Crosstalk between colorectal cancer cells and tumour-associated macrophages

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Abstract

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of death from cancer worldwide. People suffering from inflammatory bowel disease, such as Crohns' disease and ulcerative colitis, are at an increased risk of developing CRC. The development of CRC is highly influenced by the tumour microenvironment. Tumour cells are able to recruit macrophages and change their behaviour. These tumour-associated macrophages (TAMs) were of the M2 phenotype ($CD206^+$). In CRC patient tissues and xenografts from mouse model, presence of increased amount of M2 macrophages was observed. M2 macrophages derived from human monocyte cell line THP-1 secreted high levels of IL-8 and leukotriene D_4 (LTD_4), which induced colon cancer cell migration, and Th2 cytokines (IL-4, IL-10) to maintain a tumoral immunosuppressive environment. Remodelling of extracellular matrix (ECM) occurs in CRC. CD47 binding to α2β1 integrin could regulate cyclooxygenase-2 expression and cell migration on collagen I surface in intestinal epithelial cells. CD47 is involved in crosstalk between colon cancer cells and macrophages by interacting with its ligand signal-regulatory protein α on macrophages. This interaction enhanced cell migration of CRC cells and TAMs. IL-8 and LTD_4 could induce CD47 expression in CRC cells. Matrix metalloproteinases (MMPs) are enzymes degrading ECM. The enhanced levels of MMP-7 and -9 mRNA expressions and increased cell invasion into matrigel were observed in colon cancer cells upon stimulation with TAM-conditioned medium. These findings provide more evidence that TAMs play an important role in CRC progression. CD47, as a key regulator of crosstalk between colon cancer cells and TAMs, could thus be a therapeutic target for CRC.

Key words: CD47, tumour-associated macrophage, matrix metalloproteinase, cell migration, colon cancer

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List of papers

This thesis is based on the following papers, referred to in the text as paper I-III:

I. CD47 regulates collagen I-induced cyclooxygenase-2 expression and intestinal epithelial cell migration. Oliver Jay Broom, **Yuan Zhang**, Per-Arne Oldenborg, Ramin Massoumi and Anita Sjölander. PLoS One 2009;4(7):e6371
II. Crosstalk between colon cancer cells and macrophages via inflammatory mediators and CD47 promotes tumour cell migration. **Yuan Zhang**, Wondossen Sime, Maria Juhas and Anita Sjölander. Eur J Cancer (2013), http://dx.doi.org/10.1016/j.ejca.2013.06.005
III. Tumour-associated macrophages induce colon cancer cell migration via matrix metalloproteinases. **Yuan Zhang**, Katyayni Vinnakota, Sayeh Savari, Gunilla Jönsson, Maria Juhas and Anita Sjölander. Manuscript

Abbreviations

15-PGDH	15-Hydroxyprostaglandin dehydrogenase
5-FU	5-fluorouracil
AA	arachidonic acid
AJCC	American Joint Committee on Cancer
APC	adenomatous polyposis coli
Arp2/3	actin-related protein 2/3
ATG16L1	autophagy related 16-Like 1
BSA	bovine serum albumin
CARD15	caspase recruitment domain-containing protein 15
CD	Crohn's disease
CK1	casein kinase 1
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
CRC	colorectal cancer
CysLT	cysteinyl leukotriene
CysLTR	cysteinyl leukotriene receptor
DC	dendritic cell
DVL	dishevelled
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
F-actin	filamentous actin
FAK	focal adhesion kinase

FLAP	5-LOX-activating protein
Fzd	frizzled
GPI	glycosylphosphatidylinositol
GSK3β	glycogen synthase kinase-3 beta
IAP	integrin-associated protein
IBD	inflammatory bowel disease
IFN-γ	interferon-γ
IRGM	immunity related GTPase family M
ITIM	immunoreceptor tyrosinebased inhibitory motif
LOX	lipoxygenase
LPS	lipopolysaccharide
LRP5/6	lipoprotein receptor-related protein 5/6
LT	leukotriene
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MET	mesenchymal-epithelial transition
MMP	matrix metalloproteinases
MT-MMP	membrane-type matrix metalloproteinase
NFκB	nuclear factor κ B
NK cell	natural killer cell
NO	nitric oxide
NOD2	nucleotide-binding oligomerization domain-containing protein 2
NSAID	nonsteroidal anti-inflammatory drug
PBS	phosphate-buffered saline
PG	prostaglandin
PI3K	phosphoinositide 3-kinase
PIP3	phosphatidylinositol triphosphate
PLA_2	phospholipase A_2

PMA	phorbol-12-myristate-13-acetate
PTX	pertussis toxin
PVDF	polyvinylidene difluoride
Rap1	Ras-related protein 1
RECK	reversion-inducing cysteine-rich protein with kazal motifs
ROI	reactive oxygen intermediate
Ror2	receptor tyrosine kinase-like orphan receptor 2
sFRP	secreted frizzled-related protein
SHP	Src-homology 2-domain-containing tyrosine phosphatase
SIRPα	signal-regulatory protein alpha
TAM	tumour-associated macrophage
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGF-β	transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
TIMP	tissue inhibitors of metalloproteinase
TLR	Toll-like receptor
TNM	tumour, node, metastasis
TSP	thrombospondin
TX	thromboxane
UC	ulcerative colitis
UICC	International Union Against Cancer
VEGF	vascular endothelial growth factor

Introduction

The tumour microenvironment is the cellular milieu in which the tumour exists, and includes the surrounding blood vessels, various cell types (such as immune cells, fibroblasts), signalling molecules, and the extracellular matrix (ECM) [1]. It is considered as an important hallmark of cancer, which contributes to the cancer development and progression [2].

Colorectal cancer (CRC) is the fourth leading cause of cancer-related death worldwide (WHO, 2008). People suffering from inflammatory bowel diseases (IBDs), such as Crohns' disease and ulcerative colitis, are at an increased risk of developing CRC [3]. The development of CRC is highly influenced by the tumour microenvironment [4].

Macrophages play a central role in host defence mechanisms [5]. However, in tumour microenvironment they represent the major inflammatory component of the stroma of many tumours (breast, prostate, glioma, lymphoma, bladder, lung, cervical and melanoma) and are able to affect different aspects of the neoplastic tissue [6]. It is well established that such tumour-associated macrophages (TAMs) play an important role in cancer progression [7]. TAMs are shown to have an M2 phenotype [8]. They secrete high levels of Th2 cytokines, growth factors and inflammatory mediators, and promote tumour growth, angiogenesis, and metastasis [6].

Remodelling of ECM occurs in development, wound healing, normal organ homeostasis, inflammation and cancer [9]. In response to aberrant expressed ECM components, various signals get activated in cells. These signals are mostly regulated via integrins. CD47, also known as integrin-associated protein (IAP) is a transmembrane protein, which binds to several types of intergrins [10]. It is involved in cell adhesion to ECM and cell migration [10]. On the other hand, CD47 is involved in crosstalk between phagocytes and other cells [11]. CD47 is capable of interacting with its ligand signal-regulatory protein α (SIRPα, CD172) on macrophages to negatively regulate phagocytosis [12]. CD47-SIRPα signalling has also been shown to play a role in cell migration [13]. Degradation of ECM is required for tissue remodelling. Thus, the most important ECM proteolytic enzymes, matrix metalloproteinases (MMPs), play a role in cancer progression [9].

In this context, the aim of this work was to investigate how TAMs affect colon cancer cell migration and metastasis. The work has focused on understanding the role of TAM-derived soluble factors, CD47 signalling and impact of enhanced MMPs expression and activation on colon cancer cell migration and invasion.

Background

Physiology of the intestine

Similar to other parts of the gastrointestinal tract, the intestines have the layout of an inner mucosal layer with epithelial cells, a submucosal layer, a layer of muscle and a serosal layer. Intestines can be divided into two parts: the small intestine and the large intestine. The small intestine is divided into three sections: the duodenum, the jejunum, and the ilium. It is the organ where most of the absorption of digested food takes place. The small intestine has specialized structures to increase the absorptive surface area. The mucosa of the small intestine is folded and has finger like projections called villi, which are covered by even smaller protrusions called microvilli. The large intestine is divided into the cecum, appendix, colon, rectum, and anal canal. Unlike the small intestine, the large intestine only absorbs small amounts of water, sodium and some fat-soluble vitamins. There are several differences in the structures of the small and large intestines. The large intestine lacks of villi, has more goblet cells, thicker layer of serosa and three thick bands of muscle instead of a continuous longitudinal muscle layer. The intestinal epithelium has great renewal capacity; almost all epithelial cells in the intestinal lining are replaced by the stem cells residing at the bottom of crypts on a weekly basis [14].

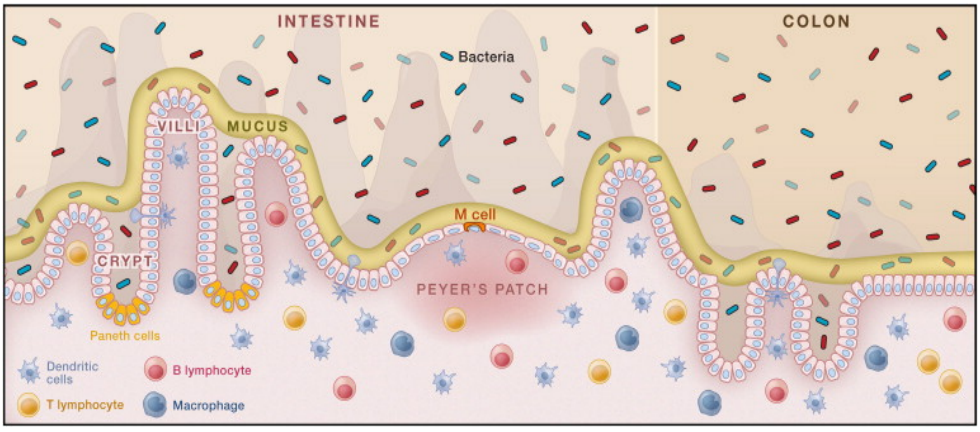

Figure 1. The gut landscape: maintaining intestinal homeostasis. Adapted from Garrett et al., 2010

The intestines of humans represent a densely populated microbial ecosystems, which are dominated by the presence of a large number of and various types of gut-friendly bacteria (Figure 1) [15]. Therefore, the intestines are challenged by low levels of inflammation. The digestive tract's immune system is often referred to as gut-associated lymphoid tissue (GALT), which is the largest lymphoid organ in the body. Dysregulation or dysfunction of GALT is thought to predispose one to inflammatory bowel disease (IBD) [16].

Inflammatory bowel disease

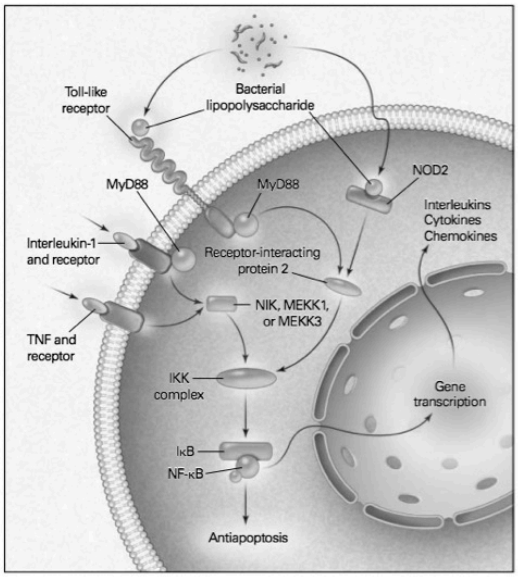

Figure 2. Common cellular pathways in IBD. Adaped from Podolsky, 2002

IBD comprises the chronic relapsing inflammatory disorders of the intestine [17]. The major forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD) [18]. CD may affect all parts of the gastrointestinal tract, but most commonly involves the distal part of the small intestine or ileum, and colon. UC results in colonic inflammation that can affect the rectum only, or can progress to involve part of or the entire colon [19]. The clinical symptoms of IBDs include diarrhea, abdominal pain, gastrointestinal bleeding, and weight loss [19].

Though the aetiology of IBD remains unclear, it is considered to arise as a result of the interaction of environmental and genetic factors. Family history is one of the risk factors for developing IBD, with a peak incidence in early adult life [17]. Multiple gene products contribute to the risk of developing IBD, and it seems the genetic contribution is more important in CD than in UC [20]. By DNA screening from IBD patients with their relatives and using genetic animal models, several genes and signalling pathways have been shown to be involved in CD and UC pathogenesis. Most of these genetic loci are linked to both CD and UC, but some are specific only to CD or UC [19, 20]. In 2001, the mutations in NOD2 gene, which encodes the nucleotide-binding oligomerization domain-containing protein 2 (NOD2), were shown to increase the risk for CD [21]. NOD2, expressed in the epithelium and in a

variety of immune cells, has important functions in innate immunity, particularly in regulating responses to intracellular pathogens and other exogenous injury-inducing stimuli [19]. Other genes linked to CD include autophagy related 16-Like 1 (ATG16L1) gene [21] and immunity related GTPase family M (IRGM) gene [22]. Genetic loci that specifically associated with UC include IL-10 and ECM1 genes [23].

Many cytokines are involved in IBD, and it has been shown that T helper 1 (Th1) cytokines are expressed in CD, whereas UC is a T helper 2 (Th2) cytokine-mediated disease [19]. The important common signalling pathways involved in IBD are shown in Figure 2 [20].

A serious long-term complication of chronic inflammation is the development of colorectal cancer (CRC). Patients with IBD have an increased risk of developing CRC. However, more is known about the risk in UC than in CD [24]. CRC is observed in 5.5-13.5% of patients with UC and 0.4-0.8% of patients with CD [25].

Colon cancer

Cancer is a broad group of diseases involving uncontrolled cell growth and spread. As a leading cause of mortality worldwide, cancer alone caused 7.6 million deaths (around 13% of all disease-related deaths) in 2008. CRC is the third most common cancer in the world and the fourth leading cause of death from cancer worldwide (WHO, 2008). The incidence of CRC has marked geographical variation, 2/3 of all CRCs are found to occur in developed countries [26].

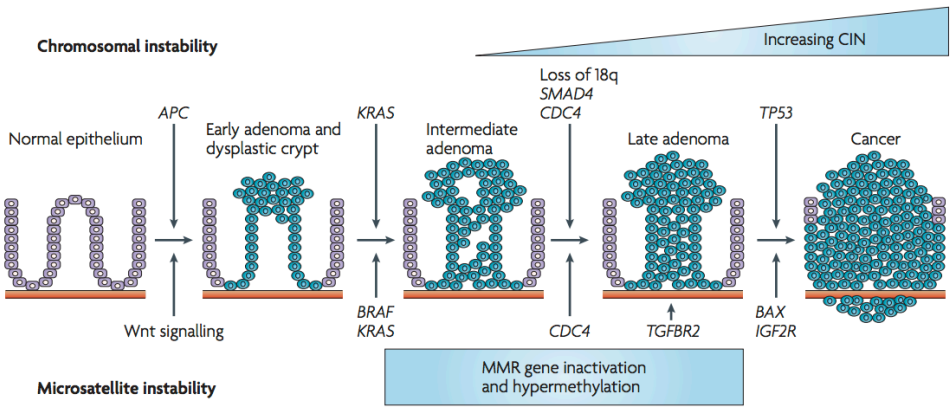

Figure 3. Adenoma–carcinoma sequence model for chromosomal instability in colorectal cancer. Adapted from Walther et al., 2009

The risk of developing CRC is increased during ageing, and it has been shown that the mean age at diagnosis of CRC is lower in males than in females [27, 28]. It is known that CRC develops in individuals with acquired or inherited genetic predisposition in response to exposure to environmental risk factors [26]. Lifestyle factors, including dietary habits, any complaint of obesity, personal habit of alcohol consumption and smoking, contribute to increased risk of CRC [29]. CRC arises as a consequence of genomic instability with an accumulation of genetic errors resulting in dysregulation of molecular pathways controlling cell migration, differentiation, apoptosis and proliferation [26]. Chromosomal instability is the most common cause by which CRC develops and is present in 65–70% of these cancers [30]. Common gene mutations in CRC include mutation of the adenomatous polyposis coli (APC) gene [31], which is a tumour suppressor gene, and small GTPase K-ras mutations [32]. The initial step in CRC tumorigenesis is that of adenoma formation, associated with loss of APC (Figure 3) [33]. Additional mutations, including mutations in transforming growth factor-β (TGF-β) [34] and p53 [32], drive subsequent malignant transformation.

Originally, colon cancer consisted of the four stages A, B, C, D according to Dukes' staging system. When Dukes' classification was first devised for rectal cancer in 1932, it was based on the extent of disease with 3 stages, as evaluated by the degree of tumour infiltration into the bowel wall (Dukes' A), through the bowel wall (Dukes' B), and the presence or absence of lymph node involvement (Dukes' C) [35]. Later on, the fourth stage D was added to this staging system for tumour with distant metastases [36]. However, this system has largely been replaced by the tumour, node, metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC), which is a more detailed system and used as the standard now for colon cancer staging [37]. In TNM staging system, primary tumour (T), regional lymph nodes (N) and distant metastases (M) are followed by a number to indicate the progressive severity (Table 1) [35, 37].

Surgery is the general treatment for colon cancer patients, especially in early stage without metastasis. However, the overall recurrence rate within 5 years is approximately 30% after surgery [38]. Approximately 50–60% of the all CRC patients will develop metastases, and 20–25% of patients with colon already have metastases at the time of diagnosis [39]. To reduce the recurrence and for metastatic colon cancer treatment, patients often receive chemotherapy and/or radiation therapy. Commonly used chemotherapeutic agents include 5-fluorouracil (5-FU) and the oral drug capecitabine, often in combination with other drugs, such as irinotecan, oxaliplatin, the vascular endothelial growth factor (VEGF) inhibitor bevacizumab, the epidermal growth factor receptor (EGFR) inhibitors cetuximab and panitumumab [40]. New drugs under clinical trails include VEGF receptor 2 blocking antibody ramucirumab [41], Akt inhibitor MK-2206 [42] and mitogen-activated protein

kinase (MAPK) kinase inhibitor selumetinib [42], which target the important signalling pathways in colon cancer. According to a research containing 119,363 patients with colon adenocarcinoma in USA, the overall 5-year CRC–specific survival was 65.2% and poor prognosis correlates with cancer stages [43]. Therefore, it is important to understand the mechanisms of colon cancer progression, find new therapeutic targets and develop new treatments for colon cancer.

Table 1. Colorectal tumour classification

Dukes	TNM stage	
-	TX	Primary tumour cannot be assessed
-	T0	No evidence of primary tumour
-	Tis	Carcinoma in situ: intraepithelial or invasion of laminaamna propria
A	T1	Tumour invades submucosa
A	T2	Tumour invades muscularis propria
B	T3	Tumour invades through the muscularis propria into pericolic tissues
B	T4a	Tumour penetrates to the surface of the visceral peritoneum
B	T4b	Tumour directly invades or is adherent to other organs or structures
-	NX	Regional lymph nodes cannot be assessed
B	N0	No regional lymph node metastasis
C	N1a	Metastasis in one regional lymph node
C	N1b	Metastasis in 2--3 regional lymph nodes
C	N1c	Tumour deposit(s) in the subserosa, mesentery or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
C	N2a	Metastasis in 4-6 regional lymph nodes
C	N2b	Metastasis in seven or more regional lymph nodes
C	M0	No distant metastasis
D	M1a	Metastasis confined to one organ or site (e.g. liver, lung, ovary, nonregional lymph node)
D	M1b	Metastasis in more than one organ/site or the peritoneum

Wnt/β-catenin signalling

Signalling by the Wnt family of secreted glycolipoproteins via the transcription co-activator β-catenin regulates cellular processes involved in embryonic development, differentiation and adult tissue homeostasis. Dysregulations of Wnt/β-catenin signalling are linked to various human diseases including cancer [44, 45].

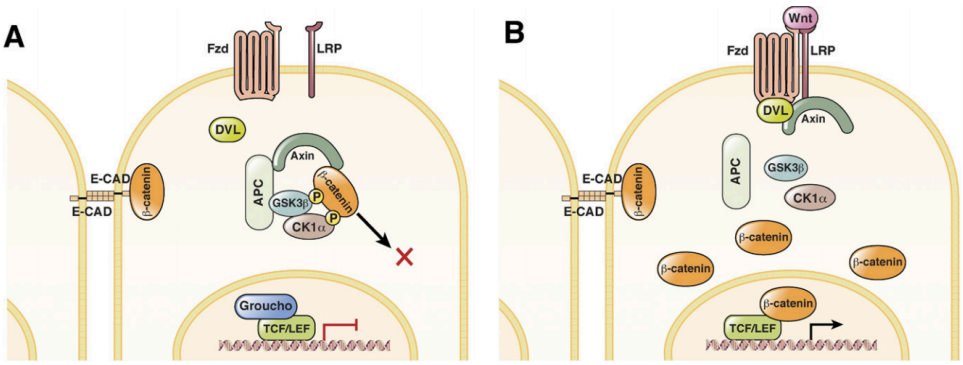

Figure 4. The Wnt/β-catenin pathway. Adapted from White et al., 2011

The Wnt proteins are a large family of secreted glycoprotein signalling molecules [46]. They act as ligands to activate the downstream Wnt/β-catenin signalling. At the core of the Wnt/β-catenin signalling pathway is the tightly regulated protein β-catenin, encoded by CTNNB1 gene. β-catenin is variably detected in 3 distinct pools: at cellular adherens junctions, where it directly interacts with E-cadherin; in the cytosolic space; and in the nucleus [45]. In the absence of Wnt ligand, cytosolic β-catenin is rapidly phosphorylated by the destruction complex, which is composed of the core proteins Axin, APC, glycogen synthase kinase-3β (GSK3β) and casein kinase 1 (CK1), and gets ubiquitinated and degraded thereafter. This process maintains a low baseline cytosolic level of β-catenin. When the Wnt isoforms bind to frizzled (Fzd) receptors and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), the cytosolic protein dishevelled (DVL) gets activated, thereby the destruction complex gets inhibited. This leads to the accumulation of β-catenin in the cytosol and translocation to the nucleus. In the nucleus, β-catenin interacts primarily with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors to activate target genes, leading to diverse cellular processes, including differentiation, proliferation, migration, and adhesion (Figure 4) [45, 47].

Binding of Wnt isoforms to either Fzd or receptors such as receptor tyrosine kinase-like orphan receptor 2 (Ror2, a receptor for Wnt-5a), can trigger β-catenin-independent downstream signalling events, including the inhibition of Wnt/β-catenin

signalling. The β-catenin-independent Wnt signalling also plays an important role in tumour progression.

Dysregulation of Wnt/β-catenin pathway occurs in various types of cancer. In colon cancer, 90% of all tumours have a mutation in a key regulatory factor of the Wnt/β-catenin pathway [31, 45], most often in APC [48] and β-catenin [49]. Thus, most of colon cancer have nuclear accumulation of β-catenin [50]. Overexpression of certain FZD receptors [51] or Wnt ligands [52] and reduced expression of secreted inhibitors of the pathway, such as secreted frizzled-related proteins (sFRPs) [53] have been reported in colon cancer. Overexpression of several components of the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway has been observed in colon cancer patient tissues [54]. The activation of this pathway can inhibit the phosphorylation of GSK3β [55]. All these changes can activate the Wnt/β-catenin signalling and benefit tumour progression.

Eicosanoids

Eicosanoid family is a large family of oxygenated C_{20} fatty acids. Eicosanoids are synthesized from naturally occurring C_{20} polyunsaturated fatty acids, within which the major precursor in most mammalian systems is the most abundant C_{20} polyunsaturated, arachidonic acid (AA) [56]. AA is liberated from the cellular membranes mostly by phospholipase A2 (PLA_2) in response to extracellular stimuli [57]. Type IV cytosolic PLA_2 ($cPLA_2$) is the key player for eicosanoid production because cells lacking $cPLA_2$ are generally devoid of eicosanoid synthesis [58].

The eicosanoid family is made up of three clans: the prostanoids including prostaglandins (PGs) and thromboxanes (TXs), which are synthesized via the cyclooxygenase (COX) pathway; the leukotrienes (LTs) and certain mono-, di- and tri-hydroxy acids, which are formed via lipoxygenase (LOX) pathways; and the epoxides which are formed by a cytochrome P-450 epoxygenase pathway (Figure 5) [56, 59].

COX exists as two distinct isoforms, COX-1 and COX-2. COX-1, expressed constitutively in most cells, is the dominant (but not exclusive) source of prostanoids for housekeeping functions, such as gastric epithelial cytoprotection and homeostasis. COX-2, induced by cytokines, shear stress, and tumour promoters, is the more important source of prostanoids formation in inflammation and cancer [60]. Although the changes of COX-1 level is unusual, it has also been reported to be upregulated in some carcinomas, such as ovarian cancer [61]. Increased expression of COX-2 has been identified in cancers, particularly in colon cancer [62, 63]. It has been shown that overexpression of COX-2 increased metastatic potential of human colon cancer cells, by upregulating the expression of matrix metalloproteinases

(MMPs) and invasion across matrigel [64]. The COX-2 specific inhibitor NS-398 is able to decrease colon cancer cell motility [65], induce apoptosis in various colon cancer cell lines [66], inhibits tumour growth and liver metastasis of colon cancer in mouse models [67]. Indeed, the nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, which are able to inhibit COX signalling, are potent preventive agents for colon cancer [68, 69]. Recently, it has been shown that daily aspirin can not only reduce the long-term incidence of adenocarcinomas but also prevent distant metastasis of colon cancer [70]. The COX pathway produce PGH_2, which in turn can be metabolized to five major prostanoids: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. These products have important roles in tissue homeostasis and tumour progression [71]. Among prostanoids, pro-inflammatory PGE_2 has a predominant role in promoting tumour growth [59]. PGE_2 is the most abundant PG that is found in various human malignancies, including colon cancer, and is associated with a poor prognosis [72]. By contrast, 15-Hydroxyprostaglandin dehydrogenase (15-PGDH), which is the key enzyme responsible for the biological inactivation of PGs, is highly expressed in normal tissues but is ubiquitously lacking in human colon cancer [73]. PGE_2 helps maintain the tumour immunosuppressive microenvironment by shifting the anti-tumour Th1 responses to Th2 responses by downregulation of Th1 cytokines and upregulation of Th2 cytokines in immune cells [74].

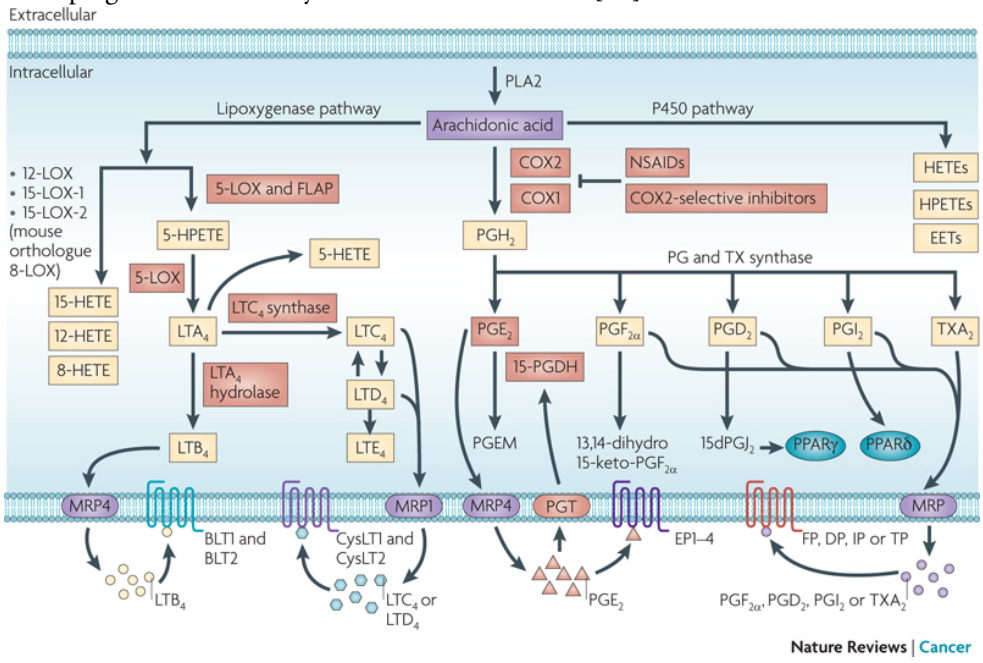

Figure 5. An overview of eicosanoid synthesis pathways. Adapted from Wang et al., 2010

The principal LOXs expressed in humans are 5-LOX, 12-LOX and 15-LOX. 5-LOX and 12-LOX stimulate angiogenesis and tumour growth, whereas 15-LOX has both pro-tumourigenic and anti-tumourigenic activity [71]. The 5-LOX enzyme interacts with a 5-LOX-activating protein (FLAP) and converts AA to the unstable LTA_4. LTA_4 is subsequently converted to biologically active LTB_4 or to the cysteinyl leukotrienes (CysLTs), LTC_4, LTD_4 and LTE_4. LTB_4 and LTD_4 are the most potent LTs [59]. In contrast to PGs, which can be produced by most of the cells in the body, LTs are made predominantly by inflammatory cells like polymorphonuclear leukocytes, macrophages, and mast cells [58]. LTB_4 can bind to two receptors, BLT_1 with high affinity and BLT_2 with low affinity [59]. It has been shown that LTB_4 can promote the growth of inflammation-induced melanoma [75]. The LTB_4 receptor antagonist, LY293111, is able to inhibit colon cancer tumour growth and induced apoptosis in vitro [76]. The action of CysLTs is mediated by two subtypes of cysteinyl leukotriene receptors (CysLTRs), $CysLT_1R$ and $CysLT_2R$ [58].

The $CysLT_1R$ has a higher affinity for LTD_4 than $CysLT_2R$, and with a much higher affinity for LTD_4 than LTC_4, whereas the $CysLT_2R$, exhibits low, but equal affinities for LTD_4 and LTC_4 [77]. Elevated circulating LTD_4 level is observed in patients with hepatocellular carcinoma [78]. The expression level of the $CysLT_1R$ is increased in colon cancer tissues and several human colon cancer cell lines [79]. The $CysLT_1R$ signalling induces COX-2 expression [80], activates MAPK/ERK signalling pathway [81] and Wnt/β-catenin signalling pathway in intestinal epithelial cells [82]. Consequently, $CysLT_1R$ signalling induces cell survival, proliferation and migration [83-85]. In contract, the $CysLT_2R$ signalling is shown to have anti-tumour effect, low expression of $CysLT_1R$ and high expression of $CysLT_2R$ mediate good prognosis in colon cancer [86, 87].

Taking together, pro-inflammatory eicosanoids, mainly the PGs and LTs, produced by tumour epithelial cells and their surrounding stromal cells, are important mediators in crosstalk between inflammation and cancer. They can directly or indirectly induce epithelial tumour cell proliferation, survival, and migration and invasion [59].

Remodelling of extracellular matrix

Extracellular matrix

The extracellular matrix (ECM) is the non-cellular component present in all tissues and organs [88]. Besides providing physical support to the cells, ECM molecules exhibit important functional roles in the control of key cellular events such as

adhesion, migration, proliferation, differentiation, and survival [89]. ECM is composed of glycoproteins and proteoglycans, and forms a complex, three-dimensional network, where the ECM components present dynamic interactions with each other [90] and with the cells of different tissues in an organ-specific manner [89].

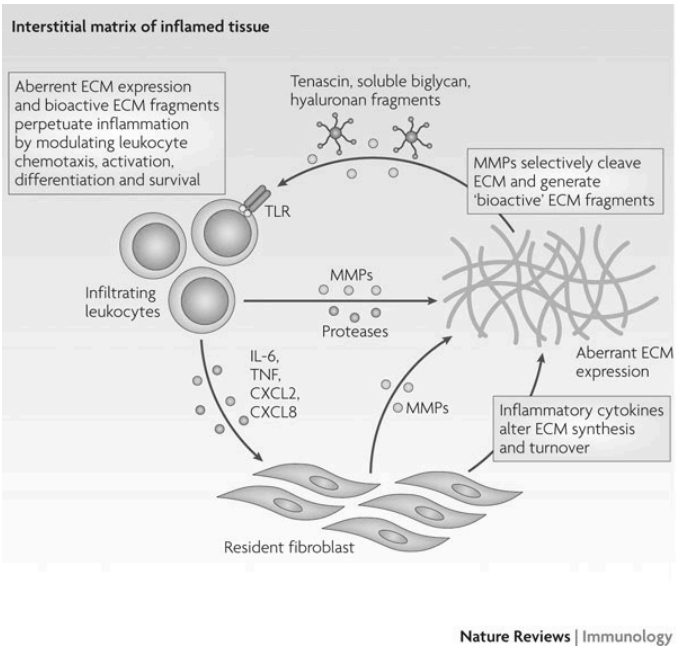

Figure 6. Remodeling of ECM under inflammation. Adapted from Sorokin, 2010

The ECM components are secreted by various types of cells (mainly fibroblasts or myofibroblasts) [91], they are tissue-specific but markedly heterogeneous [88]. Collagen, which is the most abundant fibrous protein within the interstitial ECM, constitutes the main structural element of the ECM, provides tensile strength, regulates cell adhesion, supports chemotaxis and migration, and directs tissue development [92]. So far, 28 types of collagen have been identified, among which type I, III, IV, and VI are most prominent [93]. Collagen I is the structural component of all tissues except cartilage [94]. Collagen III is the dominant collagen type of granulation tissue [95]. Collagen IV, which is nonfibrillar collagen, is the structural component of basement membrane [96]. Collagen VI belongs to association collagens and is the dominant structural component of connective tissue [97].

Remodelling of ECM occurs during development, wound healing and normal organ homeostasis, as well as in a number of pathologies, including fibrotic disorders, hypertension, atherosclerosis and cancer [9, 98, 99]. Abnormal ECM dynamics lead to dysregulated cell proliferation and invasion, failure of cell death, and loss of cell differentiation [100]. The changes of ECM are regulated by two opposing forces: synthesis and deposition on one hand, and proteolytic breakdown on the other [90]. For example, under chronic inflammatory conditions, infiltrated immune cells secrete cytokines and proteases, including MMPs, which alter ECM synthesis and/or selectively cleave ECM domains. Both aberrant ECM expression and cleaved ECM

fragments can influence the inflammatory response by modulating immune cell chemotaxis, activation, differentiation or survival, thereby contributing to the perpetuation of the inflammatory response, in some cases through the activation of Toll-like receptor 2 (TLR2) and/or TLR4 (Figure 6) [101].

Therefore, integrins, which mediate ECM-cell interaction, and MMPs, which are the most prominent proteolytic enzymes involved in the dissolution of the ECM, play very important roles in regulating ECM remodelling and signal transduction.

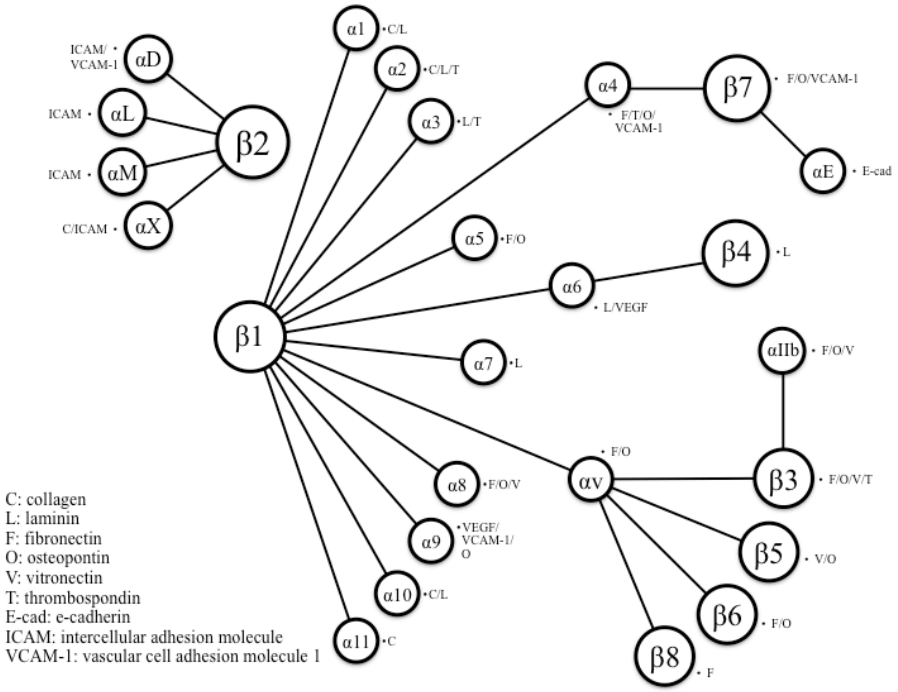

Figure 7. Integrin subunits combinations and ligands.

The integrin family

The integrins are heterodimeric transmembrane proteins, which mediate the attachment of cell to cell or cell to ECM and signal transduction. Integrins are composed of 18 α subunits and 8 β subunits that can be non-covalently assembled into 24 combinations with overlapping ligand specificity [102] (Figure 7). Thus, different integrin expression patterns of a cell determine which ECM molecules the cell can bind, as well as the downstream signalling [102]. Because ECM components

may be recognized by more than one integrin, competitive or cooperative binding among different integrin heterodimers adds an additional layer of complexity to cellular responses to the ECM [103].

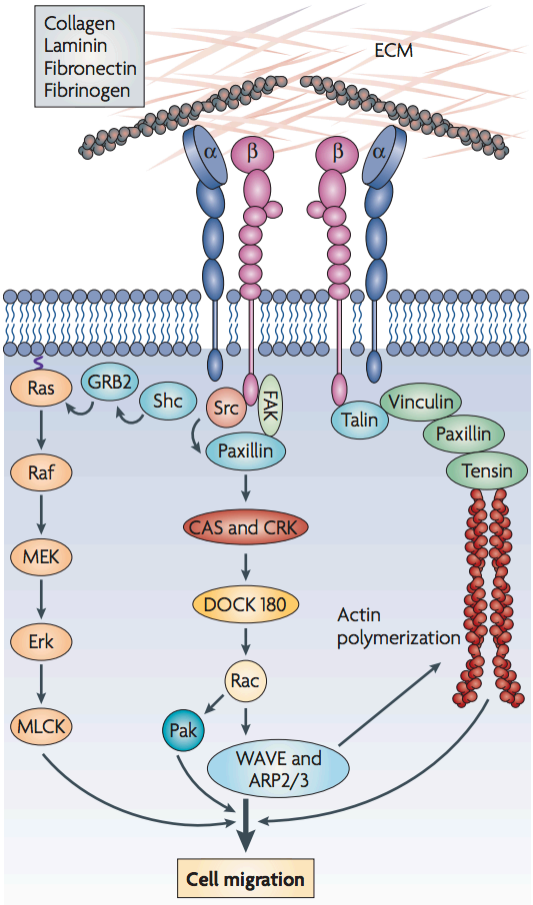

Figure 8. Integrin regulation of cell migration and focal adhesions. Adapted from Avraamides et al., 2008

Integrins transmit signals bi-directionally. The inside-out signalling is induced by the binding of talin and kindlin to the cytoplasmic domains of integrin β subunits, which activates the ligand binding function of integrins [104]. The alternative to this is outside-in signalling, which is induced the interaction between integrins and their ligands. The outside-in signalling allows the cell to sense the extracellular environment and react correspondingly [104, 105]. The outside-in signalling is dependent on the adaptors or scaffold proteins that link integrins to kinases, such as focal adhesion kinase (FAK) and the proto-oncogene tyrosine-protein kinase Src, since enzymatic activities are not present in the cytoplasmic tails of integrins [106]. FAK and Src act together as adhesion-associated complex. The signals from growth-factor receptors, integrins, or from the upstream Src-family kinases activate this complex by phosphorylate FAK, and facilitate cell adhesion, cell migration and survival [107].

Both inside-out and outside-in signals are important at every stage of cancer. The inside-out signalling is important for ECM remodelling that facilitates invasive growth and metastasis of tumours [108]. The outside-in signalling performs as transducers of chemical and mechanical signals that control tumour cell responses to ECM [108] and is involved in regulation of cell spreading, retraction, migration, proliferation, and survival [104]. The intergrin signalling pathways regulate cell migration and focal adhesions as shown in Figure 8 [109].

CD47

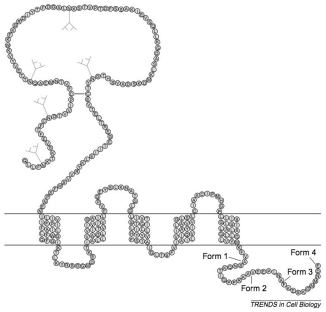

Figure 9. Structure of CD47. Adapted from Brown et al., 2001

CD47, also known as integrin associated protein (IAP), was first recognized as a 50-kD molecule that expressed on the plasma membranes of all hematopoietic cells and associated with a β3-integrin [110]. The structure of CD47 is shown in Figure 9 [10]. It is a cell surface protein of the immunoglobulin (Ig) superfamily, which has an extracellular amino-terminal IgV like domain, highly hydrophobic carboxy-terminal region with 5 membrane-spanning segments and a short intracellular tail [111]. CD47 associates with integrins via the extracellular Ig domain [112], and the membrane-spanning domain stabilizes this association [113]. Four splice forms of the cytoplasmic domain have been found. The fourth variant, which has the longest intracellular tail, is the predominant form present in the intestines [114].

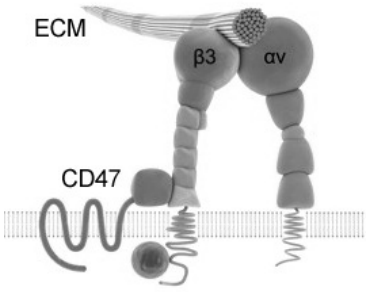

Figure 10. CD47 binds to αvβ3 integrin. Adapted from Legate et al., 2009

CD47 does not interact with all integrins. By now, the broadly expressed vitronectin receptor αvβ3 (Figure 10) [105], the fibrinogen receptor αIIbβ3 and the collagen receptor α2β1 have been co-precipitated or co-purified with CD47 [10].

Besides integrins, CD47 is able to bind to other ligands, such as Signal-regulatory protein α (SIRPα) [115]. SIRPα is a member of SIRP family, which also belongs to Ig superfamily. It is highly expressed by myeloid cells and neurons, but expression has also been found on endothelial cells and a subpopulation of B cells [116]. SIRPα has three extracellular Ig-like domains and several tyrosines in its cytoplasmic domain. The tyrosine-phosphorylated form of SIRPα binds the Src-homology 2 (SH2)-domain-containing tyrosine phosphatases, SHP-1 and SHP-2. It has either positive (SHP-2) or negative (SHP-1) regulatory effects on cellular responses, such as cell growth, migration and differentiation, induced by growth factors, oncogenes or insulin [117]. CD47 and SIRPα, associated via their Ig-like domains (Figure 11) [118], induce phosphorylation of immunoreceptor tyrosinebased inhibitory motifs (ITIMs) in the cytoplasmic domain of SIRPα. Phosphorylated ITIMs bind to SHP-1 [119] and negatively regulate macrophage fusion [11, 120]. In recent years, the association of CD47 and

SIRPα has been demonstrated to play a "don't eat signal" and negatively regulate macrophage phagocytosis [12, 121]. CD47-SIRPα association is involved in homeostasis of T cells, natural killer (NK) cells and dendritic cells (DCs) [122, 123]. In summary, this association is involved in regulating leukocyte activation, adhesion, migration, phagocytosis, xenotransplant rejection, hematopoietic stem cell engraftment and neuronal network formation [13, 118]. CD47-SIRPα association is considered as a validated target for therapies of leukemia and solid tumours [13, 124-126].

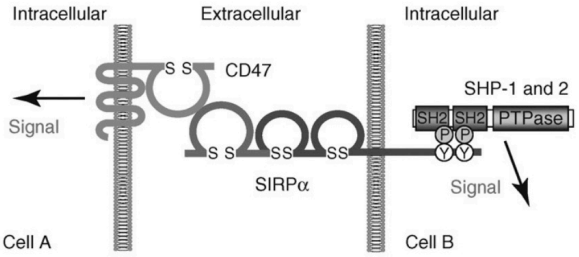

Figure 11. The CD47–SHPS-1 signalling complex. Adapted from Matozaki et al., 2009

Another CD47 ligand is thrombospondin (TSP). TSPs are a family of secreted glycoproteins that participate in cell-ECM communication [127]. All 5 isoforms of TSP have CD47-binding sequence, therefore it is believed that CD47 is a receptor for all TSP family members [10]. The prototypic member of TSP family, TSP-1, is a multifunctional protein. It interacts with various adhesion receptors, including CD36, αv integrins, β1 integrins and CD47 [128], as well as numerous proteases involved in angiogenesis. [129]. It has been shown that TSP-1 plays an inhibitory role in angiogenesis and tumour growth [130].

All forms of CD47 bind to heterotrimeric G proteins of the $G\alpha_i$ family, which are sensitive to pertussis toxin (PTX) and mediate most of CD47 signalling [10]. By binding different ligands, CD47 is involved in many developmental, immunological and pathological processes [10].

Matrix metalloproteinase

As mentioned before, degradation of ECM, which is mediated via various matrix-degrading enzymes, is required during ECM remodelling. The MMP family members are the most prominent proteases, which are implicated in the proteolytic degradation of the ECM and thereby play a central role in tissue remodelling [131]. MMPs are are zinc-dependent endopeptidases and belong to the metzincin family of enzymes, which exploit a zinc ion in their active sites. By now, 28 members were identified in this family, within which at least 23 members were found in human [132]. Each of the vertebrate MMPs has distinct but often over-lapping substrate specificities, and

together they can cleave numerous extracellular substrates, including virtually all ECM proteins [133].

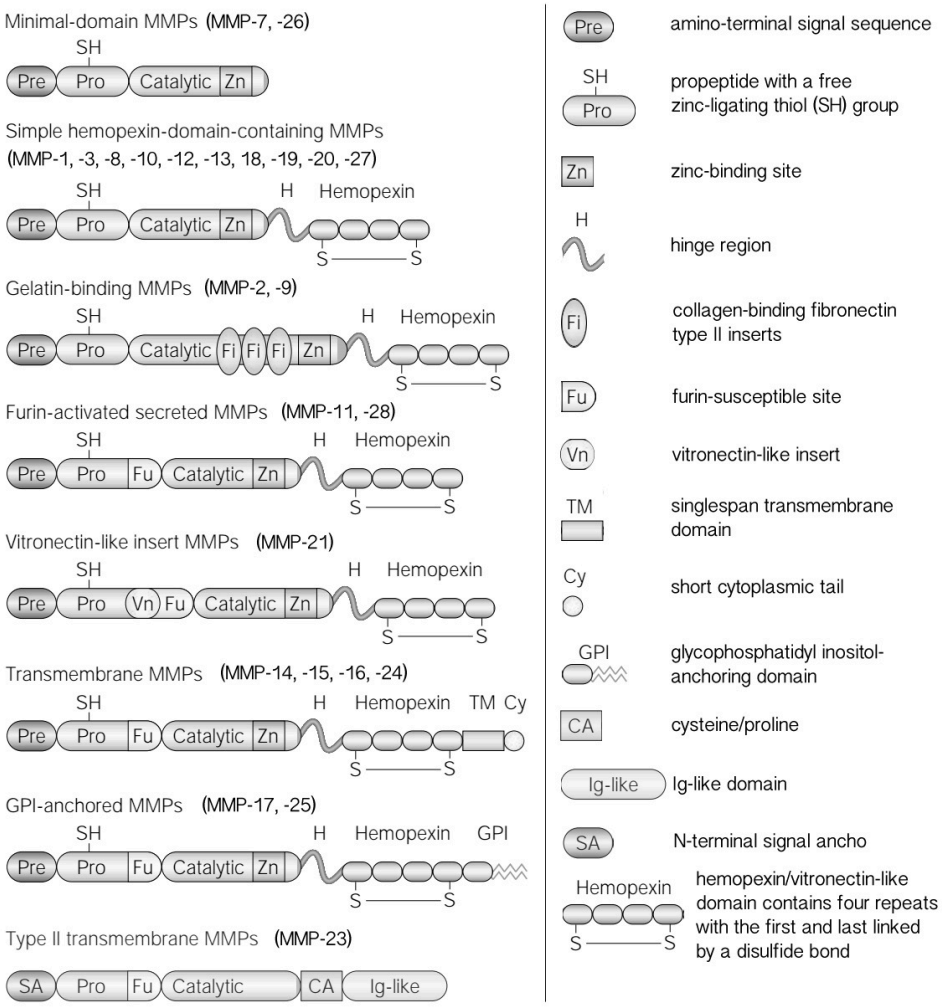

Figure 12. The protein structure of the MMPs. Adapted from Sternlicht et al., 2001 and Egeblad et al., 2002

Historically, the MMPs were divided into collagenases, gelatinases, stromelysins and matrilysins on the basis of their specificity for ECM components. However, as the list of MMP substrates has increased, the MMPs are now grouped according to their structure [134]. There are eight distinct structural classes of MMPs: five are secreted and three are membrane-type MMPs (MT-MMPs) with the protein structure shown in Figure 12 [134]. With the exception of MMP7, MMP26 and MMP23, all MMPs have a hemopexin/vitronectin-like domain, which mediates

protein-protein interactions and contributes to proper substrate recognition, activation of the enzyme, protease localization, internalization and degradation. The hinge region that mediates interactions with tissue inhibitors of metalloproteinases (TIMPs), cell-surface molecules and proteolytic substrates. The gelatin-binding MMPs and the furin activated secreted MMPs contain inserts for intracellular activation. MT-MMPs include (type I) transmembrane MMPs, the glycosylphosphatidylinositol (GPI)-anchored MMPs and MMP-23, which is a type II transmembrane MMP. [132-134].

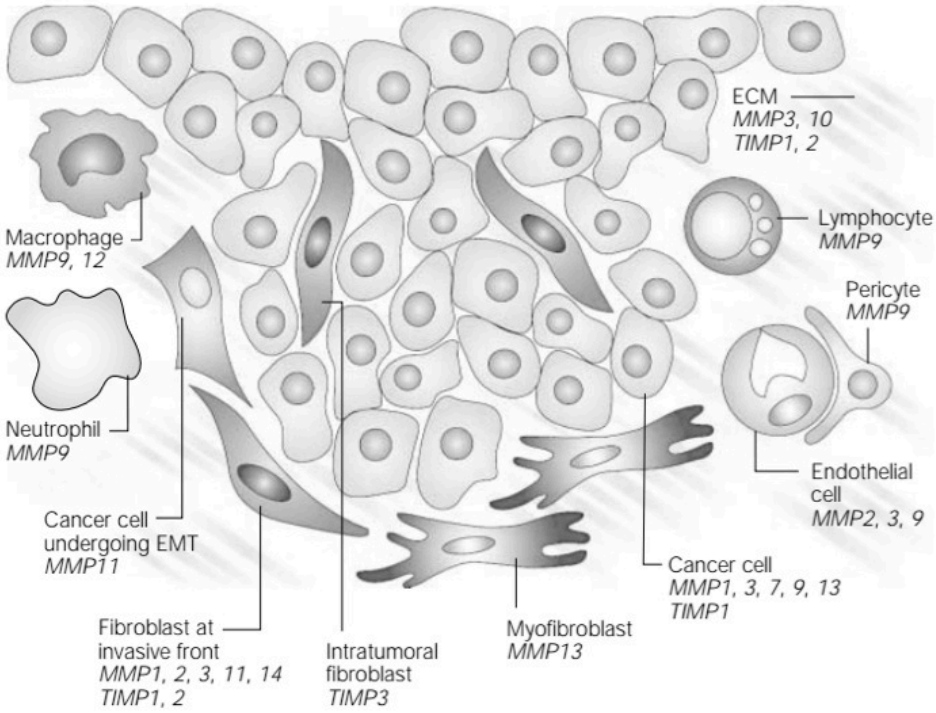

Figure 13. Expression of MMPs and TIMPs in breast tumours. Adapted from Egeblad et al., 2002

Most of the MMPs are released in a biologically inactive pro-enzyme form and need to be activated [135]. The activity of MMPs can be regulated by gene transcription, mRNA stability, translational control, cell compartmentalisation, zymogen activation via proteolysis, and inhibition by endogenous inhibitors [136]. The most investigated endogenous MMP inhibitors are TIMPs -1, -2, -3 and -4, which reversibly inhibit MMPs. They differ in tissue-specific expression and ability to inhibit various MMPs [134]. As an example, Figure 13 [134] shows the expression of different MMPs and TIMPs in breast cancer. Other important MMP tissue inhibitors

include α2-macroglobulin, TSP-1, -2 and RECK, which is the only known MT-MMP inhibitor [134].

Besides the function of degrading ECM components, MMP proteolysis can produce specific substrate-cleavage fragments with independent biological activity, regulate tissue architecture through effects on the ECM and intercellular junctions, and modify the activity of signalling molecules, both directly and indirectly [132]. MMPs play important roles in mammary development, bone modelling and remodelling, wound healing, angiogenesis, inflammation and cancer [132, 134]. The expression and activity of MMPs are increased in almost every type of human cancer, and this correlates with advanced tumour stage, increased invasion and metastasis, and poor prognosis [134]. In colon cancer, over-expression of several MMPs has been demonstrated, including MMP-1, -2, -3, -7, -9, -13 and MT1-MMP [137].

In this context, the remodelling of ECM and signal transduction from and to ECM, contributes to cancer via various mechanisms, especially via modulating cancer cell migration and invasion.

Cell migration

Directed cell migration is an integrated molecular process that is essential for mammalian development and homeostasis. Under physiological or pathological conditions, cell migration is required in many processes, such as mounting an effective immune response, repair of injured tissue, as well as tumour development and metastasis [138].

Cells often migrate in response to specific external signals, such as chemotaxis (movement to a concentration gradient of chemoattractants), haptotaxis (movement to a gradient of immobilized ligands) and mechanotaxis (movement induced by mechanical forces) [139].

Cell migration with lamellipodia

To migrate, a cell first acquires a characteristic polarized morphology in response to extracellular signals. At the cell front, actin assembly drives the extension of flat membrane protrusions called lamellipodia and filopodia [140]. Protrusion formation is an essential step during cell migration [141]. At the leading edge of the lamellipodium, the cell forms nascent adhesions, which connect the ECM to the actin cytoskeleton and anchor the protrusion. Then the assembly of filamentous actin (F-actin) stress fibres occurs, to tract the cell body forwards. Finally, the cell retracts its

trailing edge by combining actomyosin contractility and disassembly of focal adhesions at the rear [140, 142, 143]. During cell movement, the endocytosis and recycling of some molecules, such as E-cadherin, integrins or growth factor receptors that play important roles in cell motility [143].

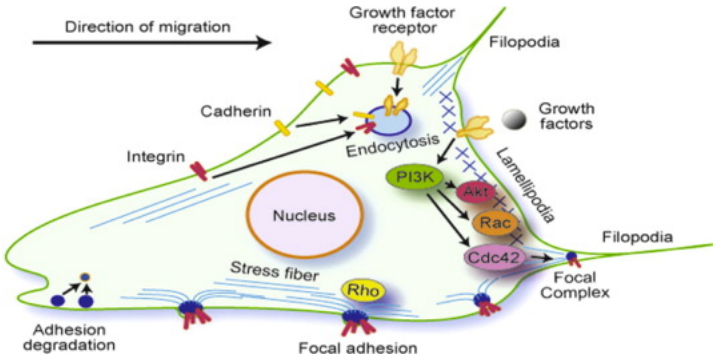

Figure 14. Lamellipodia-mediated cell migration in breast cancer cell. Adapted from Jiang et al., 2009

The small GTPases Rho family plays a center role in lamellipodia-mediated cell migration. The formation of nascent adhesions and the breakdown of and focal adhesions are controlled by Rac, Rho, Rho-associated protein kinase (ROCK), Myosin II and FAK [144]. Rac is required at the front of the cell to regulate actin polymerization and membrane protrusion, whereas Rho is thought to regulate the contraction and retraction forces required in the cell body and at the rear [145]. Filopodia can be induced by Cdc42, which activates heptameric actinpolymerizing complex actin-related protein 2/3 (Arp2/3) [146]. PI3K activation in response to extracellular stimuli leads to accumulation of phosphatidylinositol triphosphate (PIP3), and in turn stimulates Rac, Cdc42 and Akt, which are involved in the formation of protrusions at the leading edge (Figure 14) [143].

Rho family is also a key regulator other migration modes. Activation of Rac triggers actin polymerization and is associated with epithelial-mesenchymal transition (EMT) process, whereas Rho activation leads to actomyosin contraction, which induces membrane blebbing and rounded amoeboid morphologies [147].

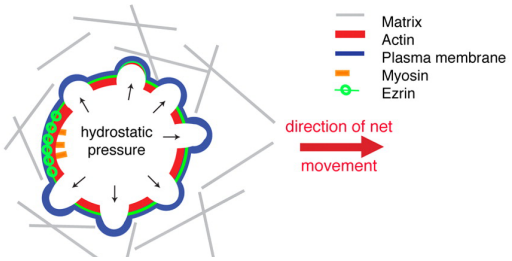

Figure 15. An amoeboid-type cell migration model. Adapted from Lorentzen et al., 2011

Amoeboid-type migration with membrane blebs

Cells migrating in three-dimensional environments and in vivo can form a wide variety of protrusion types, including lamellipodia and membrane blebs [141], which are produced by contractions of the

actomyosin cortex [148]. Blebs are often considered to be a hallmark of apoptosis; however, for tumour cells and a number of embryonic cells, blebbing migration seems to be a common alternative to the lamellipodium-based motility [148]. Non-mesenchymal types of cancer cell movement often display amoeboid motility with amoeboid-like rounded cell morphologies and membrane blebs (Figure 15) [149].

Epithelial-mesenchymal transition

Tumour cells in can adopt a mesenchymal migration mode, characterized by elongated cell shape via EMT process [150]. Cancer cells facilitate motility in complex environments by switching between non-mesenchymal and mesenchymal modes.

Epithelial cells establish close contacts with neighbour cells ECM through adherens junctions, desmosomes, and tight junctions. The epithelial cell layer has the capacity to function as barriers or in absorption. Conversely, mesenchymal or stromal cells are loosely organized in a 3D extracellular matrix and comprise connective tissues adjacent to epithelia. The EMT is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells [151]. EMT is classified into 3 subtypes (Figure 16) [152, 153]. Type 1 is embryological EMT and occurs in gastrulation and migration of neural crest cells. Type 2 EMT occurs in wound healing and can result in fibrosis when there is persistent inflammation [153]. Tumours are considered as wounds that never heal, since wound healing and tumour stroma formation share many important properties [154]. One of these properties could be EMT. Type 3 EMT occurs in epithelial tumour cells, which metastasize. After invading, tumour cells can transition back to mesenchymal-epithelial transition (MET) to proliferate and generate tumours at distant sites [153].

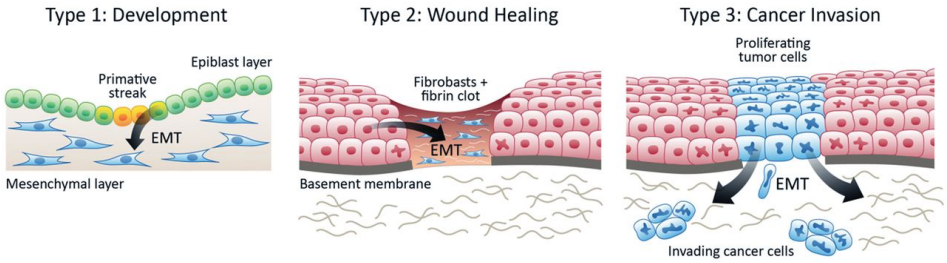

Figure 16. Three types of EMT. Adapted from Scanlon et al., 2012

Various proteins are involved in EMT, including cell-surface proteins, cytoskeletal proteins, ECM proteins, and transcription factors. At the molecular level, EMT is generally characterized by loss of E-cadherin and increased expression of

several transcriptional repressors of E-cadherin expression, such as Twist, Slug and Snail [155]. However, in clinic, evidence of a full EMT phenotype in cancer processes is generally lacking [156].

The idea that EMT has a role in cancer has also benefited from its link with the loss of E-cadherin expression, which is a well-established condition for malignant cancer progression [157]. EMT is not only involved in metastatic events in cancer, but also in other events highly relevant to tumour progression, including resistance to cell death and senescence, therapeutic resistance, immunosuppression and acquiring stem cell-like properties [151]. Several signalling pathways associated with growth and progression of cancer are also involved in EMT progress. The MAPK/ERK pathway has been shown to activate Snail, Slug and Twist, both of which are transcriptional repressors of E-cadherin [158]. Activation of the PI3K/Akt signalling has emerged as a central feature of EMT [159]. Smad-mediated TGF-β signalling is able to induce EMT of cancer cells, thereafter, this signalling play an important role in promoting cancer cell invasion and metastasis [160]. The majority of human colon cancer cases carriy mutations that lead to the activation of Wnt signalling, which has a pivotal role in EMT [161].

Macrophage

Macrophages are phagocytes derived from monocytes. They are found in all tissues and have roles in development, homeostasis, tissue repair and immunity [162]. Macrophages function in both non-specific defence (innate immunity) and initiate specific defence mechanisms (adaptive immunity) [163]. Macrophages are remarkably plastic cells, which are found in different tissues and assume different phenotypes.

Macrophage polarization

Generally, macrophages are classified in to M1 (classically activated) phenotype and M2 (alternatively activated) phenotype, as shown in Figure 17 [164]. Macrophages are polarized to M1 phenotype in response to interferon-γ (IFN-γ) and some bacterial moieties, such as lipopolysaccharide (LPS). M1 macrophages produce reactive nitrogen, oxygen intermediate and IL-12. They are involved in Th1 responses and mediating resistance against intracellular parasites and tumours. In contrast, M2 polarization was originally discovered as a response to the Th2 cytokine IL-4 [165]. M2 macrophages show more phagocytic activity, high expression of scavenging, mannose and galactose receptors, production of ornithine and polyamines through the arginase pathway, and a phenotype of low expression of IL-12 and high expression

of IL-10, the IL-1 decoy receptor and IL-1 receptor antagonist. In general, these cells participate in polarized Th2 responses, help with parasite clearance, dampen inflammation, promote tissue remodelling and tumour progression and have immunoregulatory functions [164].

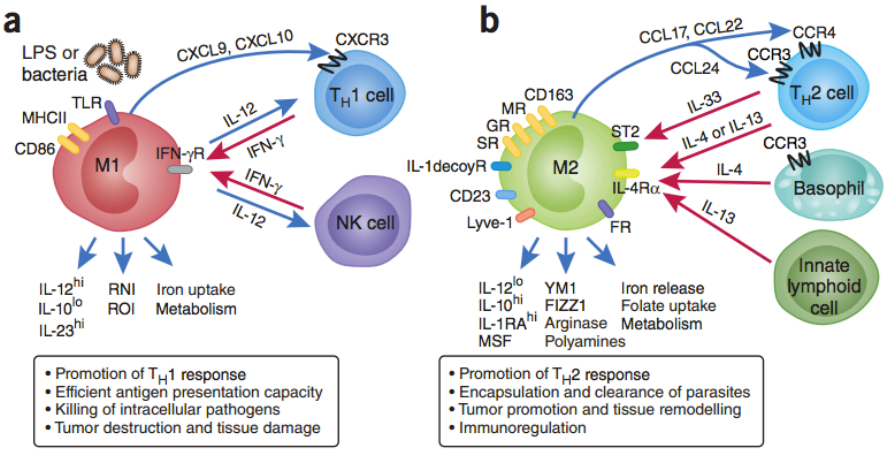

Figure 17. Macrophage activation and polarization. Adapted from Biswas et al., 2010

Tumour-associated macrophage

Macrophages are major inflammatory components of stromal tissue of various types of cancer. From early-stage tumour nodules that are beginning to vascularize to late-stage tumours that are invasive and metastatic, tumours are always infiltrated and/or surrounded by monocytes [166]. Tumours can recruit macrophages via a number of chemoattractants including macrophage colony-stimulating factor (M-CSF or CSF-1), the CC chemokines, (CCL2, CCL3, CCL4, CCL5, and CCL8) and VEGF [167]. These macrophages, which are termed TAMs, were then "educated" to benefit tumour progression.

TAMs are believed to have an "M2-like" phenotype, and share some but not all the signature features of M2 macrophages [164]. For example, M-CSF, TGF-β and IL-10, give rise to M2-like functional phenotypes that share properties with IL-4- or IL-13-activated macrophages [168]. TAM summarise a number of M2-like functions, involved in tuning inflammatory responses and adaptive immunity, scavenge debris, promote angiogenesis, tissue remodelling and repair. The production of IL-10, TGF-β and PGE_2 by cancer cells and TAMs contributes to a general suppression of anti-tumour activities [8]. As M2 macrophages, TAMs are poor

producers of nitric oxide (NO) [169] and reactive oxygen intermediates (ROIs) [170]. Expression levels of inflammatory cytokines, such as IL-12, IL-1β, TNF-α, IL-6, are low in TAMs [171]. TAMs express various molecules, which affect tumour cell proliferation, angiogenesis and metastasis (Figure 18) [8, 172]. These molecules include cytokines [6], chemokines [173], growth factors, such as EGF, VEGF [8] eicosanoids [174], and MMPs, especially MMP-9 [7].

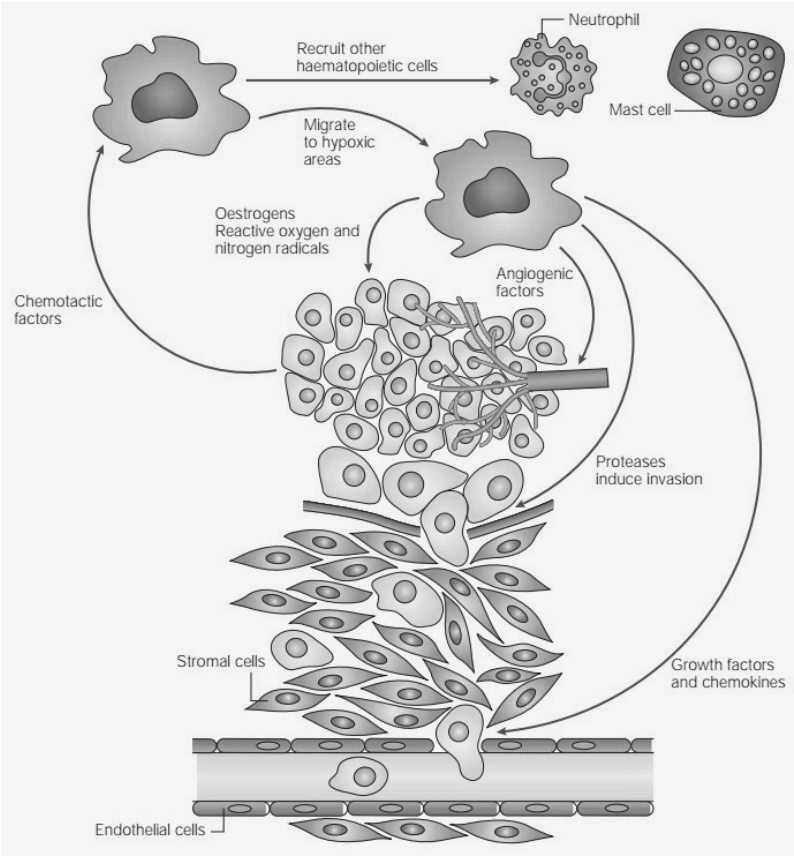

Figure 18. TAMs promote tumour progression and metastasis. Adapted from Pollard, 2004

Present investigations

Aim

The general aim of this thesis has been to investigate the relationship between inflammatory tumour microenvironment and colon cancer, focusing on the role of CD47 signalling and TAMs in colon cancer cell migration and invasion. The specific aims were the following:

- To investigate the functional role of CD47-α2β1 integrin binding in epithelial cell migration.
- To explore the role of TAM-derived factors and CD47-SIRPα interaction in colon cancer cell migration.
- To further understand the mechanism of colon cancer cell invasion mediated by elevated MMP expression and activation in response to TAM stimulation.

Materials and methods

Cell culture

The human embryonic intestinal epithelial cell line, Int 407, human colon cancer cell lines SW480, SW620, HCT-116 and human monocyte cell line THP-1 (obtained from the American Type Culture Collection) were cultured following the supplier's instructions. THP-1 cells were differentiated by treatment with 100 nM phorbol-12-myristate-13-acetate (PMA) for 7 days. The adhered cells were further differentiated with 100 ng/ml LPS for 72 h and 20 ng/ml IFN-γ during the last 48 h (M1) or 20 ng/ml IL-4 for 48 h (M2).

Patient samples

Formalin-fixed and paraffin-embedded colon cancer and control colon specimens from colorectal cancer patients were obtained from the archives of the Department of Pathology at Malmö University Hospital. Tissues from 72 patients with varying grades and stages of disease were included. Staging of the tumours was done using Dukes' classification Fresh biopsies were obtained for mRNA analysis. The biopsy samples were placed in RNAlater (Qiagen, Hilden, Germany) and frozen by submersion in liquid nitrogen. The matched control samples from normal colon tissues were surgical specimens from the same patients. Specimens were obtained with informed consent after ethical approval was granted by the Ethics Committee of Lund University.

Tumour xenograft study

The Regional Ethics Committee for Animal Research at Lund University, Sweden (M205-10) approved the present animal study. Female 6- to 8-week-old athymic nude mice (BalbCnu/nu) were purchased from Taconic Europe A/S (Ry, Denmark). To generate subcutaneous human colon cancer xenografts, 2.5×10^6 low-passage HCT-116 or SW480 cells suspended in 100 µl phosphate-buffered saline (PBS) were injected into both flanks of the mice. Twenty-one days after tumour cell inoculation all mice were sacrificed, and the tumours were removed. Tumour tissues were immediately fixed in 10% buffered formalin and then embedded in paraffin for further immunohistochemical analysis.

Immunohistochemistry

Formalin-fixed, paraffin-embedded archival colon cancer human specimens and mice xenografts were stained with specific antibodies. All stained tissues were visualised by incubation with secondary peroxidase-conjugated antibodies. After immunostaining, all slides were manually counterstained with Mayer's haematoxylin. Slides were

scanned with the ScanScope CS (Aperio, Vista, CA, USA) at 10× and 40× magnification. Cell numbers were counted with NIS-Elements Advanced Research software (Nikon, Tokyo, Japan).

RT-PCR

RNA from cells and tissue samples was isolated following the protocol of the Qiagen RNeasy Plus Mini Kit. Amplification was performed in Mx3005P system (Agilent Technologies, Inc., CA, USA), and reactions were analysed with MxPro software and normalised against the housekeeping gene HPRT1.

Immunofluorescent staining

Cells on coverslips were fixed for 15 minutes with 4% paraformaldehyde in PBS at room temperature. The coverslips were then blocked with 3% bovine serum albumin (BSA)/PBS solution for 30 min. The cells were incubated with specific primary antibodies for 1 h, washed with PBS and incubated with Alexa 488 or Alexa 546 conjugated secondary antibody for 1 h. After washing with PBS, the cells were incubated with DAPI for 3 min and mounted in fluorescent mounting medium (Dako, Glostrup, Den- mark). The slides were photographed with a fluorescent microscope or confocal microscope.

Transfection with CD47 siRNA oligomers

Cells were cultured for 3 days to 50–60% confluence. The cell media was aspirated and the cells detached and scraped into 3 ml of serum and antibiotic free medium containing 50 nM siRNA against CD47 or a scrambled control siRNA with lipofectamine 2000. After 4 hours, the transfection medium was diluted with normal growth medium without antibiotics and the cells were allowed to grow for an additional 48 hours period.

Immunoprecipitation

The cells were allowed to adhere onto collagen I coated or control dishes for 1 hour at 37°C. The cells were lysed in the lysis buffer containing 1% octyl-b-D-1-thioglucopyranoside. The lysates were then incubated for 20 minutes on a rotator at 4°C, after which cell debris was removed by centrifugation at 9,000 x g for 10 minutes. Protein G agarose was used to pre-clear the lysates after which the different cell lysates were adjusted to the same protein content. These lysates were then incubated with CD47 antibody or control IgG antibodies overnight on a rotator at 4°C. Protein G was then added and the lysates incubated at 4°C for 1 hour. After three washes with lysis buffer the final pellets were re-suspended in sample buffer, boiled and analysed by Western blotting.

Western blotting

Whole cell lysates and membrane fractions of SW480 and SW620 cells were prepared, and western blotting was performed as described in [175]. The polyvinylidene difluoride (PVDF) membranes were blocked for 1 h at room temperature with either BSA/PBS or non-fat dried milk/PBS. The membranes were incubated overnight at 4°C with specific primary antibodies. The membranes were washed and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies. Proteins were detected after incubation with an Immun-Star Western Chemiluminescence Kit (BioRad, Hercules, CA, USA) using a Bio-Rad ChemiDoc XRS+ System. Bio-Rad Image Lab software was used for densitometric analysis, and the value obtained from the non-stimulated control was set to 100.

Wound healing assay

Cells were serum starved for 2 h with/without pretreatment. A sterile pipette tip was used to make a scratch in the cell monolayer and non-adherent cells were removed by gentle washing. The cells were allowed to migrate for 18 h in serum free medium at 37°C. For the co-cultured cell wound healing assay, M2 macrophages were differentiated in the wells of an ibidi culture-insert (ibidi, Martinsried, Germany). On day 4, 2.5×10^6 SW480 cells were added to the dishes outside of the insert. Thereafter, SW480 cells and macrophages were cultured for an additional 5 days. On day 9, the culture medium from the macrophages was aspirated before the insert was carefully removed. SW480-conditioned medium was collected, centrifuged to remove cell debris and added back to the dishes. Non-adherent cells were gently washed in PBS. The cells were allowed to migrate for 24 h at 37°C. Pictures were taken with a Nikon DS-Fi1 microscope using a 10× objective and analysed with NIS-Elements Basic Research software. The area of the wound was measured with Image J or Adobe Photoshop CS4 software.

Flow cytometry

The differentiated M1 and M2 macrophages were detached using 0.02% versene and were washed twice in 0.5% BSA/PBS before blocking the human FcRs with 20 µg/ml heat-aggregated human IgG. Next, 0.5×10^5 cells were suspended in 100 µl 0.5% BSA/PBS and incubated with specific conjugated antibodies (30 min each at 4°C). Cells were examined with a FACS-Calibur using the software Cell Quest and analysed using FCS Express Version 4 (De Novo Software, Los Angeles, CA, USA). Forward and side scatter gates were set to include all viable cells.

Measurement of secreted cytokines by multiplex assay

To remove cell debris, the conditioned media were collected and centrifuged at 1000 rpm for 5 min. Electrochemiluminescence assays were performed on macrophage-

conditioned medium in duplicate using a 10-plex human TH1/TH2 detection kit (Meso Scale Discovery, Gaithersburg, MD, USA) according to the manufacturer's instructions. The plates were read using the Meso Scale Discovery SECTOR Imager 6000 and analysed using Discovery Workbench and SoftMax PRO 4.0 software.

Visualizing gelatinase activity

200 µl of Oregon green 488-conjugated gelatin (0.2mg/ml in PBS) was added to glass coverslips. The coated coverslips were kept in dark for 30 min at room temperature. After washing with PBS, 1×10^5 colon cancer cells and/or M2 macrophages were added onto the coverslips. After 6 h incubation in the dark at 37°C, the cells were incubated with DAPI (1:1000) for 3 min and mounted in fluorescent mounting medium, after fixation and blocking as previously described. The slides were photographed with a Nikon Eclipse 80i microscope using a PlanApo 40× objective (Nikon) and NIS-Elements Advanced Research software.

Gelatin zymography for MMP-2 and MMP-9

MMP-2 and MMP-9 activities in SW480 and SW620 colon cancer cells stimulated with M2 macrophage conditioned media were assessed through gelatin zymography. The cells were seeded at a density of 1×10^6 cells and allowed to grow to 70-80% confluence. They were further incubated for 24 h with/without M2-conditioned media containing 1.5% fetal bovine serum. 50 µl of the supernatants were mixed with 2× non-reducing Laemmli buffer and incubated at room temperature for 10 min before being electrophoresed directly on a 10% SDS-polyacrylamide gel containing 0.1% gelatin (type A from porcine skin, Sigma). After the run, the gel was washed 4 times for 20 min at room temperature with 2.5% Triton X-100 solution, and then transferred to a Ca^{2+} containing development buffer and incubated for 20 h at 37°C with gentle agitation. Subsequently, the gels were stained for 1 h with Coomassie brilliant blue (Sigma) followed by destaining with Methanol: Acetic acid for 30 min until excess stain was removed and clear bands appeared against a blue background. The gel was then scanned with ChemiDoc XRS+ imaging system (BioRad).

3D cell migration

Cells (250,000) were added on top of a collagen I containing (3 mg/ml) gel placed in the upper well of a Boyden chamber. The lower well contained serum free medium and was separated from the upper well by a polycarbonate PVPF membrane with 8.0 mm diameter pores. After 18 hours of incubation at 37°C, the cells that were attached to the upper side of the membrane or present in the collagen I gel were removed with a cotton swab, and the remaining cells were fixed with 4% paraformaldehyde, for 15 minutes. The cells in the membrane were subsequently stained with a 1% crystal violet/10% methanol solution at room temperature for 15 minutes. The membranes

were washed in PBS after which the remaining dye was solubilised using a 10% SDS solution and the absorbance was measured at 590 nm.

Invasion assay

10 µl of Matrigel (BD Biosciences) was added into the wells of an ibidi µ-Slide Angiogenesis (ibidi, Martinsried, Germany) and allowed to polymerize as a 0.8 mm thick gel. The cells were then trypsinized and 5000 cells were added on top of the matrigel in 1.5% FBS containing RPMI 1640 medium or M2-conditioned medium. After incubation at 37°C for 18 h, the medium was removed from the wells. Cells were fixed and blocked as described above and stained with Alexa Fluor 488 Phalloidin (Dako, Glostrup, Denmark) for 1 h and DAPI for 3 min. The slides were photographed as z-stack images (5 µm step) with a Zeiss LSM 700 confocal microscope and Zen 2012 software (Carl Zeiss Microscopy GmbH, Jena, Germany). The distances from the gel surface to each cell were measured.

Statistical analyses

SPSS software 16.0 was used for all immunostaining analyses. Univariate survival analysis was performed by Kaplan-Meier analysis with a log-rank test to determine the risk of death. Survival time was measured from the date of surgery to the date of death or 80 months of follow-up. The death information is from the Swedish Cause of Death Registry and the Swedish Cancer Register. The overall survival was calculated as colon cancer-specific death. Deaths due to other causes were censored at the time of death. Prizm software 5.0d (GraphPad Software, San Diego, CA, USA) was used for other statistical analyses. All the data are presented as the mean ± standard error of the mean (SEM), and statistical significance was determined as $P < 0.05$ by a two-way analysis of variance (ANOVA; labelled #), Column statistics or a two-tailed Student's t-test (labelled *). All means were calculated from data from at least three independent experiments.

Results and discussion

Collagen mediated COX-2 expression and cell migration is regulated through the CD47-α2β1 integrin signalling (paper I)

The ECM is one of the most important regulators of cellular and tissue function in the body. Remodelling of the ECM is essential for physiological conditions such as development; wound healing and normal organ homeostasis, as well pathological conditions including cancer [9]. The signal transduction from ECM to cells is mainly mediated via integrins [176].

Previous findings from our lab have shown that the inflammatory mediator LTD_4 regulates the adhesive properties and migration of colon cancer cells Caco-2 by upregulating COX-2 and stimulating PGE_2-induced expression of α2β1 integrins on collagen I surface [177]. In IBD, intestinal inflammation leads to mucosal ulceration and subsequent tissue repair with remodelling of the ECM [178]. Our lab has previously shown that COX-2 expression is enhanced via α2β1 integrin signalling in human intestinal epithelial cells, when the cells were exposed to collagen IV (found mainly in basement membrane) or collagen I (found mainly in stromal tissue). Activation of PKC, the small GTPase Ras and nuclear factor κ B (NFκB) was required in collagen-induced COX-2 expression [179]. In this study intestinal epithelial cells were placed onto a collagen I coated surface to further investigate this signalling pathway, based on previous findings.

First, increased COX-2 expression in intestinal epithelial cells on collagen I surface was observed, and this induction was mediated by a PTX sensitive $G\alpha_i$-protein. Collagen I binding integrin has been described as the α2β1 integrin [180]. CD47 is known to binds to α2β1 integrin and associate with a PTX sensitive G-protein. Thus, CD47 could be involved in this signalling. CD47-α2 integrin binding was demonstrated by immunoprecipitation. Pre-incubation of functional blocking antibody against CD47, transfection of dominant negative form of CD47 or CD47 siRNA to the cells could significantly block COX-2 expression after the cells were plated on a collagen I coated surface.

CD47 was shown to play a role in neuronal development via activation of Cdc42 and Rac [181], which are also important regulators of cell migration [146]. Thus, the role of CD47 in cell migration was investiged. In the cells pre-treated with CD47 blocking antibody, cell adhesion to collagen I was enhanced, whereas cell migration on collagen I was downregulated. With pre-treatment of COX-2 specific inhibitor or CD47 siRNA transfection, migration of the cells on collagen I was reduced in both 2D and 3D cell migration assays.

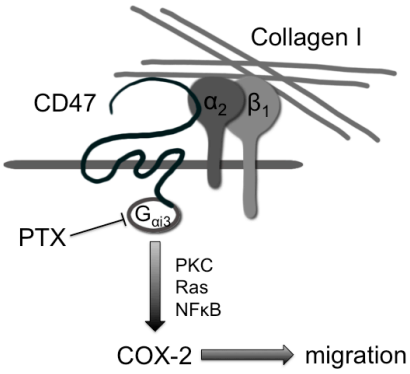

Figure 19. Cell migration and COX-2 expression is regulated by CD47-α2β1 signalling via $G\alpha_{i3}$ protein on collagen I surface.

One possible explanation of the effect seen on cell adhesion and migration could be the morphological change. Rounded morphology with membrane blebs was observed after the cells were plated onto collagen I surface, as compared to the spread morphology was seen when the cells were plated onto fibronectin. Membrane blebs were observed during cytokinesis and during cell migration in 3D cultures and in vivo. For tumour cells and a number of embryonic cells, blebbing migration seems to be a common alternative to lamellipodia-mediated migration [148]. Immunofluorecent staining showed clear expression of CD47, α_2 integrin and Rho A in the membranes blebs, and the formation of these blebs was CD47 and COX-2 dependent.

In this paper, we emphasized the importance of the ECM signal transduction in controlling cell migration and expression of eicosanoids. Taking into account previous findings, we demonstrated signalling pathway as shown in Figure 19.

Crosstalk between TAMs (M2 phenotype, $CD206^+$) and tumour cells promotes colon cancer cell migration via TAM-derived factors and CD47-SIRPα association (paper II)

As phagocytes differentiated from monocytes in tissue, macrophages function in both non-specific defence (innate immunity) and initiate specific defence mechanisms (adaptive immunity) [163]. Activated macrophages are classified into M1 (classical activated) macrophages and M2 (alternatively activated) macrophages. M1 macrophages produce inflammatory cytokines and mediate Th1 responses, whereas M2 macrophages are involved in Th2 responses and have immunoregulatory functions [182]. As a major inflammatory component of stroma, macrophages could be recruited into tumour tissues [167]. These cells, so-called TAMs, are shown to play a role in promoting tumour progression and metastasis [172]. The aim of this study was to investigate the role of TAMs in colon cancer cell migration.

Colon cancer represents a strong link between chronic inflammation and tumour progression [183]. In both tissues from colon cancer patients and mouse xenograft tumours from a human colon cancer cell line, high infiltration of M2

macrophages ($CD68^+$, $CD206^+$) was found, especially in later stages of colon cancer and bigger mouse xenograft tumours. In tumour tissue, these macrophages were seen in between cancer cells. This localization indicated a probable interaction between TAMs and colon cancer cells.

Human colon cancer cell line SW480 was used in this study. To create a cell line model for investigating the function of TAMs in colon cancer, human monocyte cell line THP-1 was differentiated into M1 or M2 macrophages. Expressions of a number of macrophage surface markers, such as CD83, CD80 and CCR7 for M1 macrophages or CD206 and CD163 for M2 [184-186] macrophages were investigated. As compared to M1 macrophages, M2 macrophages expressed higher levels of CD206 and SIRPα, secreted higher levels of IL-4, -8 and -10, lower levels of IL-1β, IFN-γ and TNF-α.

Cell migration of SW480 was enhanced after stimulation with M2-conditioned medium or under co-culture conditions with M2 macrophages in a time dependent manner. The contribution of M2 macrophage-derived factors in this cell migration was further investigated.

IL-8, through its receptors CXCR1 and CXCR2, mediates Ca^{2+} release, contraction and cell migration [187]. In wound healing assay, SW480 failed to respond to M2-conditioned medium stimulation in the presence of CXCR1 blocking antibody. Macrophages are the source of LTs [188], which are important inflammatory mediators in IBD and colon cancer. Our lab has previously shown that LTD_4 increases motility of human intestinal epithelial cells [189]. In this study, significantly down-regulated migration of SW480 cells was observed after M2-conditioned medium, by using $CysLT_1R$ inhibitor.

SIRPα expression was higher in M2 macrophages as compared to M1 macrophages. It was shown that CD47 associated with its ligand SIRPα could negatively regulate phagocytosis [190], as well as positively regulate cell migration [13]. Expression of CD47 was higher in colon cancer patient tissue than normal tissue. In a co-culture system, CD47 blocking antibody and CD47 siRNA significantly reduced cell migration of SW480 and M2 macrophages moving towards each other, and the addition of a SIRPα blocking antibody further reduced cell migration. CD47 expression in SW480 cells was significantly increased upon IL-8, LTD_4 and M2-conditioned medium stimulation. These findings demonstrated the importance of CD47 in colon cancer cell migration in crosstalk with TAMs.

M2 macrophages secreted high levels of IL-4 and IL-10, which are Th2 cytokines and provide M2 macrophage–polarizing signals [191]. Interestingly, IL-10 secretion from colon cancer cells SW480 was much higher than in M2 macrophages. IL-10 was known to promote tumour development, growth, and metastasis [192].

After co-culturing with SW480 cells or stimulation with IL-10, the expression pattern of CD206 and SIRPα in M1 macrophages became similar to that in M2 macrophages. These findings supported the notion that IL-10 contributed to M2 macrophage differentiation.

In summary, high content of TAMs was observed in colon cancer tissue. TAMs play an important role in colon cancer cell migration by secreting IL-8, LTD_4 and mediating CD47-SIRPα interaction. Targeting this interaction could have potential therapeutic benefits in colon cancer.

Colon cancer cell invasion induced by elevated MMP gene expression and activation in response to stimulation by TAM-conditioned medium (paper III)

Tumour metastasis is the main reason for high mortality in cancer patients [193]. ECM remodelling is crucial for tumour malignancy and metastatic progression [9]. MMPs, a family of zinc-dependent proteases, are proteolytic enzymes intimately linked to tumour invasion and progression. Although additional functions of MMPs have emerged recently, physiologically they are known to degrade the extracellular matrix, facilitating tumour cell invasion and metastasis [194]. In colon cancer, over expression of several MMPs has been demonstrated, including MMP-1, -2, -3, -7, -9, -13 and MT1-MMP [137]. However, it is still unclear how these MMPs are regulated. In this study, we explored how TAMs promote colon cancer cell invasion through MMPs.

First, the enhanced mRNA expressions of MMP-7 and -9 were observed in colon cancer patient tissue and tumours from mouse xenograft model using colon cancer cell line SW480. To compare the differences between primary tumour and metastatic tumour, 2 colon cancer cell lines were used: SW480 and SW620, which were established from the same colon cancer patient with a primary adenocarcinoma (SW480) or a lymph node metastases (SW620). In both cell lines, the mRNA levels of MMP-2, -7 and -9 were elevated after M2-conditioned medium stimulation.

Since MMPs have a pro-form and active-form, in addition to changes at the gene expression level, the activity of MMPs were investigated. Gelatin zymography was performed to determine their ability of degrading ECM. An increased activity of MMP-9 was observed in both SW480 and SW620 cells after M2-conditioned medium stimulation.

Degradation of ECM was essential for ECM remodelling and was linked to enhanced cell migration and invasion [9]. The invasion assay was performed with a 3D matrigel. Z-stack pictures taken with a confocal microscope showed increased

invasion of both SW480 and SW620 cells in the presence of M2-conditioned medium. This increase of invasion could be partly blocked by using specific inhibitors to MMPs.

Besides degrading ECM, MMPs could also induce EMT, which contributes to carcinoma invasion and metastasis [195]. It was shown that a broad range of MMPs are involved in the induction of EMT in breast cancer [196]. MMP-9 cooperated with the transcription factor Snail to induce EMT [197]. EMT was characterized by the loss of E-cadherin expression and gain of vimentin expression [198]. Thus the expression levels of several EMT-related proteins were measured. In membrane fractions from SW480 cells cultured in M2-conditioned medium, elevated vimentin expression was observed after 24 h and 72 h. Whereas decreased β-catenin expression was observed after 72 h. Expression of E-cadherin in SW480 cells was inherently low, and could not be detected in SW480 cells cultured in M2-conditioned medium.

This study reported that the conditioned medium from TAMs increased mRNA levels and activities of MMP-2, -7 and -9 in colon cancer cell lines SW480 and SW620. Increased invasion of SW480 and SW620 cells was observed in the presence of this conditioned medium, which could be partly blocked with specific MMP inhibitors. The M2-conditioned medium could also affect the expression of several EMT-related proteins. These preliminary findings provided an approach to understand the mechanism of colon cancer invasion and metastasis induced by TAMs.

Summary

In summary, I have shown the following:

- CD47, binding to α2β1 integrin, plays a role in regulating COX-2 expression and cell migration on a collagen I surface.
- Colon cancer tissue is enriched with TAMs (M2 phenotype, $CD206^{+}$).
- IL-8 and LTD_4 secreted by TAMs contribute to colon cancer cell migration and facilitate CD47 signalling.
- CD47-SIRPα interaction plays a role in cell migration of colon cancer cells and TAMs.
- Enhanced mRNA expression and activity of MMPs regulating colon cancer cell invasion in the presence of TAM-conditioned medium.

Popularized summary

Colorectal cancer (CRC) is a cancer caused by uncontrolled cell growth in colon or rectum. CRC is the third most common cancer and the fourth leading cause of cancer related death in the world. It is a multifactorial disease, both genetic predisposition and environmental play a role in the development of CRC. CRC is closely linked to chronic inflammation. Patients with inflammatory bowel disease (IBD) have an increased risk of developing CRC. Tumours are surrounded by the cellular milieu called tumour microenvironment, which is an important hallmark of cancer. In this study, we investigated how colon cancer crosstalk with its microenvironment.

Extracellular matrix (ECM) maintains the morphology of cells, as well as mediates various signalling pathways. Under inflammatory or tumoral conditions, remodelling of ECM often occurs. Matrix metalloproteinases (MMPs) are the predominant ECM-degrading enzymes. MMPs and other factors abound in tumour microenvironment alter ECM synthesis and/or selectively cleave ECM domains. These processes can influence cell proliferation and invasion, failure of cell death, and loss of cell differentiation. When cells are exposed to aberrantly expressed ECM proteins, various downstream signalling pathways will be activated. Signals from ECM can be transduced intracellularly by integrins and CD47. In the first article, CD47, associated with integrin, was shown to play a role in ECM mediated cell migration of intestinal epithelial cells. This increased migration was regulated by inflammatory mediator cyclooxygenase-2 (COX-2).

Macrophages are important phagocytes involved in host defence. They are also major components of inflammatory tumour microenvironment. Tumour cells can recruit macrophages and change their behaviour. These tumour-associated macrophages (TAMs) perform crosstalk between colon cancer cells via CD47 associated with signal-regulatory protein α (SIRPα). This association and TAM-derived factors was shown to contribute to colon cancer cell migration. TAM-derived factors can also induce mRNA expression and activity of MMPs in colon cancer cells. This might be one explanation how TAMs can induce colon cancer cell invasion.

In summary, these new findings provide an important clue for better understanding of the crosstalk between tumour microenvironment and colon cancers cells, and help to identify new therapeutic targets for colon cancer treatment.

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