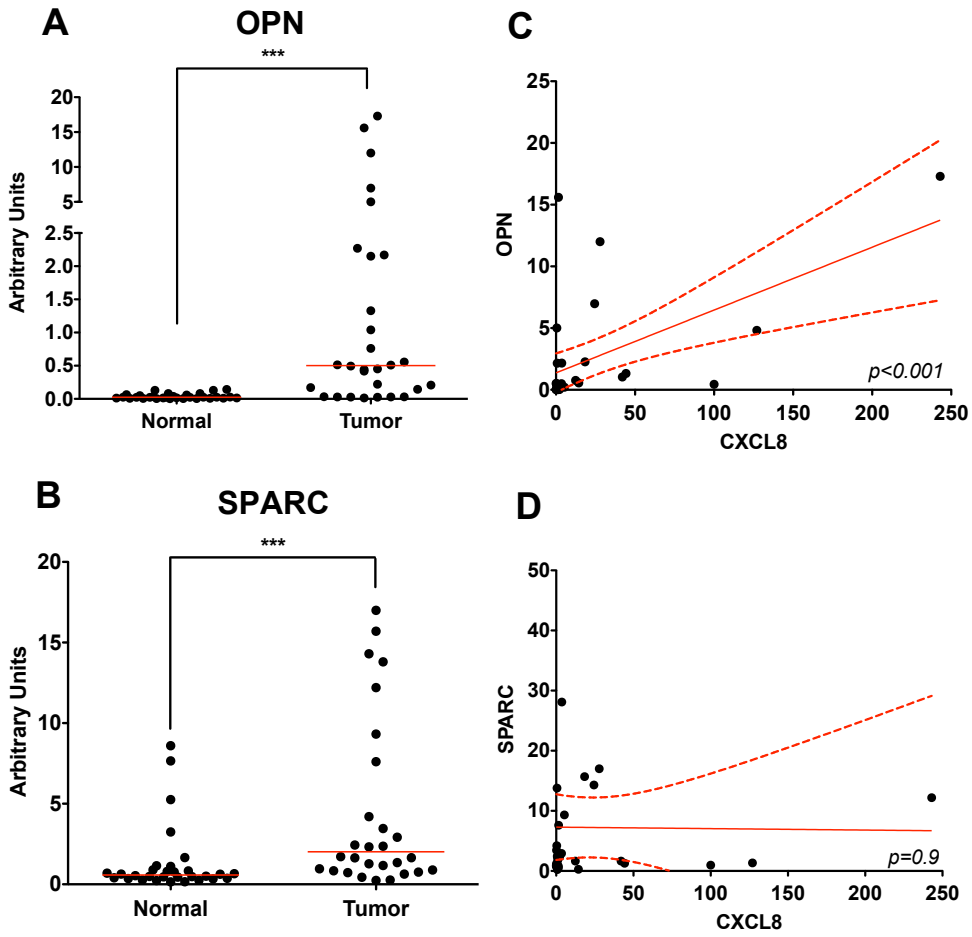


Fig 20: CXCL8 correlation with OPN and SPARC in CRCs

mRNA expression of Osteopontin (OPN) (panel A) and SPARC (panel B) in CRC tumor samples and in adjacent normal colonic mucosa has been analysed by RT-PCR. Statistical significance is shown, both OPN and SPARC are significantly increased in tumor samples. Panels C and D show the linear regression analysis of CXCL8 and OPN (C) or SPARC (D). mRNA CXCL8 significantly correlates with mRNA OPN in the tumor tissues.

Ligand CX_3CL1 and receptor CX_3CR1 axis in cancer

Our results indicated that the chemokine CX_3CL1 and its receptor CX_3CR1 are also up regulated in CRC samples. Unlike other chemokines, CX_3CL1 (originally named Fractalkine or Neurotactin) is a transmembrane protein that can function as an adhesion molecule as well as a chemokine when cleaved by specific proteases. CX_3CL1 is expressed by a large number of cell types, including neurons, endothelial cells and epithelial cells. CX_3CL1 has a single receptor, CX_3CR1, which is expressed primarily on monocytes, DC, NK cells and T-cells, mediating both adhesive and chemoattractive functions. Several studies indicated that Fractalkine is involved in the pathogenesis of different inflammatory conditions, including atherosclerosis [234], glomerulonephritis [235], rheumatoid arthritis [236], primary biliary cirrhosis [237] and IBD [238]. In particular, CX_3CL1 expression is increased in Chron's disease mucosa [239]. A recent study showed that the interaction between Fractalkine and its receptor could promote tissue damage in colitis conditions, by promoting inflammatory cell infiltration [240].

The role of CX_3CL1 and CX_3CR1 has been investigated in different tumor types. Mastubara et al. demonstrated that high expression of CX_3CL1 and CX_3CR1 is associated with a better prognosis in patients with hepatocellular carcinoma [241]. CX_3CL1 expression has been frequently associated with an increased infiltration of CX_3CR1^+ immune cells. Studies on lung carcinoma, melanoma and gastric adenocarcinoma showed that the overexpression of CX_3CL1 resulted in an increased infiltration of CX_3CR1^+ immune cells, suggesting that Fractalkine has an antitumor activity inducing both innate and adaptive immune responses [242-244]. On the contrary, it has been demonstrated that the CX_3CL1-CX_3CR1 system has a crucial tumor-promoting role in human glioblastomas via its impact on glioma-infiltrating macrophages [245]. Recently our group showed that CX_3CL1 expression was inversely correlated with patient overall survival, and that glioblastoma-derived neurospheres, containing a mixed population of stem and progenitor cells, were positive for both Fractalkine and its receptor [246].

Few studies have investigated the role of CX_3CL1 in colorectal cancer. The murine cell line CT26 transduced with CX_3CL1 grew more slowly and was less

metastatic in mice [247]. In addition, CX_3CL1 expression scores were positively correlated with TIL infiltration and good prognosis in human CRC [248,249].

Besides immune cells, CX_3CR1 has been demonstrated to be expressed also by tumor cells. Our group reported that pancreatic adenocarcinoma cells expressed CX_3CR1 and its expression correlated with neural tropism of pancreatic cancer cells [219,220]. In addition, CX_3CR1 expression by tumor cells has been associated with the metastatic potential of different tumors, such as prostate cancer [250,251], breast cancer [252] and glioma [253].

Expression of CX_3CL1 and CX_3CR1 in human CRC

As mentioned above, some studies indicated a correlation between CX_3CL1 expression, tumor infiltrating cells and prognosis in human cancer patients, including colorectal cancer. The expression of CX_3CR1 by colon cancer epithelial cells, however, has not been investigated. We therefore decided to investigate a large cohort of CRC tumor samples for the expression of the CX_3CL1/CX_3CR1 axis.

We first analysed by immunohistochemistry the expression of CX_3CL1 in a series of 144 microsatellite stable (MSS) human colorectal cancers, 18 adenomas and 18 normal colonic tissues. CX_3CL1 was weakly expressed by normal colonic epithelial cells, and its expression significantly increased in pre-cancerous lesions (adenomas) and stage 1 CRC (fig. 21A). Moreover, vessels' endothelial cells within tumor tissue also stained positive for CX_3CL1. CX_3CL1 expression's score by tumor epithelial cells was calculated as indicated in "Material and Methods". Interestingly, CX_3CL1 expression strongly decreased in advanced non-metastatic tumors (stage 3) (fig. 21B).

To better understand the role of CX_3CL1 in the pathogenesis of human colorectal cancer, we decided to analyse the expression of its receptor in our human CRC case list (fig. 20A). The expression of CX_3CR1 by epithelial cells was low in healthy colon, as well as in pre-cancerous lesions, and strongly increased in tumor tissues (fig. 20B). As for CX_3CL1, CX_3CR1 expression decreased from stage II to stage III, although this difference was not statistically significant (fig. 21B).

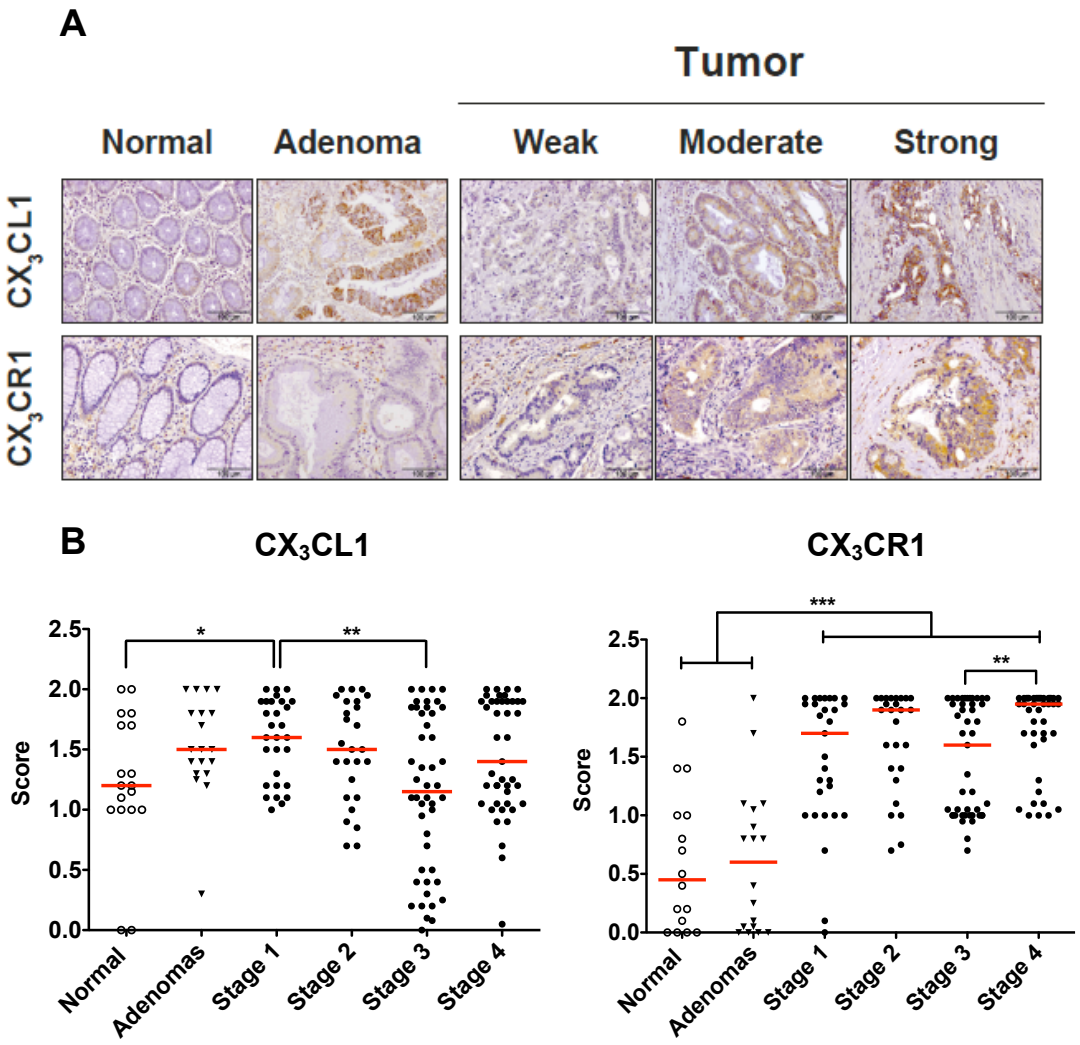


Fig. 21: Expression of CX_3CL1 and CX_3CR1 in human colorectal cancer.

Immunohistochemical analysis of CX_3CL1 and CX_3CR1 expression was performed on 18 healthy colonic tissues, 18 pre-cancerous lesions and 144 MSS human CRCs . Expression patterns are shown (panel A). Panel B represents the distribution of CX_3CL1 and CX_3CR1 expression among tumor stages. Red bars indicate median values. Magnification: 200X; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

CX_3CL1 and CX_3CR1 expression correlation with patients' clinical and pathological features

The expression of CX_3CL1 and CX_3CR1 by human colorectal cancer epithelial cells was correlated with patients' clinical and pathological features. Based on CX_3CL1 and CX_3CR1 expression scores, the CRCs case list was divided into two groups, using the median value of all cases as cut-off (1.4 and 1.7, respectively).

Data are shown in tab. 2A. CX_3CL1 expression was significantly reduced in stage III tumors (lymph node involvement) (univariate, OR 0.41, p=0.01; multivariate, OR 0.32, p=0.009). In addition, CX_3CR1 expression is increased in primary stage IV metastatic tumors (univariate, OR 2.74, p=0.01). No correlation was found regarding tumor site, local invasion, cell type, grading and vascular invasion (tab. 2B).

CX_3CL1 and CX_3CR1 expression was also correlated with patients' outcome: patients with stage I-III tumors showing higher expression of CX_3CL1 or CX_3CR1 had a significantly increased disease-specific survival (p=0.05) and a barely lower recurrence (disease-free survival, p=0.096 and p=0.08, respectively) (fig. 22).

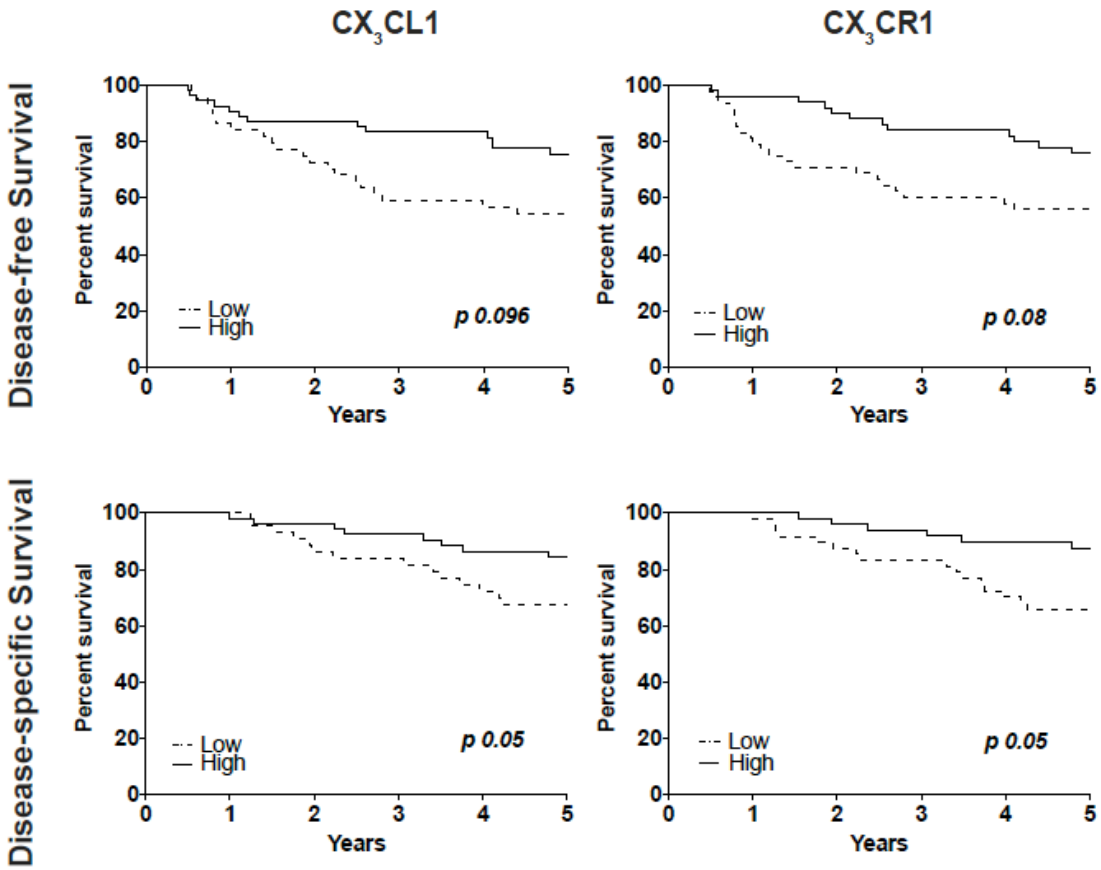


Fig. 22: CX_3CL1 and CX_3CR1 expression correlation with patients' survival

Kaplan-Meier curves indicate disease-free and disease-specific survival in patients with stage I-III tumors, based on CX_3CL1 or CX_3CR1 median expression.

A

		CX_3CL1 expression		Univariate Analysis		Multivariate Analysis	
		Low (n=66)	High (n=78)	OR (95% C.I.)	P	OR (95% C.I.)	P
Age	*(years, mean ±SD)*	61.3±11.6	65.7± 11	1.04 (1.00-1.07)	**0.02**	1.05 (1.01 – 1.09)	**0.01**
Sex	*Male*	39	36	1.00 Ref.			
	Female	27	42	1.69(0.87-3.27)	0.12		
Tumor Site	*Distal*	19	21	1.00 Ref.			
	Proximal	30	33	1(0.45-2.2)	0.99		
	Rectum	17	24	1.28(0.53-3.07)	0.58		
Local Tumor Invasion	*pT1*	2	4	1.00 Ref.			
	pT2	9	18	1.00 (0.15-6.53)	1.00		
	pT3	46	48	0.52(0.09-2.99)	0.46		
	pT4	9	8	0.44(0.06-3.11)	0.41		
Nodal Status	*No*	20	40	1.00 Ref.		1.00 Ref.	
	Yes	46	38	0.41 (0.21-0.82)	**0.01**	0.32 (0.14 – 0.75)	**0.009**
Distant Metastasis	*M0*	45	56	1.00 Ref.			
	M+	21	22	0.84 (0.41-1.72)	0.64		
Tumor Cell type	*ADC*	58	74	1.00 Ref.			
	Variant	8	4	0.39 (0.11-1.37)	0.14		
Vascular Invasion	*No*	42	56	1.00 Ref.			
	Yes	24	22	0.69 (0.34-1.39)	0.3		
Grading	*G1-G2*	52	69	1.00 Ref.			
	G3	14	8	0.43 (0.17-1.1)	0.08		

B

		CX_3CR1 expression		Univariate Analysis		Multivariate Analysis	
		Low (n=60)	**High (n=84)**	**OR (95% C.I.)**	**P**	**OR (95% C.I.)**	**P**
Age	*(years, mean ±SD)*	63.3±11.8	63.9± 11.2	1.00 (0,98-1,03)	0.75		
Sex	*Male*	35	40	1.00 Ref.			
	Female	25	44	1.54 (0.79-3.00)	0.21		
Tumor Site	*Distal*	18	22	1.00 Ref.			
	Proximal	25	38	1.24(0.56-2.77)	0.59		
	Rectum	17	24	1.16 (0.48-2.78)	0.75		
Local Tumor Invasion	*pT1*	2	4	1.00 Ref.			
	pT2	12	15	0.63 (0.10-4.01)	0.62		
	pT3	34	60	0.88(0.15-5.07)	0.89		
	pT4	12	5	0.20(0.03-1.53)	0.12		
Nodal Status	*No*	26	34	1.00 Ref.			
	Yes	34	50	1.12 (0.57-2.2)	0.73		
Distant Metastasis	*M0*	49	52	1.00 Ref.			
	M+	11	32	2.74 (1.25-6.03)	**0.01**		
Tumor Cell type	*ADC*	57	75	1.00 Ref.			
	Variant	3	9	2.28 (0.59-8.80)	0.23		
Vascular Invasion	*No*	39	59	1.00 Ref.			
	Yes	21	25	0.79 (0.39-1.6)	0.51		
Grading	*G1-G2*	50	71	1.00 Ref.			
	G3	9	13	1.02 (0.40-2.56)	0.97		

Tab. 2. CX_3CL1 and CX_3CR1 expression and correlation with patients' clinical and pathological features

CX_3CL1 (**A**) and CX_3CR1 (**B**) expression scores were correlated with patients' clinical and pathological features. Univariate and multivariate analysis has been performed.

Correlation between CX_3CL1 expression and $CD3^+$ and $CD68^+$ infiltrating cells

The function of immune cells in the prognosis of human colorectal cancer has been extensively studied, with a particular attention to lymphocyte infiltration. Given the chemotactic effect of CX_3CL1 on CX_3CR1^+ immune cells, we investigated the role of CX_3CL1 in the recruitment of lymphocytes and macrophages to colorectal cancer tissues.

The same case list of specimens studied for CX_3CL1 and CX_3CR1 expression were analysed by immunohistochemistry for the presence of $CD3^+$ and $CD68^+$ cells at the tumor invasive front. Representative images of low and high infiltrated tumors are shown in fig. 23.

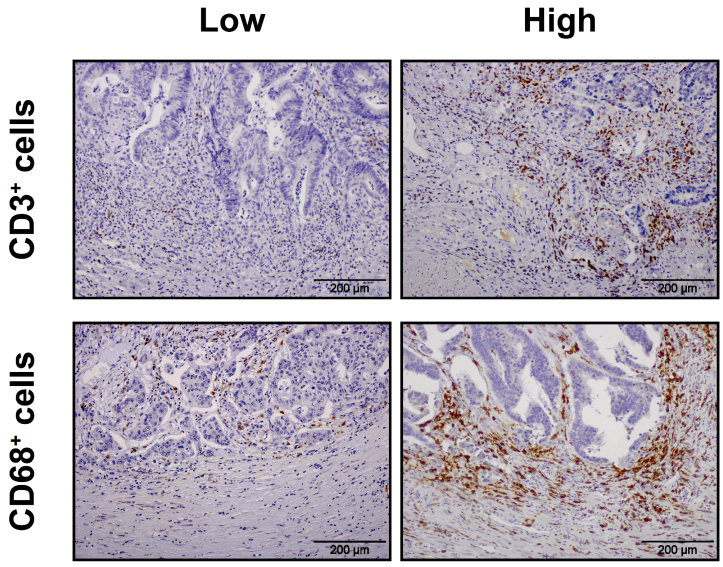


Fig. 23: Expression of $CD3^+$ and $CD68^+$ cells at tumor invasive burden
Immunohistochemical analysis of $CD3^+$ and $CD68^+$ cells at tumor invasive margin. Magnification: 200X

Using an *ad hoc* image-analysis software we calculated the density of $CD3^+$ and $CD68^+$ cells (percentage of immune-reactive area) specifically at the tumor invasive margin. The density of $CD3^+$ cells increased in stage III (not significant) and strongly decreased in stage IV ($p<0.01$) (fig. 24A). The density of $CD68^+$ cells progressively decreased from stage II to stage IV (fig. 24A).

Subsequently, based on the percentage of $CD3^+$ and $CD68^+$ cells at the tumor invasive margin, the CRC case list was divided into two groups, using the median values as cut-off (2.15 and 4.24, respectively). According to patients' clinical and

pathological features, stage IV tumors (presence of distant organ metastasis) showed a reduced level of $CD3^+$ cell infiltration (univariate, OR 0.41, p=0.02) (tab. 3A). Moreover, high levels of $CD68^+$ cell inversely correlated with tumor vascular invasion (univariate, OR 0.36, p=0.007; multivariate, OR 0.44, p=0.04) and grading (univariate, OR 0.27, p=0.02) (tab. 3B).

In line with previous studies, high levels of $CD3^+$ cells at the tumor invasive front correlated with lower recurrence (p=0.04) and a tendency to increased disease-specific survival, although this difference did not reach statistical significance (fig. 24B). No correlation between $CD68^+$ cell infiltration and patients' outcome was found, although high-infiltrated tumors showed a tendency to a lower recurrence and a better survival (fig. 24B).

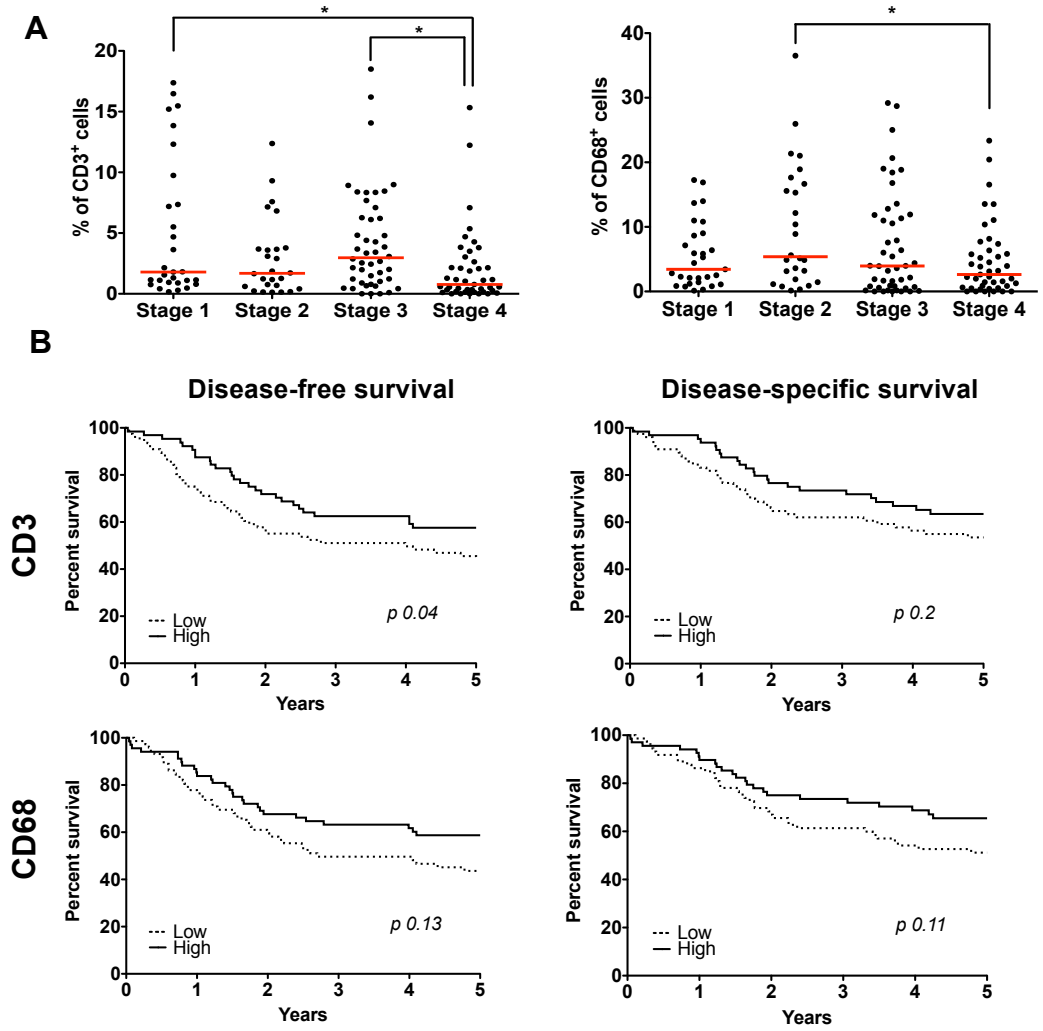


Fig. 24: Expression of $CD3^+$ and $CD68^+$ cells at tumor invasive burden

(**A**) $CD3^+$ and $CD68^+$ cell distribution among tumor stages. Data have been calculated as the percentage of the immune-reactive area. Red bars indicate median values. (**B**) Kaplan-Meier curves show disease-free and disease-specific survival based on $CD3^+$ and $CD68^+$ cell expression. * *p<0.05*

		CD3 expression		Univariate Analysis		Multivariate Analysis	
		Low (n=79)	High (n=65)	OR (95% C.I.)	P	OR (95% C.I.)	P
Age	*(years, mean ±SD)*	64.5±10.8	62.7±12.2	0.99 (0.96-1,02)	0.35		
Sex	*Male*	40	35	1.00 Ref.			
	Female	39	30	0.88 (0.46-1.7)	0.7		
Tumor Site	*Distal*	21	19	1.00 Ref.			
	Proximal	34	29	0.94 (0.43-2.1)	0.88		
	Rectum	24	17	0.78 (0.33-1.89)	0.58		
Local Tumor Invasion	*pT1*	5	1	1.00 Ref.			
	pT2	12	15	6.25 (0.64-60.9)	0.11		
	pT3	55	39	3.55 (0.4-31.5)	0.26		
	pT4	7	10	7.14 (0.68-75.2)	0.10		
Nodal Status	*No*	35	25	1.00 Ref.			
	Yes	44	40	1.27 (0.65-2.48)	0.48		
Distant Metastasis	*M0*	49	52	1.00 Ref.			
	M+	30	13	0.41 (0.19-0.87)	**0.02**		
Tumor Cell type	*ADC*	70	62	1.00 Ref.			
	Variant	9	3	0.38 (0.1-1.45)	0.16		
Vascular Invasion	*No*	52	46	1.00 Ref.			
	Yes	27	19	0.8 (0.39-1.62)	0.53		
Grading	*G1-G2*	65	56	1.00 Ref.			
	G3	13	9	0.80 (0.31-2.02)	0.64		

B

		CD68 expression		Univariate Analysis		Multivariate Analysis	
		Low (n=76)	**High (n=68)**	**OR (95% C.I.)**	**P**	**OR (95% C.I.)**	**P**
Age	*(years, mean ±SD)*	64.9±11.9	62.2±10.8	0.98 (0.96-1,01)	0.15		
Sex	*Male*	43	32	1.00 Ref.			
	Female	33	36	1.47 (0.76-2.83)	0.25		
Tumor Site	*Distal*	23	17	1.00 Ref.			
	Proximal	32	31	1.31 (0.59-2.91)	0.51		
	Rectum	21	20	1.29 (0.54-3.1)	0.57		
Local Tumor Invasion	*pT1*	5	1	1.00 Ref.			
	pT2	13	14	5.38 (0.55-52.4)	0.15		
	pT3	52	42	4.04 (0.45-35.9)	0.21		
	pT4	6	11	9.17 (0.86-97.7)	0.07		
Nodal Status	*No*	28	32	1.00 Ref.			
	Yes	48	36	0.66 (0.34-1.28)	0.22		
Distant Metastasis	*M0*	50	51	1.00 Ref.			
	M+	26	17	0.64 (0.31-1.32)	0.23		
Tumor Cell type	*ADC*	66	66	1.00 Ref.			
	Variant	10	2	0.2 (0.04-0.95)	**0.04**		
Vascular Invasion	*No*	44	54	1.00 Ref.		1.00 Ref.	
	Yes	32	14	0.36 (0.17-0.75)	**0.007**	0.44 (0.2-0.96)	**0.04**
Grading	*G1-G2*	58	63	1.00 Ref.			
	G3	17	5	0.27 (0.09-0.78)	**0.02**		

Tab. 3. CD3 and CD68 expression and correlation with patients' clinical and pathological features

CD3 (**A**) and CD68 (**B**) expression scores were correlated with patients' clinical and pathological features. Univariate and multivariate analysis has been performed

Since subsets of either lymphocytes or monocytes express CX_3CR1, we investigated whether the expression of CX_3CL1 by tumor epithelial cells influenced the recruitment of immune cells at the tumor site. Double immunofluorescence staining, revealed that only a limited number of $CD3^+$ and $CD68^+$ cells are positive for CX_3CR1 (fig. 25). Linear regression analysis showed that there is no correlation between the score of CX_3CL1 and the percentage of $CD3^+$ or $CD68^+$ cells at tumor invasive margin, indicating that tumor epithelial cell derived-CX_3CL1 is not directly responsible for the recruitment of these cells to colorectal cancer tissues (fig. 25).

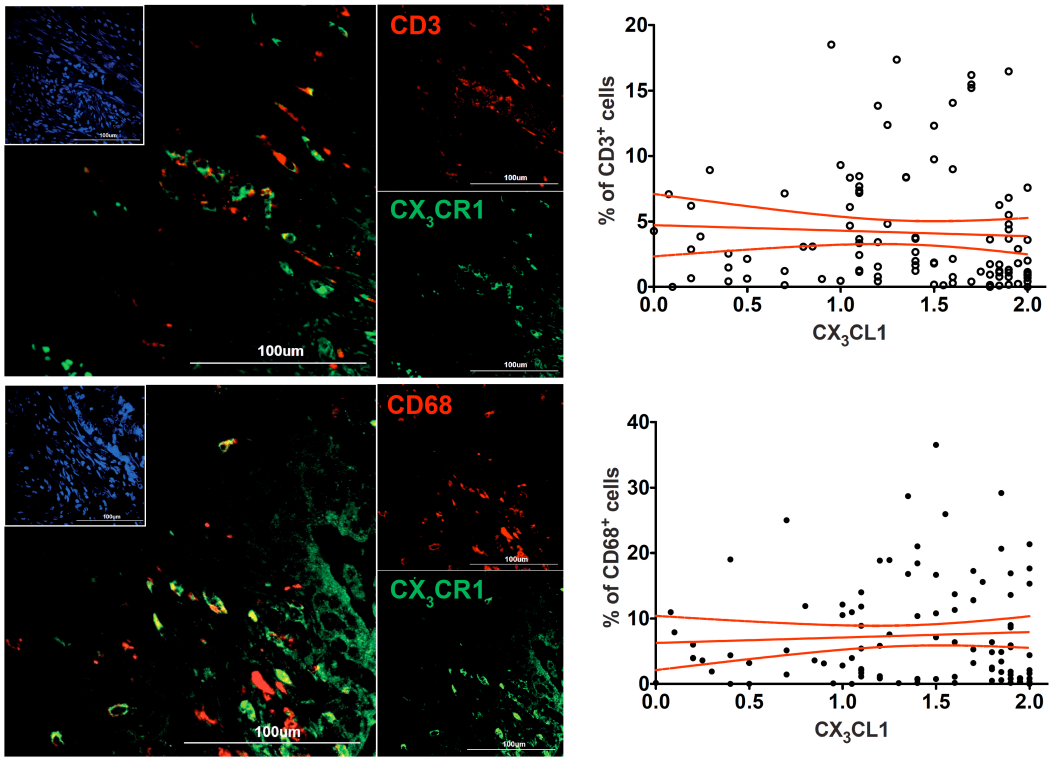


Fig. 25: Correlation between CX_3CL1 expression and $CD3^+$ and $CD68^+$ expression at tumor invasive margin

Linear regression test has been used to analyse the correlation between tumor cell derived-CX_3CL1 and $CD3^+$ and $CD68^+$ expression by infiltrating cells at tumor invasive margin. Double immunofluorescence was performed to check the expression of CX_3CR1 in $CD3^+$ and $CD68^+$ cells. Magnification: 400X.

CX_3CL1-CX_3CR1 axis in human colorectal cancer

The immunohistochemistry data showed that tumor cells may express both CX_3CL1 and its receptor CX_3CR1. These results were confirmed by immunofluorescence analysis. In addition, we observed different spots of colocalization, indicating a possible tumor cell-cell interaction mediated by CX_3CL1-CX_3CR1 axis (fig. 26A). To better investigate the role of the CX_3CL1-CX_3CR1 axis in tumor progression, we compared tumors with high expression of both CX_3CL1 and CX_3CR1 (positive-axis tumors) with tumors with low expression of both CX_3CL1 and CX_3CR1 (negative-axis tumors). Double-positive tumors showed a better disease-specific survival (p=0.003) and a lower recurrence (p=0.02) in stage I-III tumors (fig. 26B).

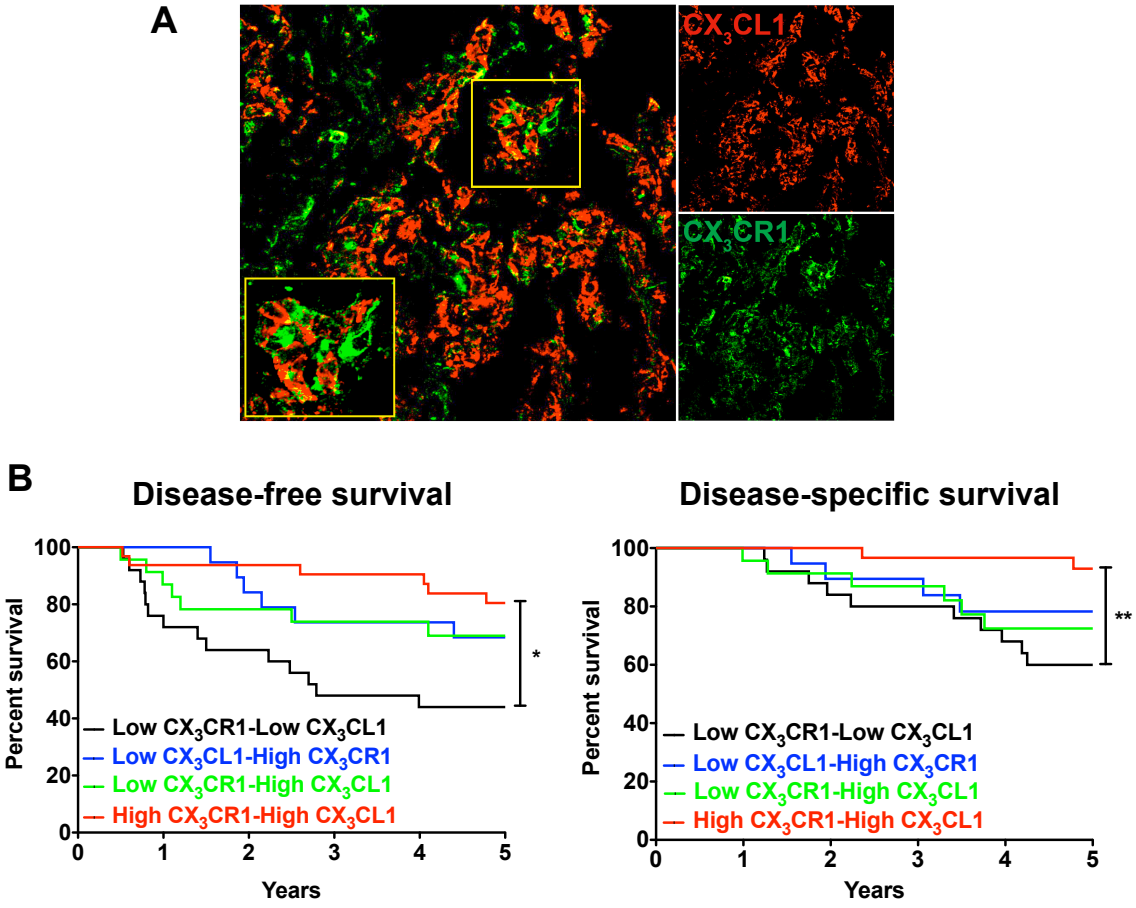


Fig. 26: CX_3CL1-CX_3CR1 axis in human colorectal cancer

(**A**) Double immunofluorescence analysis on frozen sections of human CRC. Several spots of colocalization were identified (merge, in set). Nomarski image shows tissue structure. Magnification: 400X. (**B**) Kaplan-Meier curves show patients' survival according to CX_3CL1-CX_3CR1 axis. * *p=0.02,* ** *p=0.003*

CX_3CL1-CX_3CR1 axis and metastasis

As our data demonstrated that the expression of CX_3CL1 decreased in tumor stage III and that the lack of the CX_3CL1-CX_3CR1 axis by primary tumor cells resulted in a worse prognosis, we investigated the role of CX_3CL1-CX_3CR1 axis in tumor metastasis. We analysed by immunohistochemistry the expression of CX_3CL1 and CX_3CR1 in 35 lymph node metastases and 18 liver metastases and compared the expression in metastatic lesions with the expression of the corresponding primary tumor. Representative images are shown in fig. 27. We observed that the expression of CX_3CL1 and CX_3CR1 in lymph node metastasis was significantly reduced in comparison to primary lesions ($p=0.03$ and $p=0.0005$, respectively) (fig. 27A). In addition, the expression of CX_3CR1 in liver metastasis was dramatically reduced ($p<0.0001$). The ligand CX_3CL1, instead, was still expressed in liver metastasis (fig. 27B). Overall, these data indicate that, at variance from the expression of CX_3CL1 and CX_3CR1 in primary lesion, tumor metastatic cells showed a strongly reduced expression of CX_3CR1, or both the receptor and ligand.

Moreover, correlation with patients' clinical and pathological features indicated that high expression of CX_3CL1 or CX_3CR1 in primary tumors reduced the risk of metachronous metastasis (i.e. metastasis occurring after surgical resection, within 12 months) (CX_3CL1: univariate, OR 0.34, $p=0.02$; CX_3CR1: univariate, OR 0.28, $p=0.007$; multivariate, OR 0.28, $p=0.008$). In particular, CX_3CL1-CX_3CR1 positive-axis tumors have a significantly lower risk of metachronous metastasis (univariate, OR 0.10, $p=0.002$; multivariate, OR 0.06, $p=0.002$) (tab.4).

As the two molecules are involved in cell-cell interaction, we hypothesized that the concomitant expression of CX_3CL1 and CX_3CR1 could increase tumor cell adhesiveness, thus preventing tumor spreading and metastasis.

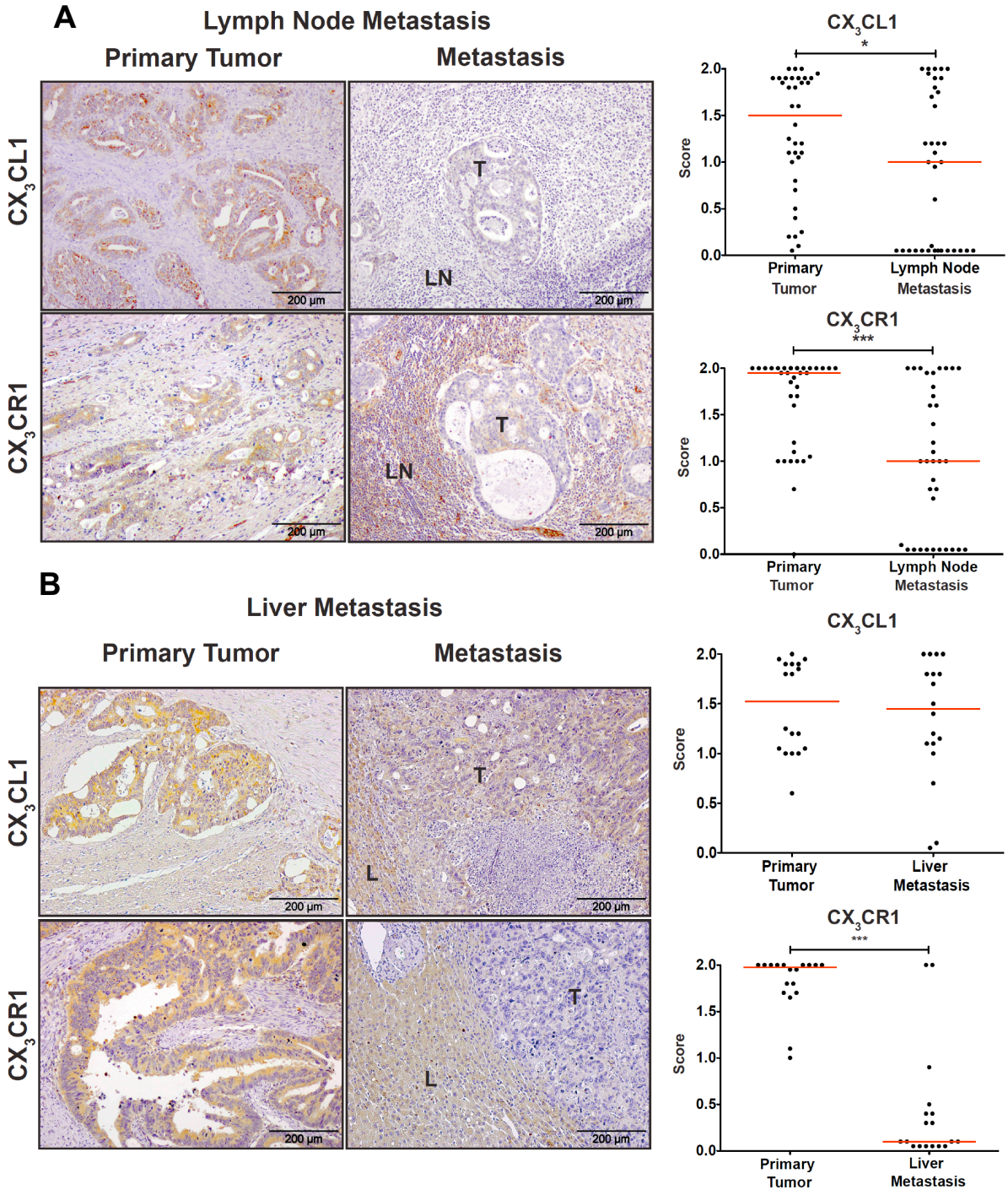


Fig. 27: CX_3CL1 and CX_3CR1 expression in metastatic lesions

The expression of CX_3CL1 and CX_3CR1 was evaluated by immunohistochemistry in lymph node (**A**) and liver (**B**) metastasis, and compared with their expression in primary tumors. Mann-Whitney analysis has been performed. Magnification: 200X; * *p<0.05;* *** *p<0.001*. **T**: Tumor; **LN**: Lymph Node; **L**: Liver.

Expression in Primary Tumors	Metachronous Metastasis	Expression		Univariate		Multivariate	
		Low	High	OR (95% C.I.)	*P*	OR (95% C.I.)	*P*
CX_3CL1	*No*	33	48	1.00 Ref.		1.00 Ref.	
	Yes	18	9	0.34 (0.14-0.86)	**0.02**	0.38 (0.14-1.03)	0.06
CX_3CR1	*No*	32	49	1.00 Ref.		1.00 Ref.	
	Yes	19	8	0.28 (0.11-0.70)	**0.007**	0.28 (0.11-0.72)	**0.008**
CX_3CL1-CX_3CR1 axis	*No*	13	29	1.00 Ref.		1.00 Ref.	
	Yes	13	3	0.10 (0.03-0.43)	**0.002**	0.06 (0.01-0.34)	**0.002**

Tab. 4. Correlation between the expression of CX_3CL1, CX_3CR1 and CX_3CL1-CX_3CR1 axis in primary tumors and the occurring of metachronous metastasis.

Role of the CX_3CL1-CX_3CR1 axis in in vitro tumor cell proliferation and adhesion

To functionally study the potential adhesive role of CX_3CL1-CX_3CR1 axis in tumor cells, we investigated the expression of both ligand and receptor in a established tumor cell lines of human colorectal origin. We first performed a screening of CX_3CL1 and CX_3CR1 mRNA levels in nine human CRC cell lines. Receptor expression was very low or undetectable in 9/9 cell lines, while the ligand was expressed mainly by DLD-1 cells and, to a lesser extent, by HT-29, SW620, SW480 and CaCo2 cells (fig. 28).

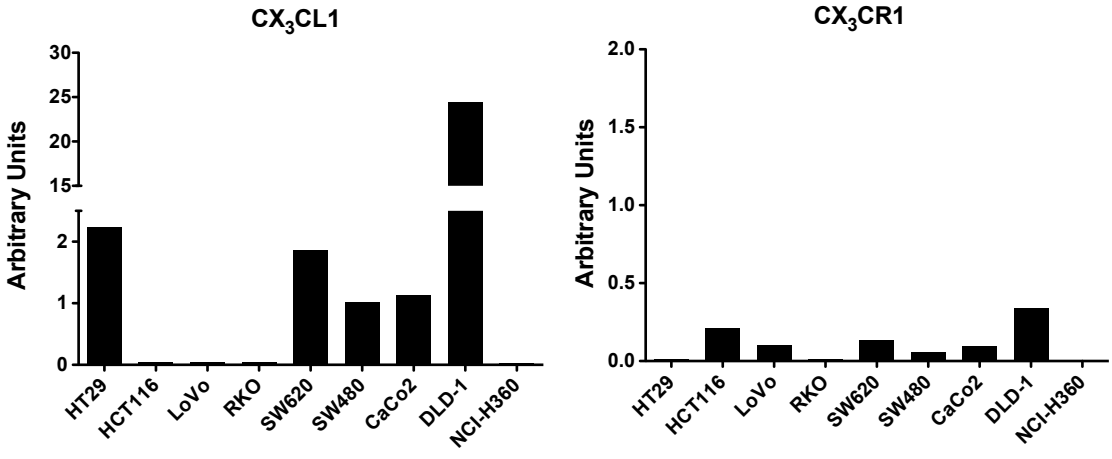


Fig. 28: Screening of CX_3CL1 and CX_3CR1 mRNA expression in nine human colorectal cancer cell lines.

We identified two cell lines negative for both CX_3CL1 and CX_3CR1: RKO and NCI-H360, that were used for transduction experiments.

We generated two different lentiviral vectors carrying human CX_3CL1 or CX_3CR1 genes fused to Cherry or GFP, respectively, at the C-terminus of the protein. Empty vectors carrying Cherry or GFP alone were used as negative controls.

Subsequently we infected both the NCI-H360 and the RKO cell lines with CX_3CL1-cherry or CX_3CR1-GFP fusion protein and their respective empty vectors (mock) (fig. 29A).

We first tested whether CX_3CL1 and CX_3CR1-infected cells showed a differential ability to proliferate *in vitro*. We performed a 7 days-long proliferation assay on CX_3CL1 and CX_3CR1-infected cells, using the corresponding empty vector-infected cells as negative control, and we did not observe any difference in the ability of cells to proliferate *in vitro*. To investigate whether the expression of CX_3CL1 by tumor cells could influence the proliferation of CX_3CR1-infected cells, we co-cultured CX_3CL1-expressing cells with cells infected with CX_3CR1 (positive loop). As negative controls, we co-cultured cells infected with the empty vectors (cherry or GFP), with cells expressing either CX_3CL1 or CX_3CR1 (negative loops), as shown in fig. 29B. Cells were mixed in 1:1 ratio. Also in this case, we did not observe any difference in cell proliferation in comparison to negative controls, suggesting that the presence of the positive loop does not significantly influence tumor cell proliferation *in vitro* (fig. 29B).

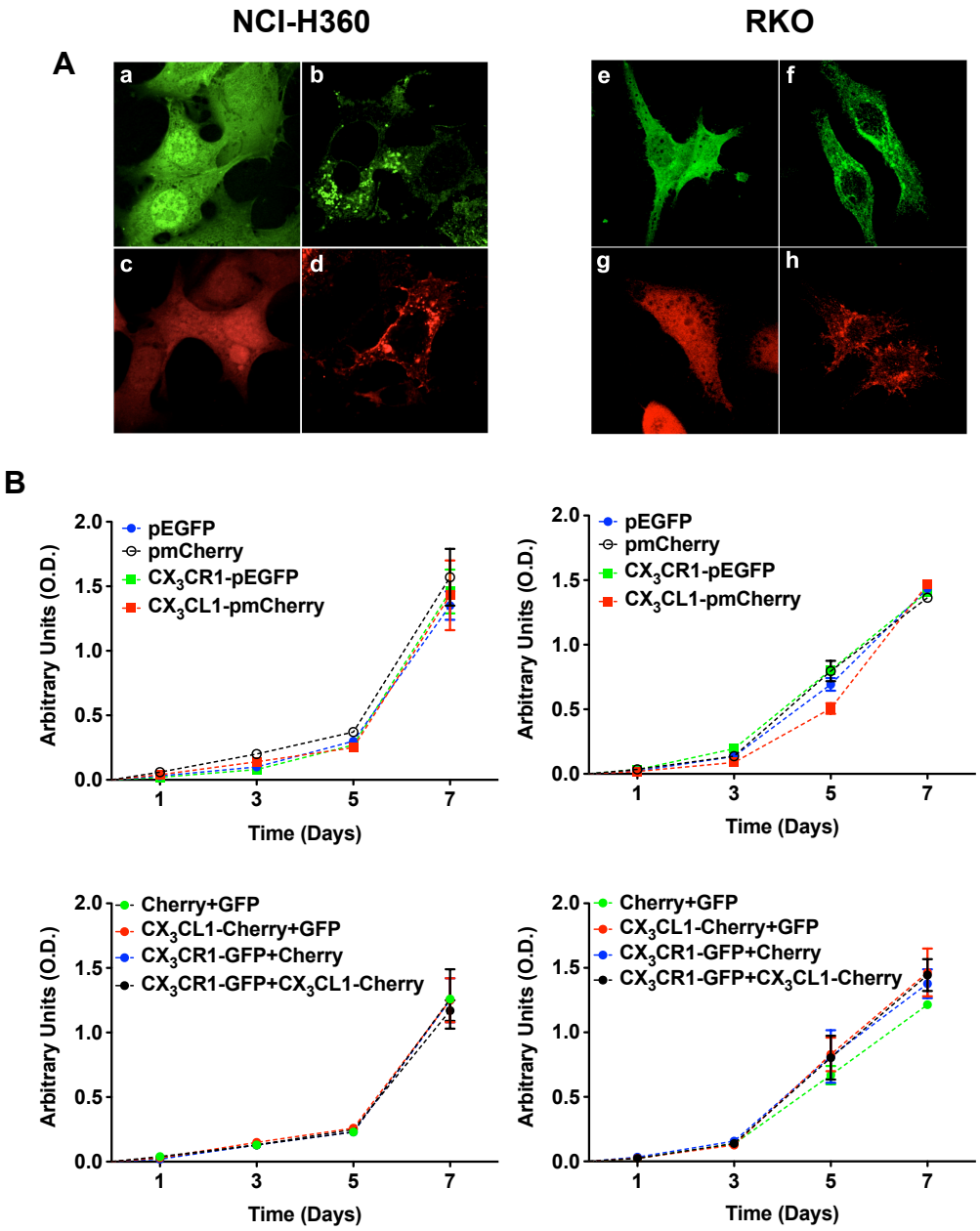


Fig. 29: Infection and proliferation assay on NCI-H360 and RKO human colorectal cancer cell lines.
(**A**) NCI-H360 and RKO cells infected with CX_3CL1-cherry, CX_3CR1-GFP and corresponding empty vectors. **a, e:** GFP; **b, f:** CX_3CR1-GFP; **c, g:** Cherry; **d, h:** CX_3CL1-cherry (**B**) Growth curve of NCI-H360 and RKO infected cells.

Subsequently, we investigated whether the ectopic expression of CX_3CL1-CX_3CR1 axis modifies tumor cell adhesiveness. We performed an adhesion assay plating CX_3CR1-GFP-infected cells or GFP-infected cells for 30 minutes on 96-well plate coated with different concentration of human recombinant CX_3CL1: we observed that the expression of CX_3CR1 significantly increased cell adhesiveness (fig. 30).

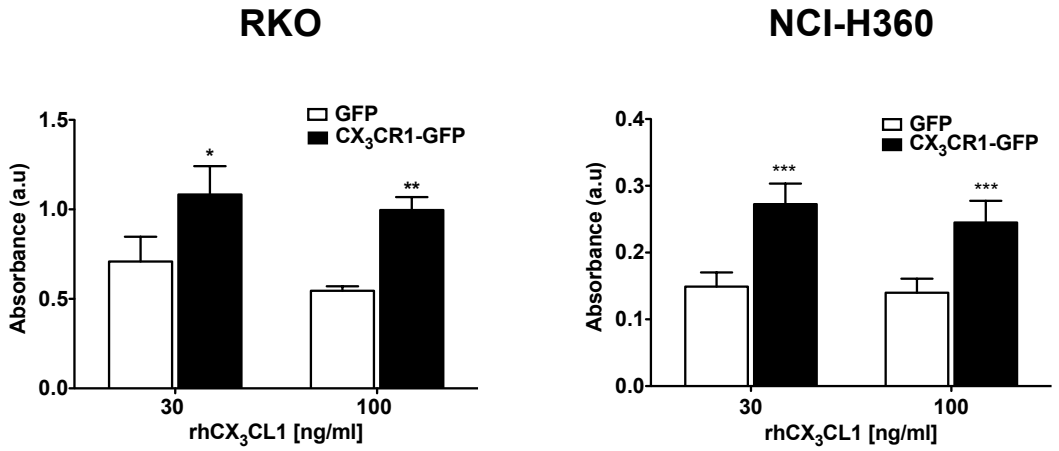


Fig. 30: Adhesion assay on rhCX$_3$CL1 coated plates

RKO or NCI-H360 cells infected with CX$_3$CR1 or the corresponding empty vector plated 96-well plate coated with different concentration of rhCX$_3$CL1. Adherent cells were measured by optical density at 570nm wavelength. ** $p<0.05$; ** $p<0.01$; *** $p<0.001$*

In addition, the expression of CX$_3$CR1 increased the adhesion of RKO cells on a monolayer of CX$_3$CL1 RKO infected cells (not shown).

Taken together, these results demonstrate that the expression of CX$_3$CL1-CX$_3$CR1 axis by tumor cells does not modify tumor cell proliferation, but is conferring enhanced adhesion capability to CX$_3$CR1 expressing cells.

Role of the CX$_3$CL1-CX$_3$CR1 axis in vivo in a mouse xenograft model of human colorectal cancer

We next tested whether the expression of CX$_3$CL1-CX$_3$CR1 axis influenced tumor growth *in vivo*. We used the same combination of cells used in *in vitro* proliferation assay in order to generate tumors with positive chemokine loop (CX$_3$CL1$^+$-CX$_3$CR1$^+$) or negative (CX$_3$CL1$^-$-CX$_3$CR1$^-$) or single positive. Also in this case, cells were mixed in 1:1 ratio.

Tumor growth was measured by caliper every two-three days, starting two weeks after the injection. During this period, we did not observe differences in the growth of the various combinations of tumors (fig. 31A). After 36 days, mice were sacrificed and tumor weight measured: as shown in fig. 31B, we did not observe any

significant difference in tumor weights, confirming the initial observation that the presence of CX_3CL1-CX_3CR1 axis is not influencing tumor growth, also *in vivo*.

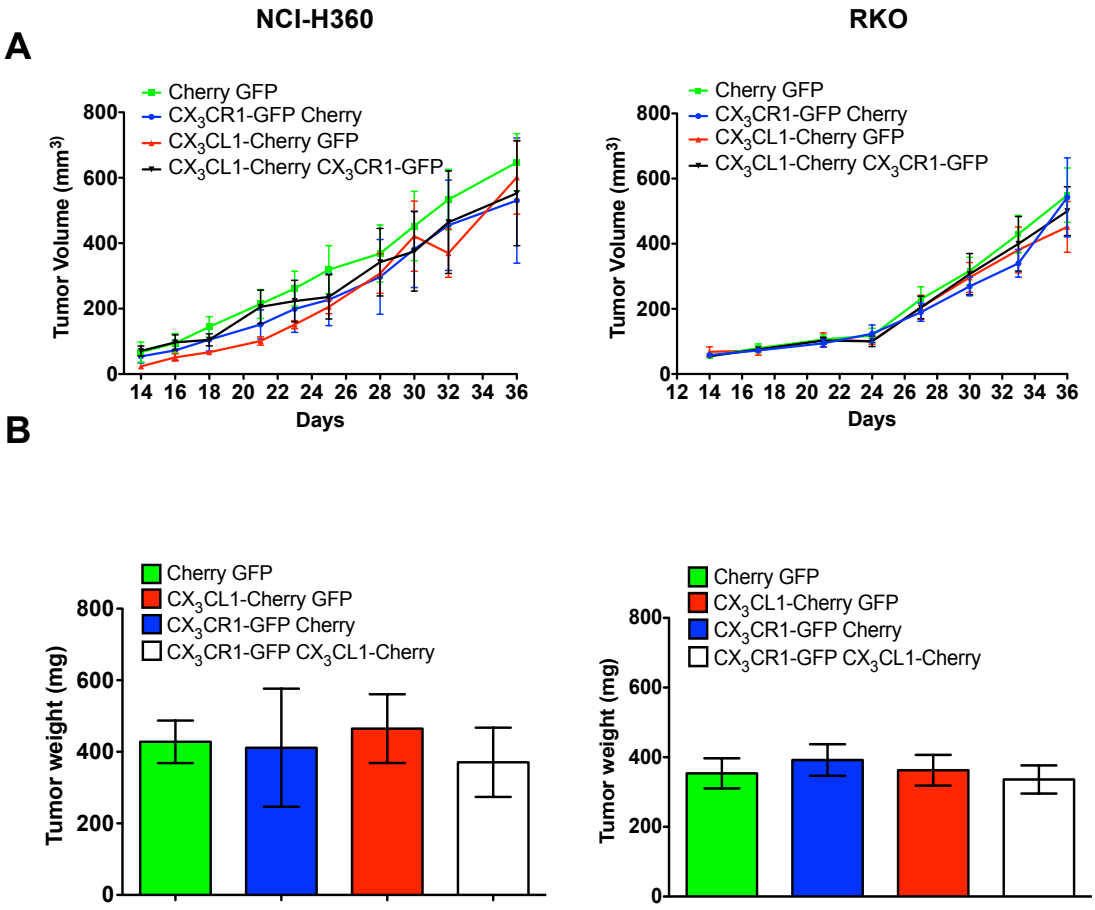


Fig. 31: NCI-H360 and RKO tumor growth *in vivo*

(**A**) Measurement of the volume of tumors generated injecting subcutaneously NCI-H360 and RKO infected cells.

(**B**) Tumor weight after mouse sacrifice, 36 days.

CX_3CL1-CX_3CR1 interaction in colorectal cancer cells

Tumors obtained with NCI-H360 infected cells were subsequently analysed by immunofluorescence. Interestingly, CX_3CL1-CX_3CR1 positive loop tumors showed several spots of colocalization in comparison to CX_3CL1-CX_3CR1 negative loop tumors (fig. 32), indicating a cell-cell interaction *in vivo* mediated by CX_3CL1-CX_3CR1 axis.

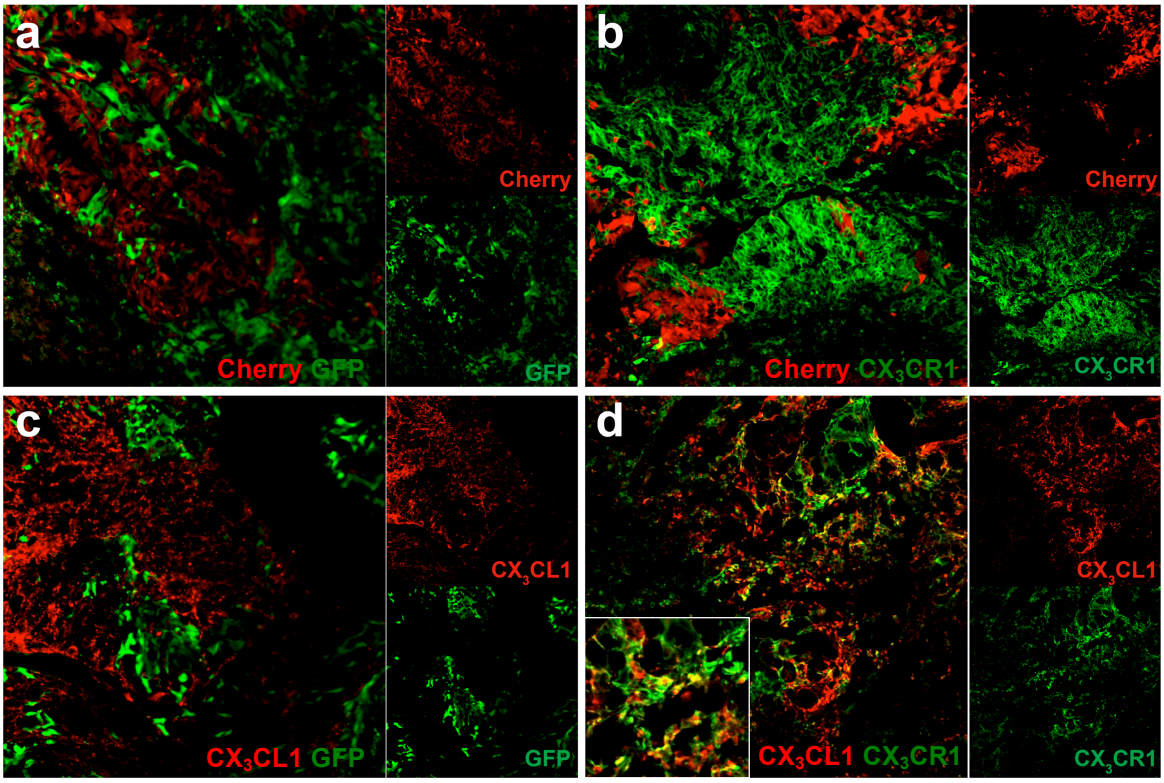


Fig. 32: CX_3CL1-CX_3CR1 axis in a xenograft mouse model of human CRCs

Immunofluorescence of the xenograft mouse model generated using NCI-H360 infected cells. Several spots of colocalization are present in the CX_3CL1-CX_3CR1 positive loop tumors (**d**), in comparison to CX_3CL1-CX_3CR1 negative loop tumors (**a**, **b**, **c**). Magnification: 200X

To further investigate the role of CX_3CL1-CX_3CR1 axis in tumor cell organization, we analysed the expression of CX_3CL1 and CX_3CR1 in NCI-H360 cells growing *in vitro* as multicellular tumor spheroids (MTS). The same combinations of NCI-H360 infected cells used in *in vivo* model were co-cultured in non-adherent condition in order to form MTS. Immunofluorescence analysis showed the distribution of NCI-H360 infected cells within MTS: in particular we observed several spots of colocalization in MTS of CX_3CL1^+-CX_3CR1^+ cells (fig. 33, panel g, h), while MTS lacking the chemokine loop (fig. 33, panel a-f) did not show colocalizing spots.

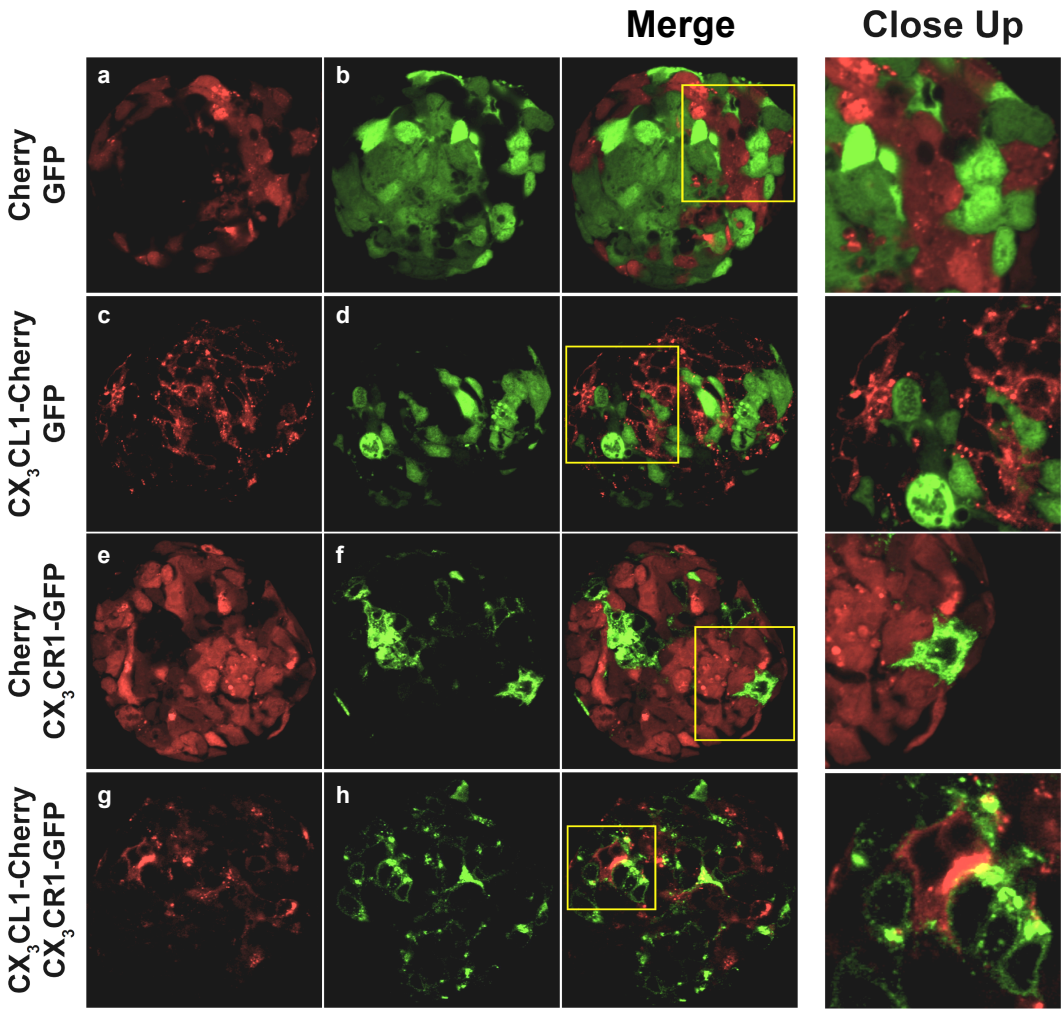


Fig. 33: Expression of CX_3CL1-CX_3CR1 axis in NCI multicellular tumor spheroids

MTS were obtained using different combination of NCI-H360 infected with CX_3CL1-cherry and CX_3CR1-GFP cells and corresponding empty vectors to mimic CX_3CL1-CX_3CR1 axis. **a,e**: cherry; **b,d**: GFP; **c,g**: CX_3CL1-cherry; **f,h**: CX_3CR1-GFP. Merge and Close up images are indicated. Magnification: 600X

To confirm ligand-receptor interaction, we performed Fluorescence Resonance Energy Transfer (FRET) assay on MTS obtained with CX_3CL1^+-CX_3CR1^+ NCI-H360 cells. Fluorescence Lifetime Imaging Microscopy (FLIM) has been used to measure FRET efficiency. The CX_3CR1-GFP fluorescence lifetime in double-positive MTS was measured in comparison to CX_3CR1-GFP fluorescence lifetime in single positive MTS (fig. 34A). FLIM analysis showed that CX_3CR1-GFP mean lifetime was reduced in the presence of CX_3CL1-cherry, indicating FRET events (fig. 34B) Comparing mean lifetime of CX_3CR1-GFP in presence or absence of CX_3CL1-cherry, the FRET efficiency was calculated as E=14%. In addition, based on FRET

efficiency and GFP-Cherry Föster Radius (51 Å), we calculated the mean distance between CX_3CR1-GFP and CX_3CL1-cherry fusion proteins (R=6.9 ± 0.9 nm). Taken together, these data strongly support the hypothesis that CX_3CL1-CX_3CR1 axis is important in tumor cell interaction mediating their adhesiveness.

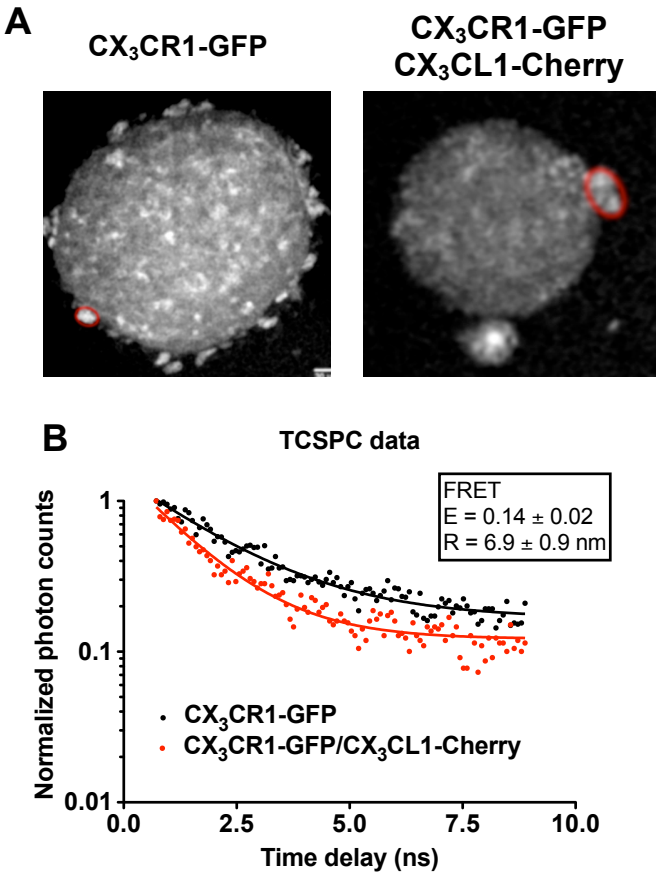


Fig. 34: FRET analysis on CX_3CL1-cherry+CX_3CR1-GFP MTS in comparison to CX_3CR1-GFP MTS
(**A**) FLIM was calculated selecting region of interest (ROI) within the MTS with high expression of GFP. Examples of ROI are indicated by red circles. (**B**) Time-correlated single photon counting data indicate a reduction in GFP lifetime in presence of Cherry protein in comparison to GFP lifetime in absence of Cherry protein. FRET efficiency (E), as well as distance between GFP and Cherry proteins (R) are indicated in the box.

Role of the CX_3CL1-CX_3CR1 axis in tumor dissemination in vivo

The crucial question is whether the expression of the CX_3CL1-CX_3CR1 axis in tumor cells is sufficient to prevent *in vivo* metastatic dissemination.

As the human cell lines were not metastatic in nude mice, we have turned to a murine model of colorectal cancer. The cell line CT26 has been infected with the

murine CX_3CL1 or CX_3CR1 cDNA. Appropriate combinations of double-positive or double-negative tumors will be injected in mice in a spleen-liver model of metastasis.

These experiments are on going, and hopefully will clarify whether double-positive tumors have indeed lower metastatic ability.

DISCUSSION

Chemokines and chemokine receptors in human colorectal cancer

In this study we have investigated the chemokine system in samples of human colorectal tumors. To have a global view of which chemokines and receptors are overexpressed in the tumor microenvironment we have used a customized TaqMan Low Density Array containing probes for 24 ligands and 10 receptors. Several CC and CXC chemokines were strongly up regulated in tumor samples compared to the corresponding normal colonic mucosa. Interestingly, a number of chemokines were constitutively expressed also in the normal tissue, such as CCL2, CCL20 and CXCL12. The significance of their expression in normal colonic mucosa is likely explained by the abundant presence, at this site, of immune effectors of both innate and adaptive immunity, to maintain the local homeostasis. These chemokines were not or only slightly elevated in tumor samples. In contrast, several inflammatory chemokines, such as CCL3, CCL4, CXCL8, and CXCL1 were significantly increased in tumor tissues. We confirmed the up regulation of these chemokines in a larger number of tumor specimens. CCL3 and CCL4 are chemotactic for monocytes/ macrophages and T cells, the major components of tumor-infiltrating leukocytes in colorectal tumors, as well as in other tumors. Of note, while the subset of $CD8^+$ T cells has been defined as protective anti-tumor effectors in CRC and their number associated with better prognosis [130], the role of macrophages is more ambiguous. Some studies have described divergent results, reporting a correlation with a favourable clinical outcome [135,208] or, in marked contrast, an association with tumor progression [137,254,255]. These contrasting results may be interpreted in view of the macrophage balance hypothesis and the distinct functions of polarized macrophage populations [132,256].

The top-expressed chemokine in the tumor microenvironment was CXCL8, a ligand attracting mainly neutrophils, as well as T lymphocytes. Recently, it has been reported that Myeloid Derived Suppressor Cells (MDSCs) express the CXCR2 receptor and may respond to CXCL8. The contribution of CXCL8 in the recruitment of MDSC, or related myeloid immature precursors, deserves further investigation.

CXCL8 and other members of the same family have been extensively studied for their role in stimulating neoplastic growth [257,258]. These chemokines may also promote tumor progression and invasion by stimulating the process of neo-

angiogenesis and the activation of matrix proteases [205,259,185] In addition to CXCL8, the receptor CXCR2 was also found up regulated in selected samples, suggesting an increased activity of the functional loop in the tumor micro-environment.

It is well established that other members of the CXC family possess anti-angiogenic activity [205]. In the tumor microenvironment, the balance between pro-angiogenic and anti-angiogenic chemokines may determine the degree of neo-angiogenesis and, consequently, tumor progression. In our study, we found high levels of two anti-angiogenic chemokines, CXCL9 and CXCL10, but no significant expression of their specific receptor CXCR3 was detected. Hence, it is likely that the pro-angiogenic effect of CXCL8 prevails.

The TaqMan array we used contained several other probes coding for inflammation-related genes. Among these was Osteopontin (OPN), a glycoprotein belonging to the large family of extra-cellular matrix proteins, which has raised much interest in cancer biology [260,261]. OPN levels are usually elevated in aggressive tumors when compared with low-grade tumors or with the normal tissue, and correlate with the presence of metastatic disease. Moreover, OPN is included among lists of genes that predict poor prognosis in patients with various types of cancer [262261]. Yet, the mechanism by which OPN supports tumor progression is not clear. Tumor cell interaction with OPN is mediated via the adhesion receptor CD44 and its variants (e.g.CD44v6) and by beta-integrins [263]. Recently McAllister et al, proposed that soluble OPN released by cancer cells could activate bone-marrow cells to be recruited at tumor site, fostering tumor progression [264]. In our study, and in line with previous reports, mRNA of OPN was strongly up regulated in tumor samples. Of interest, a linear correlation was found between the expression of OPN and CXCL8. As mentioned above, these two mediators share some important functions, such as cell mobility and cell survival via integrin activation [232].

Overall, this initial screening lead to the identification of the most expressed genes in tumor samples, and was therefore very informative for the selection of a more restricted panel of candidate molecules for further investigation. These data may be helpful for the consideration of candidate targets for novel therapeutic interventions directed to specific inflammatory molecules, and to identify novel prognostic markers of CRC.

CX_3CL1-CX_3CR1 axis in human colorectal cancer

Among the chemokines and chemokine receptors up regulated in colorectal cancer, we have identified CX_3CL1 and CX_3CR1. As discussed above, CX_3CL1 is a unique chemokine that combines properties of both chemoattractants and adhesion molecules. CX_3CL1 has a single receptor, CX_3CR1, which is expressed primarily on monocytes, dendritic cells, NK cells and T-cells. It is becoming increasingly clear that CX_3CL1 not only mediates the recruitment and activation of immune cells, but can also exert multiple effects on non-immune cells: several recently published studies demonstrated a role for Fractalkine in the metastatic process of different tumors, such as breast cancer, prostate cancer and glioma [250,252,253].

In this study, we considered for the first time the role of CX_3CL1 and CX_3CR1 expression by tumor epithelial cells in the progression of human colorectal cancer. Our data demonstrated that the CX_3CL1-CX_3CR1 concomitant expression on tumor epithelial cells positively correlates with a more favourable patient prognosis. In addition, we did not find any correlation between CX_3CL1 expression by tumor epithelial cells and the density of $CD3^+$ and $CD68^+$ cells at the tumor invasive front, suggesting that the good prognosis associated to the expression of CX_3CL1-CX_3CR1 axis is independent from the anti-tumor immune response.

The role of CX_3CL1 and CX_3CR1 in cancer has been usually associated with the recruitment of immune cells at the tumor site. *In vivo* gene-transfer studies have demonstrated an anti-tumor immune response mediated by CX_3CL1. Guo et al. [242] demonstrated the infiltration of $CD8^+$ T cells, $CD4^+$ T cells and dendritic cells into CX3CL1-transduced Lewis lung carcinoma (3LL-FK), concluding that 3LL-FK induced T-cell-dependent antitumor immunity through chemoattraction and activation of dendritic cells. In a subsequent study, the same group demonstrated that NK cells also play an important role in the antitumor response induced by 3LL-FK [243]. In B16-F10 melanoma and C26 adenocarcinoma cancer model, Xin et al. also confirmed a marked infiltration of $CD4^+$ T cells, $CD8^+$ T cells, dendritic cells and NK cells into the CX_3CL1-transduced tumors, thus suggesting that CX_3CL1 induces both innate and adaptive immunity [265]. However, in the EL-4 lymphoma model, the antitumor effect of CX_3CL1 was dependent only on NK cells but not T cells [266]. Recently, increased expression of CX_3CL1 has been correlated with a better

prognosis and an increased number of both $CD8^+$ T cells and NK cells in human gastric adenocarcinoma [244].

CX_3CL1 is expressed by intestinal epithelial and endothelial cells, and regulates the attraction and migration of intestinal lymphocytes in healthy and diseased intestinal mucosa. Both intestinal intraepithelial lymphocytes and lamina propria lymphocytes have been shown to express CX_3CR1 [239]. Patients affected by inflammatory bowel diseases (IBD), such as ulcerative colitis or Chron's disease, showed an increased expression of CX_3CL1 by intestinal epithelial cells and microvascular endothelial cells, and had a significantly increased number of CX_3CR1^+ T cells, predominantly $CD8^+$ T cells [238]. Recently, in a mouse model of acute colitis, it has been demonstrated that CX_3CL1-CX_3CR1 axis was responsible for the recruitment of $F4/80^+$ macrophages and $DEC205^+$ dendritic cells into the damaged tissues [240].

In colorectal cancer, high expression of Fractalkine resulted in a better prognosis and correlated with an increased number of TILs (predominantly $CD8^+$ T cells), both in mouse model [247] and in human samples [248,249]. The role of TILs in colorectal cancer has been clearly demonstrated, indicating that lymphocytes localized at the tumor invasive front strongly correlated with good prognosis [130,110,150], in particular in node-negative colorectal cancer [111].

Also in our cohort of patients, high $CD3^+$ T cell infiltration was associated with good prognosis, confirming previous results, but, in contrast with other studies, we did not find any correlation between CX_3CL1 expression by tumor epithelial cells and the density of $CD3^+$ T cells at the tumor invasive front. This finding indicates that tumor cell-derived CX_3CL1 is not directly responsible for the recruitment of lymphocytes to CRC tissues. This discrepancy could be explained by the fact that we analysed the expression of Fractalkine restricted to the epithelial compartment, without considering the production by endothelial cells, well known producers of this chemokine [239]. Given the well-described role of activated endothelial cell-derived CX_3CL1 in the recruitment, adhesion and transmigration of CX_3CR1^+ cells from the circulation to the inflamed tissues, it could be possible that the expression of Fractalkine by endothelial cells play the major role in the recruitment of $CD3^+$ cells into colorectal cancer tissues. Indeed, we found that almost all vessels identified in the colorectal cancer tissues showed a strong Fractalkine expression by endothelial cells (not shown).

In addition, we also investigated whether CX_3CL1 was responsible for the recruitment of $CD68^+$ macrophages into colorectal cancer tissues. In spite of an abundant literature on many pro-tumor functions of macrophages in several tumor types, their role in colorectal cancer, as mentioned above, is still controversial. In our study the high density of $CD68^+$ cells, at the tumor invasive front, resulted in a trend to lower recurrence and better survival, but the data did not reach statistical significance. Also in this case, we did not find any correlation between the expression of CX_3CL1 by tumor epithelial cells and the density of $CD68^+$ cells.

Significance of the CX_3CL1- CX_3CR1 axis in human CRC

As already discussed, CX_3CR1 is primarily expressed on different immune cell populations, and it is directly involved in the migration, adhesion and extravasation of leukocytes from the circulation into the inflamed tissue. In addition to this role in the formation of the cancer microenvironment, a number of studies investigated its expression by neoplastic cells, suggesting that CX_3CR1 could be involved in the metastatic process of different solid tumors. CX_3CR1 expression by tumor cells has been investigated in prostate [251], breast [268] and pancreatic cancer [219]. Fatatis et al. demonstrated that prostate epithelium already expressed CX_3CR1 in healthy conditions, although malignant transformation seems to increase its expression levels [250]. Moreover, the expression of CX_3CR1 on the surface of prostate cancer circulating cells increased their adhesion to the CX_3CL1-expressing bone marrow endothelial cells, influencing the formation of prostate cancer bone metastasis [250,251]. Recently, the same group demonstrated that a similar phenomenon occurred in bone metastatic process of breast cancer cells [252]. In addition, Andre et al showed that CX_3CR1 expression on breast cancer cells predicted the occurrence of brain metastasis, although it was not associated to overall survival or disease-free survival [268]. Our group demonstrated that the expression of CX_3CR1 by human pancreatic cancer cells was involved in the neural tropism of pancreatic ductal adenocarcinoma to intra-pancreatic peripheral nerves, and was a relevant and independent risk factor to predict an early local tumor relapse in resected patients [219,220]. CX_3CR1 is expressed also by human glioblastoma neoplastic cells, and its

expression is an early event during malignant transformation [269]. Interestingly, in this type of tumor constitutive expression of CX_3CL1 in CX_3CR1^+ glioblastoma negatively regulates tumor cell invasiveness, promoting their aggregation [253]. Of interest, our group demonstrated that cancer stem and progenitor cells isolated from glioblastoma surgical specimens already expressed both CX_3CL1 and CX_3CR1, indicating that the presence of the CX_3CL1-CX_3CR1 loop is an early event in the tumorigenesis process [246].

The expression of CX_3CR1 by colorectal cancer epithelial cells has not been previously investigated. In this study we demonstrated that CX_3CR1 was slightly expressed by healthy colonic mucosa and pre-cancerous lesions, and its expression increased during tumor progression, especially in primary tumors of stage IV patients. According to patients' follow up (tumors at stage I-III, excluding stage IV) we found that high CX_3CR1 expression correlates with a better disease-specific survival ($p=0.05$) and a barely significant lower recurrence in stage I-III tumors ($p=0.08$).
A similar finding was obtained considering the expression of the ligand. Patients with high CX_3CL1 had a significantly increased disease-specific survival ($p=0.05$) and barely significant lower recurrence ($p=0.096$). In addition, we found that high expression of CX_3CL1 in the primary tumors significantly decreased the risk of metachronous metastasis.

Our immunohistochemistry data demonstrated that expression of CX_3CL1 and CX_3CR1 was heterogeneous among neoplastic cells, and variable among tumor stages. We therefore considered only tumors with high expression of both Fractalkine and its receptor (positive-axis tumors) and compared the clinical data with tumors having low expression of both (negative-axis tumors). We found that patients with positive axis tumors had a better disease-free and disease-specific survival in comparison to patients with negative-axis tumors.

In fact, the occurrence of metachronous metastasis, within 12 months after surgical resection, was significantly higher in negative-axis tumors in comparison to positive-axis tumors. These data demonstrate that the presence of a functional CX_3CL1-CX_3CR1 axis in primary CRC tumors significantly reduces the risk of metachronous metastasis.

We next compared the expression of CX_3CL1 and CX_3CR1 in primary tumors with their expression in corresponding lymph node or liver metastasis: we found that CX_3CR1 was dramatically reduced in both lymph node and liver metastasis compared to primary tumors. The expression of CX_3CL1 was also strongly reduced in lymph node metastasis. Of note, this decrease is likely to occur already in the primary lesions, as axis-negative tumors have higher risk of metastasis. It cannot be excluded, however, that the down-regulation of Fractalkine and its receptor in the metastatic lesion could be due to a different microenvironment, unable to preserve their expression.

Given the adhesive function of these molecules, we hypothesized that the CX_3CL1-CX_3CR1 interaction is instrumental to maintain the adhesiveness between tumor cells, and thus preventing tumor spreading and metastasis.

Our *in vitro* data appear to support this hypothesis: the overexpression of CX_3CR1 in two different colorectal cancer cell lines significantly increased their adhesiveness on rhCX_3CL1-coated plates, or CX3CL1-expressing cells. Furthermore, in the 3D culture system, co-culture of CX_3CL1-expressing cells with CX_3CR1-cells (positive-axis tumors) showed several spots of co-localization in immunofluorescence analysis. The interaction between the two molecules was clearly confirmed in the FRET experiments.

To further demonstrate this hypothesis, we undertook experiments with human tumor cell lines transduced with either the ligand or receptor. By appropriate mixing of cells to mimic axis-positive or axis-negative tumors, we have demonstrated that the presence of CX_3CL1-CX_3CR1 axis does not influence tumor cell proliferation, both *in vitro* and *in vivo*. In addition, positive-axis tumors grown *in vivo* showed several spots of colocalization, as observed in the 3D-culture model *in vitro*.

The human cell lines did not produce metastasis, even when implanted in the spleen (spleen-liver model). We therefore switched to a syngeneic tumor model of colorectal cancer (CT26 cells) and over-expressed the mouse form of CX_3CL1 or CX_3CR1. Appropriate combinations of CT26-infected cells will be injected in the spleen in order to generate positive-axis and negative-axis tumors. These experiments are on going.

Another possible explanation is that Fractalkine could be involved in the regulation of inflammatory mediators in CRC tissues. Experimental evidence established that the CX_3CL1-CX_3CR1 axis plays a major role in the neuron/microglial crosstalk and in neuroprotection under conditions of inflammation/injury [270,271-273]. In a mouse model of colitis, the ablation of the CX_3CR1 gene reduced the DSS-enhanced expression of the pro-inflammatory cytokines IL-6, TNF-α and IFN-γ [240]. Experiments to evaluate the putative anti-inflammatory effect of Fractalkine on colorectal cancer cells or monocyte-derived inflammatory mediators are on going.

Conclusive remarks

Up to date, colorectal cancer still represents the second cause of cancer-related death in western countries. Although surgery and chemotherapy have drastically changed the clinical course of the disease, it is not infrequent that tumor recurrence eventually occurs, even for early stage tumors. Indeed, there is a lack of diagnostic and prognostic biological indicators for the clinical outcome of these patients. Our data demonstrated that the absence of CX_3CL1 or CX_3CR1 expression by tumor cells could be used as an indicator to identify patients at higher risk of disease recurrence and requiring more efficient or prolonged therapeutic treatment. The observation that positive-axis tumors have lower recurrence rate compared to negative-axis tumors, could be an important prognostic parameter, useful to orientate doctors in the choice of the pharmacological treatment, especially in early stage tumors. The analysis of CX_3CL1 and CX_3CR1 expression in primary tumors could be used, together with the $CD3^+$ infiltrating cell screening, as a useful tool to increase prognostic accuracy in colorectal cancer patients.

Of note, our results suggest opposite biological significance for the involvement of chemokines in tumors, as compared to the vast background of available data. In fact, chemokines and their receptors are considered key players of the cancer-related inflammation and their presence at the tumor site is usually associated with tumor progression and neo-angiogenesis [59,68,185]. In marked contrast, our data demonstrate that expression of the intact chemokine-receptor loop by

neoplastic CRC cells is associated with lower degree of tumor recurrence and metastasis. Thus, the results so far appear to indicate that this peculiar trans-membrane chemokine and its receptor may have biological activities in CRC distinct from that of other chemokines.

If confirmed with a mechanistic explanation, these findings may shed light on a new paradigm in the field of chemokines and cancer biology.

MATERIALS AND METHODS

Patients

All patients included in the study underwent resective surgery for colorectal cancer at the IRCCS Istituto Clinico Humanitas, Rozzano, Milan, Italy, from 1997 to 2003.

For each patient, demographics and complete clinical data at diagnosis were made available through the institutional intranet. A clinical database was prepared by investigators who were blinded to the results of molecular and immunological phenotyping of the cancers. Tissue specimens were reviewed by a single pathologist (MR), who was also unaware of the molecular phenotyping. The absence of metastasis at diagnosis was assessed definitively in all patients by combining histopathological findings, surgical records (including intra-operative liver ultrasonography), and perioperative imaging (abdominal CT and chest radiography in all patients). The observation period started immediately after surgery. To monitor postsurgical tumour recurrences, thoraco-abdominal CT (mean 1·5 [SD 0·7] per patient per year), abdominal ultrasonography (1·7 [0·5]), and chest radiography (1·5 [0·5]) were done according to common protocols for surveillance.

Microsatellite status was determined preliminarily for all cancers included in the study by testing instability at mononucleotide repeats.

The study was approved by the ethical committee of the Istituto Clinico Humanitas, and written informed consent was obtained by the referring physician at the time of surgery.

Molecular Biology

TaqMan Low Density Array (LDA)

Eight colon cancer samples and their corresponding normal tissues were used for LDA analysis. The LDA contains 8 sample-loading lines, each connected by micro channel to 48 miniature reaction chambers for a total of 384 wells per card. Gene specific exon-spanning primers and TaqMan probes were factory-design and embedded in each well. We chose 96 genes from Applied Biosystems Assays-on-

Demand™ Gene Expression Products: 5 housekeeping genes (HPRT, 18S, GAPDH, B2M, ACTB) and 91 inflammation-related genes. The LDA in this study was configured into 4 identical 96-gene sets (2 samples in duplicate).

A total of 100 μL reaction mixture with 100ng of cDNA template and 50 μL 2 X TaqMan Universal PCR Master Mix (Applied Biosystems) was added to each line of LDA after vortex and brief centrifugation. Each reaction cell contained 1 μL reaction mixture with 1ng of mRNA. LDA were sealed with a TaqMan low density array sealer (Applied Biosystems) before centrifugation.

The PCR amplification were done in the micro-fluidic card sample block of an ABI Prism® 7900HT Fast Real-Time PCR System (Applied Biosystems). The amplification protocol was used as below: 2 min at 50 °C to activate uracil-DNA glycosylase, 10 min at 94.5 °C, 40 cycles of denaturation at 97 °C for 30 s, and annealing and extension at 59.7 °C for 1 min.

The relative amount of each target gene mRNA to the mean of the five housekeeping genes (HPRT, 18S, GAPDH, B2M and ACTB) was calculated as $2^{-\Delta Ct}$, where $\Delta Ct = Ct - Ct_{\text{mean of housekeeping genes.}}$ The fold-change of each target gene mRNA to the corresponding normal tissue was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{target gene in tumor tissue}} - \Delta Ct_{\text{target gene in normal tissue}}$. The threshold cycle Ct was automatically given by the SDS2.2 software package (Applied Biosystems).

RT-PCR and Quantitative Real Time PCR

Total RNA was isolated both from cells or tissue specimens using TRI reagent (Ambion). Total RNA was quantified by Nanodrop Spectrophotometer ND-1000 and its quality was examined by 1.5% agarose gel electrophoresis. DNAse treatment (Turbo DNA-free™ kit, Ambion) was performed to avoid genomic DNA contamination. One microgram of total RNA was reverse-transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's instructions. Tumour samples as well as cell lines cDNA were analysed by SYBER Green-based Quantitative Real-Time PCR on ABI Prism® 7900HT Fast Real Time PCR System (Applied Biosystem). 18S was used as internal control to normalise

sample.

All gene specific primers were domestically designed.

The sequence is indicated below:

18S:

Forward: 5'-CGC CGC TAG AGG TGA AAT TC-3'

Reverse: 5'-CTT TCG CTC TGG TCC GTC TT-3'

CX_3CL1:

Forward: 5'-TCT GCC ATC TGA CTG TCC TG-3'

Reverse: 5'-TGA TGT TGC ATT TCG TCA CA-3'

CX_3CR1:

Forward: 5'-GGG ACT GTG TTC CTG TCC AT-3'

Reverse: 5'-GAC ACT CTT GGG CTT CTT GC-3'

CCL3:

Forward: 5' TGC AAC CAG TTC TCT GCA TC 3'

Reverse: 5' AAT CTG CCG GGA GGT GTA 3'

CCL4:

Forward: 5' TTA CTA TGA GAC CAG CAG CCT CT 3'

Reverse: 5' CAG CAC AGA CTT GCT TGC TT 3'

OPN:

Forward: 5' CGC AGA CCT GAC ATC CAG T 3'

Reverse: 5' GGC TGT CCC AAT CAG AAG G 3'

SPARC:

Forward: 5' GTG CAG AGG AAA CCG AAG AG 3'

Reverse: 5' TGT TTG CAG TGG TGG TTC TG 3'

CXCL8:

Forward: 5’ CTG CGC CAA CAC AGA AAT TA 3’

Reverse: 5’ TTG AAG AGG GCT GAG AAT TCA 3’

The threshold cycle Ct was automatically given by SDS2.2 software package (Applied Biosystem).

PCR

PCR were performed using Phusion High-Fidelity DNA polymerase (Thermo-Scientific) in supplied buffer with 200 μM dNTPs, 0.5 μM of each primer, 10-50 ng of the DNA template and 0.02 U/μl of DNA polymerase. Generally, a protocol with denaturing at 95°C, annealing at 60-65 °C, depending on primer pairs used, and elongation at 72°C for 30-35 cycles was performed.

Plasmid

CX_3CL1 and CX_3CR1 were cloned by RT-PCR amplification of cDNA isolated from DLD-1 cells and human monocytes, respectively. The full-length cDNAs of CX_3CL1 and CX_3CR1 were cloned into pmCherry-N1 and pEGFP-N1 (Clontech) respectively. CX_3CL1 and CX_3CR1 TGA stop codon were deleted to generate a Cherry- or GFP-fusion proteins. For CX_3CL1, XhoI and BamHI restriction enzymes were used. For CX_3CR1, HindIII and BamHI restriction enzyme were used.

The following primers were used:

CX_3CL1:

Forward: 5’ CCGCTCGAACCATGGCTCCGATATC 3’

Reverse: 5’ CGGGATCCCGCACGGGCACCAGGAC 3’

CX_3CR1:

Forward: 5’ CCCAAGCTTGGGATGGATCAGTTCCCTGAATC 3’

Reverse: 5’ CGGGATCCCGGAGAAGGAGCAATGCATCTC 3’

To generate Lentiviral vector, CX_3CR1-GFP, CX_3CL1-cherry and pmCherry inserts were cut from pEGFP-N1 or pmCherry-N1, and cloned into pRRLsinPPTGFPpre lentiviral vector, using BglII and BsrGI restriction enzymes

Transformation of competent cells

Fresh competent cells (Promega) were thawed on ice prior to the addition of 2 μl of DNA ligation mix in 50 μl of cells. A mock ligation (plasmid alone with ligase enzyme) was included in one transformation as negative control. Cells were incubated with DNA on ice for 30 minutes and then subjected to a heat shock for 45 seconds at 42 °C. The cells were then returned to ice for 2 minutes. Subsequently, the cells were incubated with 950 μl LB (Luria-Bertani medium: 10g/l casein hydrolysate peptone, 5 g/l yeast extract, pH 7.5) at 37°C for 1 hour before being plated onto LB plate (LB plus Bacto agar 15 g/l) in the presence of the required antibiotics (usually Ampicillin 100 μg/ml or Kanamycin 50 μg/ml). The plates were incubated at 37°C overnight.

Small and Large scale plasmid preparation

Cells picked from individual transformed colonies were used to inoculate 4 ml LB plus antibiotics and grown overnight at 37°C. Cells containing transfected DNA were expanded into 250 ml LB plus antibiotics overnight at 37°C. Plasimd DNA was isolated from these cells using PureYield™ Plasmid Miniprep (or Midiprep) System (Promega) according to the manufacturer’s instructions.

Agarose gel electrophoresis

DNA samples were loaded on 0.5-2% agarose gels along with DNA markers. Gels were made in TAE (4.8g/l Tris base, 1.14% acetic acid, 0.74 g/l Na_2EDTA $2H_2O$) buffer containing o.3 μg/ml ethidium bromide and run at 80-100 V until desired separation was achieved. DNA bands were visualized under a UV lamp.

Enzymatic modification of the DNA

Restriction analysis: to examine DNA and prepare DNA fragments for sub-cloning. Plasmid DNA was digested for 1-2 hours at 37°C with 10 units of the required restriction enzyme (New England Biolabs). The volume of each digestion was adjusted to a final volume of 50 μl with water, bovine albumin serum (BSA) when needed and the appropriate buffer.

De-phosphorylation: the 5' ends of open cloning vectors were de-phosphorylated using Antartic Phosphatase (New England Biolabs), to prevent self-ligation, according to manufacturer's instruction.

Gel extraction: when required, the DNA bands (vectors or inserts) were gel purified from 0.5-2% agarose gels using the Wizard® SV Gel and PCR Clean-UP System (Promega) according to manufacturer's instructions.

Ligation: the vector DNA (100 ng) and the insert were mixed together with water, ligation buffer and the T4-DNA ligase according to manufacturer's instructions (Promega).

Mammalian Cell Culture

Cell culture

All cell lines were grown in pyrogen-free condition in a humified atmosphere of 5% CO_2 and 95% air at 37°C. The cells were grown in tissue culture plastic plates (Falcon), passaged when confluent by trypsinization (Trypsin/EDTA, 0.05/0.05 W/V) and seed at the appropriate density.

Colorectal cancer cell lines DLD-1, HCT116, HT29, SW620, SW480, LoVo, RKO, and NCI-H360 were cultured in RPMI 1640 medium, while CaCo2 cells were cultures in DMEM medium. In both cases, medium was supplemented with 10% fetal calf serum (Lonza, BioWhittaker) and 2mM Ultraglutamine1 (Lonza, BioWhittaker).

Multicellular tumor spheroids (MTS) were obtained plating cells on 2% agarose in PBS (without calcium and magnesium)-coated plate, in order to culture cells in not adherent conditions.

293T cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Lonza, BioWhittaker), 2mM Ultraglutamine1 (Lonza, BioWhittaker), 100U/ml penicillin/streptavidin (Lonza, BioWhittaker), and used for lentiviral infection.

Transfection procedures

Calcium Phosphate procedure: 10 μg DNA was diluted in 439 μl of H_2O, and mixed to 61 μl of 2M $CaCl_2$. The mixture was added, drop-wise, to 500 μl of 2X HBS (HEPES-buffered Saline: 50 mM HEPES pH 7.1, 280 mM NaCl, 1.5 mM Na_2HPO_4, final pH 7.1). After 15 minutes incubation, the precipitate was added to cells plated on 10 cm dishes and removed after 8-16h, depending on cell lines.

Lipofectamine transfection: Lipofectamine transfection was performed according to manufacturer's instructions (Invitrogen). Briefly, cells were plated at 95% confluent 24h before the transfection. The right amount of DNA, diluited in OptiMem medium, was added to Lipofectamine diluited in OptiMem medium and

incubated for 20 minutes at room temperature (RT). The mixture was added to cells and removed after 6-16h, depending on cell lines.

Lentiviral infection

Lentiviruses were produced by transfecting 293T cell line (plated at a density of 750.000 cells per 10 cm diameter dish three days before) with the packaging plasmid (pCMV-ΔR8.74), the envelope plasmid (containing *VSV-G* gene) and with 10 μg of DNA of interest. Supernatants were collected 48 hours after transfection, filtered (0.45 μm), and used to infect NCI-H360 and RKO cells. After the addition of 5 μl of polybrene, 1000X stock solution, to 5 ml of every supernatant, it was added to each plate of cells. Cells were centrifuged for 1 hour at 900 rpm (acceleration 3, breaking 0) and then incubated in a humified atmosphere of 5% CO_2 and 95% air at 37°C. Supernatant were removed approximately after 24 hours and a second cycle of infection was repeated. After 48 hours, cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Lonza, BioWhittaker) and 2mM Ultraglutamine1 (Lonza, BioWhittaker). Infected cells were selected based on GFP or Cherry fluorescence.

MTT assay

In this assay, viable cells convert the soluble dye MTT to insoluble blue formazan crystals. The intensity of the dye is directly proportional to the number of viable cells representing cellular growth rate. NCI-H360 and RKO cells, infected with the different constructs, were plated into 96 well plates. 20 μl of MTT (5 mg/ml in PBS 1X) were added to each well (200 μl medium) and left at 37°C for 2-4 hours, in the dark. After aspiration of the medium, 100 μl of DMSO was added to each well and crystals were dissolved pipetting up and down. The absorbance of this solution was measured at 570 nm.

Adhesion assay

96-flat bottom well plates (Costar) were coated with different concentration of human recombinant Fractalkine (R&D Systems) diluted in PBS and incubated overnight at 4°. Cells were trypsinized and resuspended in adhesion buffer (RPMI 1640, 1% fetal calf serum, 2mM Ultraglutamine1, 0.5% BSA) at the concentration of 200000 cells/ml. Coated well were washed twice in PBS. 100 μl of cell suspension were plated in each well and incubated for 30 minutes in a humified atmosphere of 5% CO_2 and 95% air at 37°C. After two washes with PBS to remove not adherent cells, 50 μl of Crystal Violet solution (Crystal Violet 1%, 25% methanol in H_2O) was added in each well for 10 min. After extensive washes in PBS to remove Crystal violet solution, cells were disrupted with dimethyl sulfoxide (DMSO) and the absorbance of this solution was measured at 570 nm.

Immunochemistry

Immunohistochemistry for CD3 and CD68

Formalin-fixed, paraffin-embedded, and 2-μm thin sections of tumour were deparaffinised and exposed or not to an antigen-retrieval system (1 mmol/L ethylenediamine tetraacetic acid, EDTA, pH 8, 98°C for 30 min) before being incubated with the specific CD68 and CD3 antibody respectively (Dako, mouse anti-human CD68; Dako, mouse anti-human CD3). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min at room temperature. Primary mouse monoclonal antibody was applied for 1 h at room temperature. Reactive sites were identified by exposure to a secondary antibody (HRP rabbit/mouse; ChemMate Dako Envision, Carpinteria, CA, USA) for 30 min at room temperature. Immunoperoxidase staining was then obtained by using diaminobenzidine as a chromogen (DAB+chromogenX-50, ChemMate, DakoCytomation, Carpinteria, CA, USA). The slides were finally counterstained with haematoxylin (Harris Hematoxylin, DiaPath, Microstain Division, Martinengo, Bergamo, Italy).

$CD3^+$ and $CD68^+$ cells density at tumor invasive margin was measured in three randomly selected and non-contiguous microscopic areas encompassing the deep front of tumour invasion. For each selected area, the cancer tissue had to represent approximately 50% of the entire microscopic field. The pathologist who selected the areas of interest was blinded to any patient clinical data. For each selected region, a digital image was captured. To measure the extent of CD3 and CD68 immunoreactivity, we used a computer-aided image analysis system with software able to discriminate the immunostained area on the basis of red, green, and blue colour segmentation, and to calculate the per cent immunoreactive area as a fraction of the total area digitally captured. Given that an accurate optical count of $CD3^+$ and $CD68^+$ cells was not feasible in the presence of very dense infiltrates or agglomerates, we did not attempt to convert area values to number of cells. For each tumour, the mean of values obtained in three distinct regions was calculated and used for the subsequent analysis of data.

Immunohistochemistry for CX_3CL1 and CX_3CR1

Formalin-fixed, paraffin-embedded tissues were deparaffinised and endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min at room temperature. For CX_3CR1 staining, sections were exposed to an antigen retrieval procedure in sodium citrate buffer (pH 6.0), 98°C for 30 min, before being incubated with specific antibody (Abcam, rabbit anti-human CX_3CR1, 1h RT), followed by a goat anti-rabbit secondary antibody (HRP rabbit/mouse; ChemMate Dako Envision, Carpinteria, CA, USA) for 30 min at room temperature. For CX_3CL1 staining, sections were directly incubated with specific primary antibody (R&D Systems, goat anti-human CX_3CL1, 1 h at room temperature), followed by a biotin-conjugated donkey anti-goat secondary antibody (SantaCruz Biotechnology, Inc.) for 30 min at room temperature. Sections were subsequently incubated with avidin (ABC, vectastain) for 30 min at room temperature. After diaminobenzidine reaction (DAB+chromogenX-50, ChemMate, DakoCytomation, Carpinteria, CA, USA), section was counter-stained with haematoxylin (Harris Hematoxylin, DiaPath, Microstain Division, Martinengo, Bergamo, Italy).

Two different operators evaluated CX_3CL1 and CX_3CR1 score independently. The operators who analysed the expression of CX_3CL1 and CX_3CR1 were blinded to any patient clinical data. To evaluate CX_3CL1 and CX_3CR1 expression, both the intensity of the staining at the percentage of tumor positive cells were taken into consideration. Regarding the intensity of the staining, tumor cells were classified as negative (0), moderate (1) and strong (2). Subsequently, the percentage of cells relative to any staining classification was assessed. For each specimen, the final score was calculated using the formula:

$$\frac{(0 \times \%_{\text{of negative tumor cells}}) + (1 \times \%_{\text{of moderate tumor cells}}) + (2 \times \%_{\text{of strong tumor cells}})}{100}$$

Confocal microscopy analysis

All the images were acquired with a laser scanning confocal microscope (FluoView FV1000; Olympus). For image analysis, Imaris X64 7.0.2 Software (Bitplane, AG) was used.

Cell lines were cultured on glass slides coated with Poly-L-Lysine, washed in PBS and fixed in 4% PFA for 15 min at RT. DAPI (Invitrogen, Molecular Probes) was used to stain nuclei. Slides were mounted with Fluor Preserve™ Reagent (Calbiochem) and analysed with confocal microscope.

Multicellular tumor spheroids (MTS) were fixed in 4% PFA overnight at 4°C. After intensive washes in PBS, MTS were incubated with DAPI to stain nuclei, and then mounted with Fluor Preserve™ Reagent (Calbiochem) and analysed with confocal microscope.

Frozen section of human colorectal cancer samples were fixed in 4% PFA for 15 min at RT. After 2 washes in 2% bovine albumin serum (BSA) in PBS, tissues were incubated with the specific primary antibody (R&D Systems, goat anti-human CX_3CL1; Abcam, rabbit anti-human CX_3CR1; Dako, mouse anti-human CD3; Dako, mouse anti-human CD68) diluted in 2% BSA, 0.3% TritonX-100, 0.1% glycine, 5% Normal Donkey Serum in PBS overnight at 4°C. After 3 washes in washing buffer (0.2% BSA, 0.05% Tween 20 in PBS), tissues were incubated with specific secondary antibody (Alexa donkey anti-goat 647, Alexa donkey anti-rabbit 488, Alexa donkey anti-mouse 647) for 1h at RT. After 4 washes in washing buffer, DAPI was used to stain nuclei. Tissues were mounted with Fluor Preserve™ Reagent (Calbiochem) and analysed with confocal microscope.

Fluorescence Resonance Energy Transfer (FRET)

FRET analysis was performed by fluorescence lifetime imaging microscopy (FLIM), using the BX51WI microscope (Olympus). FLIM measurements were obtained illuminating the sample with a Chameleon Ultra laser (Coherent, CA) with 80 MHz repetition rate tuned at 820 nm for two-photon excitation. Fluorescence emission was detected with FLIM X16-78 Mhz TCSCP Detector (LaVision BioTec).

Measurements were performed on the confocal system previously described with a 20X water immersion objective (XLUMPlanFLn, N.A.1.00, Olympus). Two photon excitation spectra were set as previously described [274]. FRET efficiency E was obtained using the formula $E=1-tau_{DA}/tau_{D}$, were tau_{DA} is the donor fluorescence lifetime in presence of the acceptor and tau_{D} is the donor fluorescence lifetime alone. The distance between the two molecules was calculated as $R_0 (1/E - 1)^{1/6}$, were R_0 is the Föster radius (5.0nm) relative to GFP-cherry couple and E is the FRET efficiency. Acquisition and instrument control was performed using Imspector Pro software (LaVision, BioTec), while data analysis was performed with GraphPad Prism5 software.

Animal experiments

Eight-week-old nude mice (Charles River) were used for the mouse model. Procedures involving animals and their care conformed to institutional guidelines in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 86/609, OJ L 358,1,12-12-1987; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All efforts were made to minimize the number of animals used and their suffering. Mice (six per group) received s.c. injection into the left and the right flank with 2 x 10^6 viable cells. Tumors were measured every three days by caliper, starting two weeks after tumor cells injection. After one month, mice were sacrificed, s.c. tumors removed and use for subsequent experiments.

Statistical analysis

The significance of the differential gene expression in normal and tumor tissues, as well as the differential expression of CX_3CL1, CX_3CR1, $CD3^+$ and $CD68^+$ cells in patients' specimens was determined using the non-parametric Mann-Whitney *U*-test. Correlation between CXCL8 and OPN or SPARC mRNA levels, as well as correlation between CX_3CL1 expression by tumor cells and the percentage of $CD3^+$ or $CD68^+$ tumor cells at tumor invasive margins was tested by use linear regression analysis. Association between CX_3CL1, CX_3CR1, $CD3^+$ cells and $CD68^+$ cells with patients' clinical and pathological features were tested using univariate and multivariate analysis. Kaplan-Meier curves of disease-specific survival (DSS) and disease-free survival (DFS) were plotted. To compare infected tumor cell adhesiveness and infected tumor cell growth, both *in vivo* and *in vitro*, students *T*-test was used. For each test, only two-sided p values lower than 0.05 were considered statistically significant. Analyses were done using Epi Info version 3.4.3 and GraphPad Prism5 software.

List of Abbreviations

CAC: Colitis-associated Cancer

CD: Chron's Disease

CIN: Chromosomal Instability

CRC: Colorectal Cancer

DFS: Disease-Free Survival

DSS: Disease-Specific Survival

ECM: Extra-Cellular Matrix

ELR: glutamic acid/leucine/arginine

EMT: Epithelial-to-Mesenchymal Transition

FAP: Familial Adenomatous Polyposis

FLIM: Fluorescence Lifetime Imaging Microscopy

FRET: Fluorescence Resonance Energy Transfer

GFP: Green Fluorescent Protein

HNPPC: Hereditary Non-polyposis Colorectal Cancer

IBD: Intestinal Bowel Disease

LDA: Low Density Array

MDSCs: Myeloid-derived Suppressor Cells

MMPs: Matrix Metalloproteases

MMR: Mismatch Repair

MSI: Microsatellite Instability

MSS: Microsatellite Stability

MTS: Multicellular Tumor Spheroid

OR: Odd Ratio

SSA: Sessile Serrated Adenomas

TAMs: Tumor-associated Macrophages

TILs: Tumor-infiltrating Lymphocytes

TNM: Tumor Node Metastasis

TSA: Traditional Serrated Adenomas

UC: Ulcerative Colitis

VELIPI: Vascular Emboli, Lymphatic Invasion, Perineural Invasion

PUBLICATIONS

This work has contributed to the following publications

Expression of chemokines and chemokine receptors in human colon cancer

Erreni, M., Bianchi, P., Laghi, L., Mirolo, M., Fabbri, M., Locati, M., Mantovani, A. & Allavena, P. (2009). *Methods Enzymol*, **460**, 105-121

Human glioblastoma tumors and neural cancer stem cells express the chemokine CX_3CL1 and its receptor CX_3CR1

Erreni, M., Solinas, G., Brescia, P., Osti, D., Zunino, F., Colombo, P., Destro, A., Roncalli, M., Mantovani, A., Draghi, R., Levi, D., Rodriguez, Y.B.R., Gaetani, P., Pelicci, G. & Allavena, P. (2010). *Eur J Cancer*, **46**, 3383-3392.

Role of CX_3CR1/CX_3CL1 axis in primary and secondary involvement of the nervous system by cancer

Marchesi, F., Locatelli, M., Solinas, G., **Erreni, M**., Allavena, P. & Mantovani, A. (2010). *J Neuroimmunol*, **224**, 39-44.

Tumor-associated Macrophages (TAM) and Inflammation in Colorectal Cancer

Erreni, M., Mantovani, A. & Allavena, P. (2011). *Cancer Microenviron*, **4**, 141-154.

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