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The Role of Inflammatory Lipid Mediators on Colon Cancer Initiating Cells (CICs)

Kishan Bellamkonda

LUND UNIVERSITY

DOCTORAL DISSERTATION
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To be defended at Auditorium (Level 3), Skåne University Hospital, Jan Waldenströms gata 47, Malmö, on Friday 16th January 2015 at 9:00 a.m.

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Abstract

Colorectal cancer (CRC) is one of the major causes of cancer globally. Recent studies proposed a role for cancer initiating cells (CICs), a small subset of replication-competent cells, in colon carcinogenesis. Although the role of inflammatory lipid-mediators in CRC progression is well known, their role in the promotion of cancer-initiating cells remains to be elucidated. For this thesis, we investigated the role of eicosanoids – leukotriene D_4 (LTD_4) or prostaglandin E_2 (PGE_2) – on CIC properties and changes occurring in the tumor environment that could possibly support CIC-induced tumor growth. To this end, we identified the CICs on the basis of ALDH expression and evaluated their *in vitro* characteristics like colony formation, radio or chemoresistance and *in vivo* tumorigenic properties in the presence of LTD_4 or PGE_2. We showed that LTD_4 and PGE_2 enriched the $ALDH^+$ cell population and augmented the colonies formation and tumor progression in xenograft mice model. The $ALDH^+$ cells were also resistant to 5-fluorouracil and radiation that is additionally augmented by both the lipid-mediators. Moreover the impact of lipid inflammatory mediators on the stemness properties of CICs was evident by increased expression of genes that confer survival and self-renewal ability to CICs. In immunodeficient mice, LTD_4 or PGE_2 treatment amplified CIC-induced tumor growth. Furthermore, LTD_4 and PGE_2 increased cell proliferation activated β-catenin signaling and up-regulated COX-2. Additionally, LTD_4 or PGE_2 drive massive inflammatory responses identified as $CD45^+$ enrichment, particularly of macrophages within tumors. The ability of $ALDH^+$ cells to form tumors in immunodeficient mice could not be challenged by radiation therapy.

In a separate series of experiments, we investigated the contribution of CICs in the development of sensitivity against montelukast, a $CysLT_1R$ antagonist. In this context we report that sensitivity of tumors against montelukast could depend on the variation in CICs content, activation of prosurvival factors such as BCL-2 and β-catenin signaling. Collectively, our data showed that LTD_4 and PGE_2 exacerbate CIC characteristics and promote tumor growth by allowing modifications in the tumor environment. New therapeutic strategies could aim to resolve not only cancer associated inflammation, but also to target CICs in order to achieve better remission and cure advanced colon cancer stages.

Key words Colorectal Cancer, Cancer Initiating Cells, Inflammatory Lipid Mediators, ALDH

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I dedicate this thesis to my beloved parents for their support and prayers

"If we knew what it was we were doing, it would not be called research, would it?"
Albert Einstein

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I. **Kishan Bellamkonda,** Wondossen Sime, Anita Sjölander. The impact of inflammatory lipid mediators on colon cancer initiating cells. 2014. Mol Carcinog. doi: 10.1002/mc.22207.*

II. **Kishan Bellamkonda,** Naveenkumar Chandrashekar, Janina Osman, Sayeh Savari, and Anita Sjölander. Eicosanoids leukotriene D_4 and prostaglandin E_2 promote colon tumorigenicity of colon cancer initiating cells in a xenograft mouse model. (Manuscript)

III. **Kishan Bellamkonda,** Sayeh Savari, Desiree Douglas, Naveenkumar Chandrashekar and Anita Sjölander. Role of colon cancer initiating cells in tumor malignancy and insensitivity against montelukast in xenograft model. (Manuscript)

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Abbreviations

AA	Arachidonic acid
ABC	ATP-binding cassette
AJCC	American Joint Committee on Cancer
ALDH	Aldehyde dehydrogenase
APC	Adenomatous polyposis coli
BCL-2	B-cell lymphoma 2
BSA	Bovine serum albumin
CDK	Cyclin-dependent Kinase
CICs	Cancer initiating cells
COX	Cyclooxygenase
CRC	Colorectal cancer
CSC	Cancer stem cells
$cPLA_2$	Cytosolic phospholipase A_2
CysLT	Cysteinyl leukotriene
$CysLT_1R$	Cysteinyl leukotriene receptor 1
$CysLT_2R$	Cysteinyl leukotriene receptor 2
CysLTR	Cysteinyl leukotriene receptor
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EP1-4	Prostaglandin E_2 receptor 1-4
EMT	Epithelial-mesenchymal transition
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FLAP	5-lipoxygenase activating protein
5-FU	5-fluorouracil
Fz receptor	Frizzled receptor
GDP	Guanine nucleotide diphosphate
GEF	GDP/GTP exchange factor
GPCR	G-protein coupled receptor
GSK-3	Glycogen synthase kinase 3-beta
GTP	Guanine nucleotide triphosphate
Hh	Hedgehog
HNPCC	Hereditary nonpolyposis colorectal cancer
HPETE	Hydroperoxyeicosatetraenoic acid
IBD	Inflammatory bowel disease
IL-6	Interleukin-6
IP3	Inositol 1,4,5-triphosphate
KLF4	Kruppel-like-factor 4
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

LEF/TCF	Lymphoid enhanced factor/T-cell factor
5-LOX	5-Lipoxygenase
LRP5/6	Lipoprotein-related protein 5 or 6
LT	Leukotriene
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor-κb
NSAIDS	Non-steroidal anti-inflammatory drugs
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-biphosphate
PGE_2	Prostaglandin E_2
STAT3	Signal transducer and activator of transcription 3
TCF	T-cell factor
TNF-α	Tumor necrosis factor-alpha
TNM	Tumor node metastasis
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor

1. Introduction

Colorectal cancer (CRC) is the leading cancer type which constitutes a major health issue globally [1, 2]. Recent studies implicate inflammation around tumors in the pathogenesis of CRC [3-5]. It is observed that an inflammation-rich microenvironment of tumors can initiate or promote the progression of CRC. In fact, the inflammatory cells and a variety of mediators in the proximity of epithelial cancer cells, stroma and blood vessels, together create an inflammatory microenvironment that in certain ways influences tumor growth [6, 7]. The mechanism how inflammation promotes cancer is unclear, however it has been assumed that ongoing inflammation stimulates tumor cells and immune cells to secrete various factors like pro-inflammatory eicosanoids, cytokines, chemokines which facilitate leukocytes infiltration. In presence of continued inflammation these leukocytes undergo functional changes which supports epithelial cell proliferation, angiogenesis, growth and metastasis [8]. These leukocytes are also the major source of lipid inflammatory mediators such as leukotriene D_4 (LTD_4) which is also known to stimulate proliferation, survival and migration of colorectal cancer cells [9, 10]. Likewise, cyclooxygenase-2 (COX-2) enzyme that produces PGE_2 is found increased in approximately 60 to 80 % of all colorectal cancer cases and their inhibition can suppress tumor growth by preventing cell proliferation and angiogenesis [10, 11]. Indeed, nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin, which inhibits COX signaling, are potential preventive agents for colon cancer [12]. Daily use of NSAIDs is shown to prevent the long-term incidence of adenocarcinomas and distant metastasis of colon cancer [13]. Overall these studies highlight the key role of inflammation in CRC onset and progression.

Although recent research focus mainly on devising a better therapeutics against cancer no treatment known is fully preventive of cancer in advanced stages. Recently much focus is given to a group of colon cancer initiating cells (CICs) with characteristic self-renewing and pluripotent ability. These CICs owing to their chemo and radioresistant properties are presumably held accountable for tumor initiation and growth [14-17]. The resistance mechanism of CICs is presumably due to its slow proliferating ability coupled with several mechanisms that assist in their survival and escape from an unfavourable environment. The tumor microenvironment seemingly plays a critical role in determining the behavior of cancer cells and other residing cells which are subjected to change with changing environment, signifying the necessity to understand the tumor microenvironment [11]. Moreover CICs leave their primary site and are believed to acquire some migratory properties that could aid in their ability to metastasize

into distant organs. Therefore, the properties of CICs like maintenance of their self renewal ability and survival could be regulated by the signaling events occurring in their microenvironment.

Although number of evidences hints the strong association of chronic inflammation with cancer, the plausible influences of the inflammatory signals on the overall property of CICs is still not fully unncovered. In this context, knowing the inflammatory changes in the tumor microenvironment in which CICs reside could provide some useful insight. Hence, an in-depth understanding of cancer stem cells interactions with other tumor cells is warranted for better understanding of cancer development. In this thesis, we investigated the possible role of inflammatory lipid mediators such as LTD_4 and PGE_2, which are abundantly present in tumor microenvironment, on the characteristic properties of CICs.

2. Background

2.1 Organization of the intestinal epithelium

The gastrointestinal system is one of the most regenerative systems in the human body. Daily billions of specialized intestinal epithelial cells are replaced by new cells to maintain the proper function of the large intestine. The intestine is lined by a sheet with invaginations, or crypts which make the surface area even larger [18]. The tubular glands and crypts of the large intestine are lined by a monolayer of epithelial cells consisting of stem cells and daughter cells at the bottom, and more specialized cells in the upper part of the crypt (Figure 1).

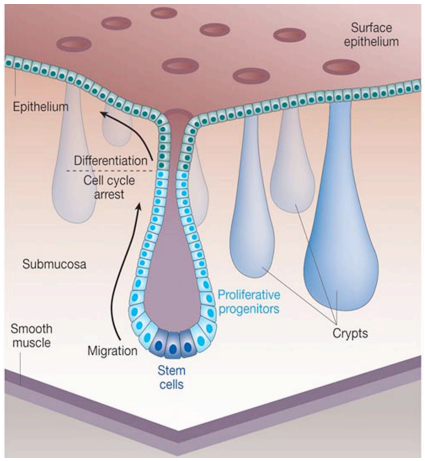

Figure 1. The anatomy of colon crypt. Reprinted by permission from Macmillan Publishers Ltd: [Nature 434, 843-850], copyright (2005)

To ensure sustainability of the epithelial sheet throughout the lifetime of an individual, stem cells divide and generate progenitors, which divide and produce more differentiated progeny like goblet cells (mucus secreting), absorptive cells and endocrine cells [19]. The cells of crypts constantly move upward and eventually terminated into the lumen. Given the complexity of such system, coupled with the high cell turnover rate, it is necessary to safeguard the mechanisms that ensure proper maintenance of cell function within the intestinal epithelium. In a normal colon there is a fine balance between cell growth and cell death, and the epithelial cells are renewed every 3-6 days. However, under certain

conditions, cell division and proliferation can take over and disrupt the balance resulting in increased gain: loss ratio of epithelial cells. This imbalance can contribute to a higher frequency of mutation, which is a risk of cancer development. An imbalance in several parameters, such as degree of differentiation and the extent of invasiveness into the surrounding membrane past the epithelial lining, constitutes a risk for CRC development.

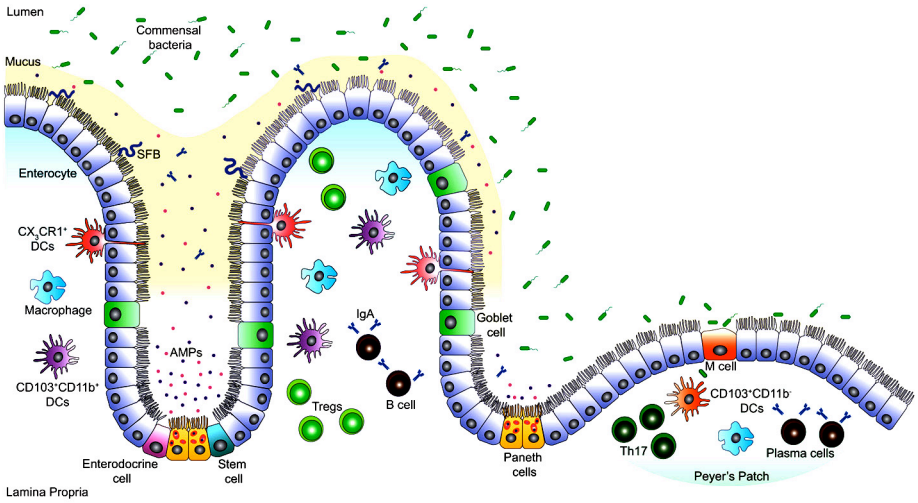

Figure 2. Representative intestinal mucosal surface. Taken from Muniz RL et. al., Front Immunol. 2012;3:310.

The human intestine is continuously exposed to low degree of inflammation due to the larger number of gut friendly bacteria it contains [20, 21]. The intestinal epithelium is protected from external pathogenic microorganism by a layer known as lamina propria that covers epithelium (Figure 2). Lamina propria hosts many immune cells; it is rich in macrophages, dendritic (DC) and lymphoid cells that make it as a prime location for immune reactions [22]. The other defense of digestive tract comes from its own immune system known as gut associated lymphoid tissue (GALT). The impairment or dysfunction of GALT leads to unbalanced inflammatory responses in the gut and predisposes one to inflammatory bowel disease (IBD) [23].

2.2 Colorectal cancer (CRC)

Colorectal Cancer (CRC), a term commonly used to refer to cancer of colon and rectum, is third most common cancer found and stands fourth in terms of leading causes of cancer related deaths globally [1, 24]. Countries of North America, Europe and Oceania reportedly have highest CRC cases, whereas sub-Saharan Africa, South American and Asian countries have lowest incidences[25]. CRC has multistep progression where mutations in oncogenes and tumor suppressor genes lead to their changed activity, which subsequently drives epithelial transformation (Figure 3) and progression from adenoma to carcinoma [26].

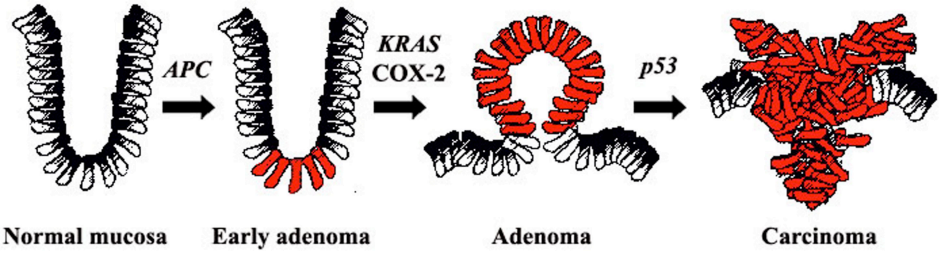

Figure 3. Sequence of development from normal mucosa to carcinoma.

CRC exists in sporadic and inherited forms such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis CRC (HNPCC). Sporadic CRC accounts for approximately 75% whereas heritable forms constitute 5% to 10% of all CRC cases [27]. Inherited and somatic mutations play an important role in CRC [28, 29]. Genetic alterations can lead to activation of oncogenes such as *KRAS*, which is mutated in approximately 50% of CRC, or to the mutational inactivation of genes like *APC* and *p53*, involved in tumor suppression [29]. The clear example for the role of mutational inactivation of genes in CRC development is Familial adenomatous polyposis (FAP), which arises due to mutated *APC* gene [3]. Under normal circumstances, the tumor suppressor protein APC together with other proteins like glycogen synthase kinase 3β (GSK-3β) and axin forms a complex in cytoplasm to which the β-catenin binds and later processed for degradation (Figure 4). However, mutation in *APC* gene inhibits β-catenin proteosomal degradation. Due to which levels of β-catenin increases in the cytosol from where it translocates into the nucleus and bind to the transcription factor TCF/LEF. These transcription factors in turn activates the expression of genes like

c-myc, *cyclin D1*and *COX-2* [30]. Interestingly, nearly 80% of sporadic CRC also carry mutational inactivation of the *APC* gene [31-33].

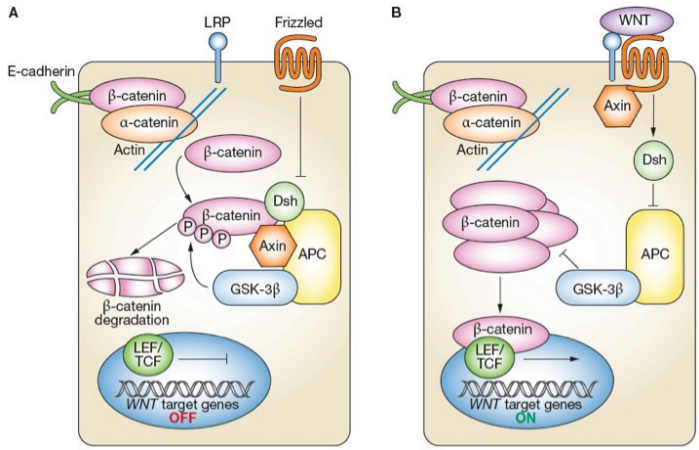

Figure 4. Wnt/β-catenin signaling pathway. Reprinted by permission from Macmillan Publishers Ltd: [Nature Clinical Practice Gastroenterology & Hepatology 3, 267-274], copyright (2006)

Other than the mutational activation of oncogenes, various factors have been suggested to be associated with colonic tumor growth. Among these, presence of inflammatory conditions near to the primary tumor site is indicated to play major roles in CRC progression [3-5]. For instance, cyclooxygenase-2 (COX-2), an intermediate of inflammation, is believed to participate considerably to CRC development. Large percentage of colorectal adenoma and carcinoma demonstrates upregulated COX-2 levels [34]. Interestingly, the direct relationship of COX-2 in CRC development was disclosed in a study on APC^{716} knockout mice [35]. In APC^{716} knockout mice, the deletion of one or both alleles of COX-2 significantly prevented the development of intestinal polyps. The contribution of inflammation in CRC development is clearly seen in individuals with inflammatory bowel diseases (IBD) who develop CRC at much higher frequency than others [36, 37].

2.2.1 CRC classification and staging

The classification of cancer patients into different stages is used to quantify the extent of disease and to provide a framework for selecting the appropriate treatment [38]. A number of staging systems exist across the world, the most common is the Tumor Node Metastases (TNM) system formed by the American Joint Committee on Cancer (AJCC). Using this system, the stage of CRC is divided into three components, primary tumors (T), regional lymph nodes (N) and

metastatic disease (M), which are combined to form stage groupings (Figure 5). The colorectal tumors are classified according to these various states: stage I, cancer present in inner lining of colon; stage II, cancer invading the muscle wall of the colon; stage III, cancer spreading to lymph nodes; and stage IV, metastatic cancer [39, 40]. Each stage can be further subdivided, indicating the intricate complexity of colorectal tumors.

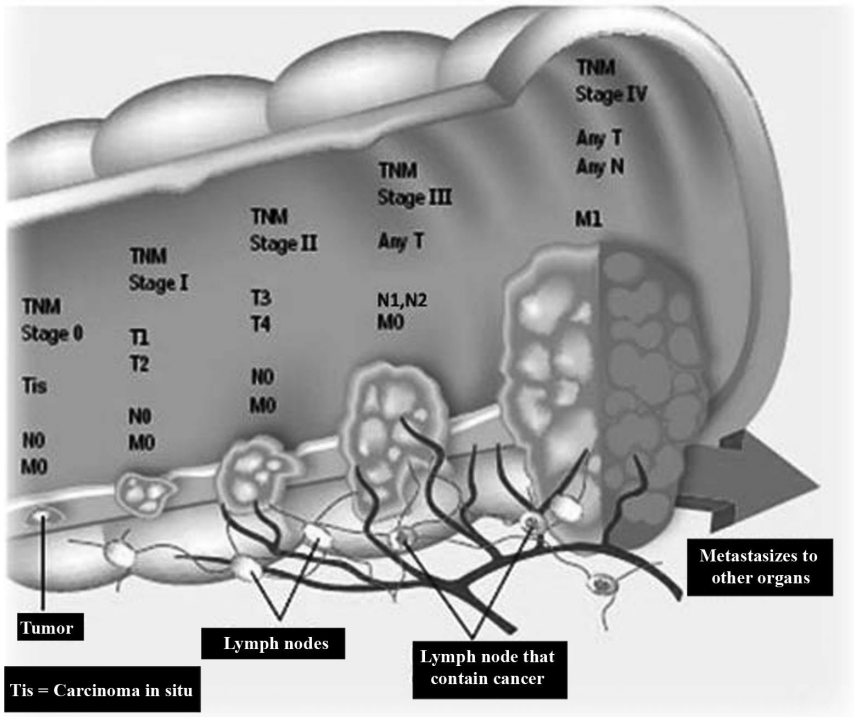

Figure 5. TNM stages for tumor classification. Taken from colorectal cancer association of Canada web page (http://www.colorectal-cancer.ca/en/just-the-facts/what-cancer/).

An older staging system for CRC also exists and is referred to as Dukes staging. The original system, proposed by Cuthbert Dukes in the 1930s [41] was for the classification of rectal cancer, but has been modified later on several occasions. Now the system covers both colon and rectal cancer and grades the growth from A to D. In this system the tumors were determined by the degree of tumor occupying the intestinal wall (Dukes' A), penetrating through the wall (Dukes' B), and into the lymph node (Dukes' C). Later on, the fourth stage D was added to this staging system for tumors with distant metastases [42]. Table 1 summarizes the different grading system used.

Table 1. Classification system of colorectal cancer.

Staging systems				
TNM Stage	T	N	M	Dukes
Stage 0	Tis	N0	M0	-
Stage I	T1	N0	M0	A
Stage II	T2	N0	M0	A
Stage IIA	T3	N0	M0	B
Stage IIB	T4	N0	M0	B
Stage IIIA	T 1-2	N1	M0	C
Stage IIIB	T 3-4	N1	M0	C
Stage IIIC	Any T	N2	M0	C
Stage IV	Any T	Any N	M1	D
TNM: (AJCC) Tumor, Node and Metastases staging system Dukes: Modified Dukes classification of colorectal cancer				

2.3 Inflammation and cancer

The crosstalk between cancer and inflammation is well established. Virchow in 1863, reported that chronic inflammation can lead to cancer [43]. Accordingly, inflammation associated with damaged tissue could stimulate cell proliferation, which over decades can form cancer. However, it is clear today that proliferation alone cannot cause cancer. Infact, sustained proliferation in an inflammatory environment that contains growth factors and inflammatory cells, principally contributes to tumor growth. The chronic inflammation associated with tissue injury over the time can lead to tumors, thus cancer is often regarded as "the wound that will not heal" [4, 44]. Even though chronic inflammation might not be required for initial onset of CRC, recent data showed inflammation as an essential component of tumor progression. For instance, patients with IBD such as ulcerative colitis and Crohn's disease develops CRC at high rate [45] reflecting that chronic inflammation rather than genetic predisposition is a concerning issue for CRC [46]. Thus in IBD associated CRC, the sequence of events leading to CRC progression seems to be inflammation-adenoma-carcinoma rather than adenoma to carcinoma transformation [46]. Another inflammatory disorder is primary sclerosing cholangitis (PSC), which also impose high risk of developing CRC [47]. It is also well recognized that cancer development in some cases can be prevented by using anti-inflammatory drugs [48, 49] and targeting inflammatory mediators can decrease the growth and metastasis of some cancers [50]. Inflammation spreads due to the factors like cytokines and chemokines produced either by tumor cells or by the inflammatory cells like macrophages [51] and mast cells [52, 53]. Many cytokines like TNFα, IL-6; chemokine IL-8 and growth

factors (e.g. VEGF) are found to be elevated in the the blood of CRC patients, and are suggested to have prognostic value [54]. TNFα and IL-1β are emerging new targets for the anticancer therapy. For instance, infliximab which is a TNFα antagonists is used to prevent tumor growth in studies with renal cell carcinoma and etanercept in ovarian cancer [55, 56]. Likewise IL-1β antagonists were also used as a treatment for some inflammatory disorders [57]. Cytokines can regulate the tumor growth and invasion by triggering transcription factors like NF-κB as in case of TNFα and IL-1β, or by STAT3 in case of IL-6 [58]. NF-κB signaling in tumor cells is also linked to the KRAS activation which overlap with the release of several pro-inflammatory mediators like IL-6 and IL-8 [59, 60]. Apart from cytokines, eicosanoids or lipid derived pro-inflammatory mediators, which are subject of our study, also promote proliferation, survival and migration of colorectal cancer cells [10, 61].

2.3.1 Inflammatory lipid mediators in cancer

Inflammatory lipid mediators or eicosanoids represent an important class of lipid mediators produced from arachidonic acid (AA) [62]. AA which is the major source of eicosanoids are generated by the actions of phospholipase A_2 (PLA_2) on plasma membrane, under external stimulus [63]. The eicosanoid family is made up of three classes (Figure 6): the prostanoids produced from cyclooxygenase (COX); leukotrienes (LTs) and certain mono-, di- and tri-hydroxy acids, formed via lipoxygenase (LOX) pathways; and the epoxides which are synthesized from cytochrome P-450 epoxygenase pathway [62, 64]. Eicosanoids play important roles in multiple diseases like IBD, asthma, arthritis, cardiovascular disease, thrombosis and in various malignancies such as colon, breast and pancreatic cancer [65]. Eicosanoids are produced by the cells of tumor itself or by the cells that surrounds tumors. Leukotrienes can be synthesized in any cell that contains all the enzymes required for their synthesis or through transcellular metabolism, which predominates in cancer tissue. For instance, epithelial and endothelial cells within tumors which lacks enzyme for LTA_4 synthesis can also produce leukotrienes by utilizing LTA_4 released from stimulated leukocytes [66]. Conversely, AA produced from epithelial cells can be used by leukocytes to generate leukotrienes. Using this transcellular biosynthesis the production of leukotrienes increases manifold which act as an additional stimulus for sustained inflammation in tumor tissue [67]. Prostaglandins are produced by most of the cells and are not reported to be generated from transcellular biosynthesis between epithelial and immune cells.

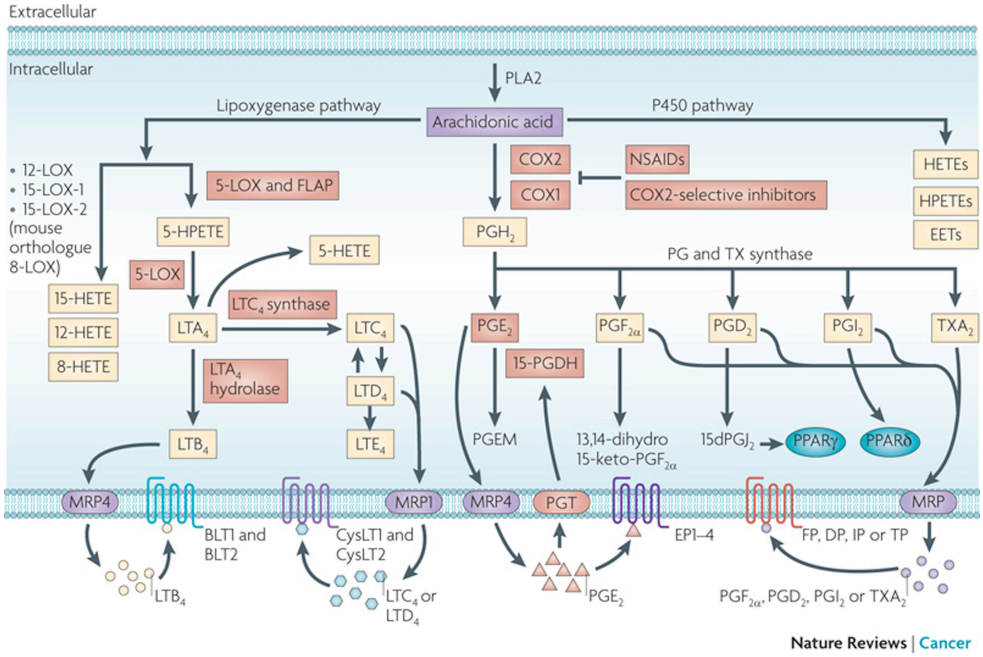

Figure 6. Eicosanoids biosynthesis pathway. Reprinted by permission from Macmillan Publishers Ltd: [Nat Rev Cancer 10, 181–193], copyright (2010).

2.3.1.1 Leukotrienes

The mammalian lipoxygenase pathway consists of three enzymes which adds an oxygen molecule to AA at 5, 12 or 15 positions to generate hydroperoxyeicosatetraenoic acid (i.e. 5-, 12- or 15-HPETE) [62]. The 5-lipoxygenase pathway (5-LOX) is involved in the biosynthesis of leukotrienes (LTs). Interaction of 5-LOX with 5-LOX-activating protein (FLAP) converts AA to LTA_4, which is subsequently metabolized to biologically active LTB_4 or to the cysteinyl leukotrienes (CysLTs); LTC_4, LTD_4 and LTE_4. The most potent LTs are LTB_4 and LTD_4 [64, 68]. LTs are mainly released from leukocytes like eosinophils, basophils, mast cells and macrophages [69].The role of leukotrienes in regulation of neoplastic transformation and growth is well documented. LTB_4 is shown to promote the growth of inflammation-induced melanoma [70]. Moreover, LTB_4 receptor antagonist, LY293111, induces apoptosis and inhibits colon cancer tumor growth [71].

Leukotrienes, particularly CysLTs mediates its actions through two cysteinyl leukotriene receptors (CysLTRs), $CysLT_1R$ and $CysLT_2R$, which belong to the G-protein coupled receptors (GPCRs) family [72]. The ligand binding to GPCRs can induce the conformational changes which promote the release of guanine

nucleotide diphosphate (GDP) from the Gα subunit of a specific intracellular heterotrimeric G-protein complex [73]. The GTP bound α subunit separates from the β and γ subunits and activates downstream signaling pathway based on associated α subunit types which could be Gαs, Gαi/o, Gαq/11 and Gα12/13 [74].

Alterations in CysLTRs are linked to several malignancies. High expression of $CysLT_1R$ is demonstrated in cancers of bladder, brain, prostate, breast, neuroblastoma and colon [75-79]. High $CysLT_1R$ tumor expression associates with breast and CRC poor prognosis. Low nuclear $CysLT_1R$:$CysLT_2R$ expression is considered as a good prognosis and linked to higher survival in CRC patients [80]. Interestingly, The $CysLT_1R$ signaling induces COX-2 expression [10], activates MAPK/ERK [81] and Wnt/β-catenin signaling pathways all of them involved in cancer development [82]. Interactions between $CysLT_1R$ and $CysLT_2R$ are also studied in intestinal epithelial cells. $CysLT_2R$ activation is demonstrated to negatively regulate the plasma membrane expression of $CysLT_1R$ by inducing $CysLT_1R$/$CysLT_2R$ heterodimer internalization in these cells [83].

2.3.1.2 Prostanoids

Prostanoids were produced from the enzymatic action of cyclooxygenase on arachidonic acid. Prostanoids includes prostaglandins and thromboxane A_2. Prostanoids mediates their actions by activation of respective receptors which are EP1–4 for PGE_2; prostaglandin $F_{2\alpha}$ receptor for $PGF_{2\alpha}$, thromboxane A_2 receptor for prostaglandin D_2 and thromboxane-A_2 and prostaglandin I_2 receptor for prostaglandin I_2. EPs activate G-proteins and signaling downstream by either increasing the intracellular levels of cyclic adenosine 3',5'-monophosphate (cAMP) as in case of EP2 and EP4, or by suppressing cAMP signals as for EP3. EP1 is also known to induce the mobilization of calcium [84, 85]. However the receptor mediated signaling events of prostanoid largely depend on the amount of ligand present and their structure, which could be subjected to change.

The prostanoid synthesizing enzyme COX exists in two isoforms, COX-1 and COX-2 which differ largely in their functions. COX-1 which is a constitutively expressed enzyme maintains the housekeeping functions of cells by production of prostanoids. Whereas, COX-2 is an inducible form which is activated in response to cytokines, stress or multiple other factors. It is the main source of PGE_2 produced during inflammation and cancer [86]. Elevated COX-2 levels are identified in cancer of colon [34, 87], breast [88], lung [89], pancreas [90], esophagus [91], ovaries [92] and carcinomas of neck and head [93]. Importance of COX-2 in colorectal carcinogenesis is also reflected in studies with APC mutated mice [94]. COX-2 knockout in APC mutant mice cause significant decline in the number and magnitude of intestinal polyps formed [95]. Notably, frequent use of

NSAIDs like aspirin (acetylsalicylic acid) is reported to inhibit COX-2 activity and subsequent tumor progression in CRC [96, 97].

PGE_2 plays a vital role in the tumor growth and progression among all prostanoids [64]. Elevated PG levels provides a poor prognosis of several malignancies including colon cancer [98]. COX-2 produced PGE_2 is demonstrated to have multiple functions in colorectal tumors like regulation of proliferation, survival and invasion [99]. Overexpression of COX-2 is also known to induce release of growth factors which assists in tumorigenic transformation of cells [100]. Moreover the studies with knockout mice for individual PGE_2 receptors have further ascertained the role of PGE_2 in colorectal tumorigenesis [101-103]. PGE_2 improves the survival of colon cancer cells by inducing PI3K–Akt–PPARδ pathway [104]. Also, PGE_2 is demonstrated to have anti-apoptotic effects possibly through upregulation of BCL-2, and NF-κB activation [105, 106]. PGE_2 induces cell proliferation in colon and lung cancers presumably by effecting Ras–Erk and GSK-3β mediated signaling events [107, 108]. Interestingly, PGE_2 stimulation of EP2 is demonstrated to induce nuclear β-catenin translocation in colon cancer cells [108, 109]. Increased β-catenin translocation to the nucleus triggers transcriptional activity of TCF/LEF [110] which modify *cyclin D1*, *c-myc*, and *COX-2* genes functions [111-113].

In summary, PGs and LTs produced by cancer cells and stromal cells are important mediators of crosstalk between inflammation and cancer. They can accelerate tumor growth by affecting survival, metastasis and proliferation of cancer cells.

2.4 Current therapies and their limitations

Treatment of CRC depends on many factors such as patient age and disease stage. In general the first line treatment for colon cancer is surgical removal of tumor and confined lymph nodes by hemi-colectomy. However, the overall recurrence rate within 5 years is approximately 30% after surgery [114]. Approximately 50–60% of all CRC patients will develop metastatic tumors, and 20–25% of patients with colon cancer were diagnosed with metastases at the time of report [115]. Chemotherapy and/or radiation therapy are often given to patients to reduce the recurrence and for metastatic colon cancer treatment. 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 VEGF inhibitor bevacizumab [116]. Recently Oxaliplatin combined to 5-FU-containing regimens is used to treat colon cancer patients at stage II and stage III [117]. The addition of monoclonal antibodies as bevacizumab

and cetuximab to adjuvant treatment is also under investigation [118, 119]. Targeted treatment with EGFR monoclonal antibodies such as cetuximab is primarily used to treat metastatic disease in CRC patients with wild type *KRAS* [116], however patients with mutation in *KRAS* do not benefit from this approach [120]. Apart from this, many new drugs which target the important signaling pathways in colon cancer are under clinical trials, such as ramucirumab (VEGFR2 blocking antibody) [121], selumetinib (MAPK inhibitor) and MK-2206 (Akt inhibitor) [122].

Despite of these advances, there are many roadblocks that hinder these therapies from fighting against tumor growth. If the primary tumor is detected early and surgically resected, the challenge comes from tumor reappearance. For instance, in 40% of CRC patients at stage II or III, the cancer recurred after primary treatment [123]. The 5 year survival expectancy in CRC patients could rise to as high as 93% if diagnosed in early stage and could fall as low as 8% in late stages. Also, chemotherapies fail to provide a permanent cure in many cases. In CRC, despite significant advances in chemotherapeutic drugs, 89% of patients with metastatic disease die [124]. Controlling the advance of colon cancer therefore remains a major challenge, especially in advanced cancer stages.

2.5 Cancer initiating cells

Over the last decades, continuous efforts have been made to understand the mechanism of cancer development, which could help in designing effective therapies. Despite all the progress and the use of newer therapies, cancer in advanced stages can not be permanently cured. Although currently used chemotherapeutic agents are capable of shrinking tumor mass, it is common to see recurrence [125]. To date, two models are proposed for cancer development; the stochastic model and the hierarchy model (Figure 7). The stochastic model projects tumors as a heterogenous population of cells and suggests that every cell within the tumor possesses similar abilities to initiate and propagate tumors. However, this model fails to explain why tumors targeted from current anticancer therapies re-grow, suggesting that there might be a distinct population of cells which can resist cytotoxicity and permits the repopulation of a tumor. This concept was later established as the “Cancer stem cell (CSC) theory or hierarchy model” which suggests that only a small subset of cells possesses tumor initiating properties.

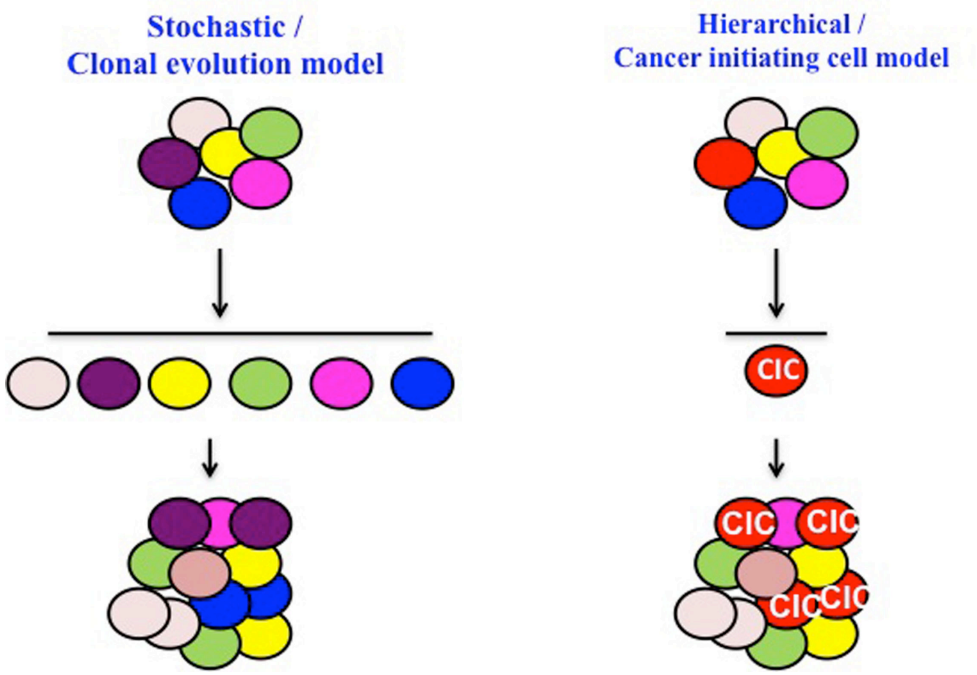

Figure 7. Cancer development model: The stochastic model states that all cells have equivalent capacity to produce tumors. In contrast cancer stem cell theory proposes a clear hierarchy of cells within tumors where only CICs can inititate tumors.

The cancer stem cells (CSCs), displaying typical characteristics of self-renewal and pluripotency, are believed to be accountable for initiating and sustaining tumor growth (Figure 8) owing to their treatment resistant properties [126, 127]. CSCs, also referred to as "tumor initiating," "tumor stem," or "cancer initiating" cells (CICs) [128, 129]. In many cases, these cells consist of only small subsets within the tumor, but have the potential to expand the bulk of the tumor. CSCs could possibly arise from normal stem cells by undergoing mutations in the self renewal genes that make them cancerous. Not only this, committed progenitors cells can also acquire self-renewal capacity through mutations during the process of differentiation and can transform into CSCs. Most of the solid tumors including colon cancer were demonstrated to contain CSCs [130].

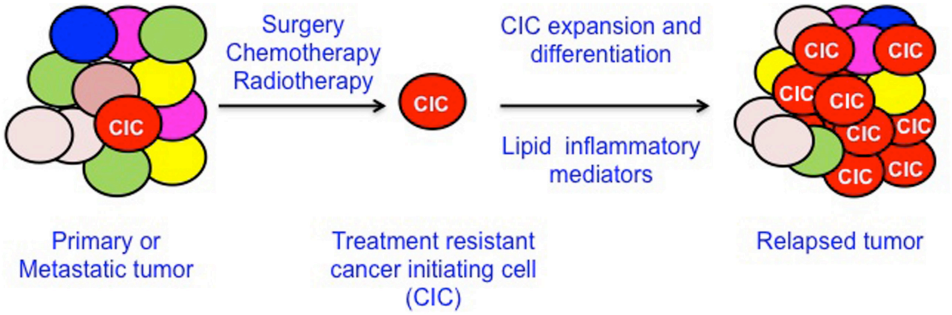

Figure 8. The standard anti-tumor therapies can target the majority of cells. However CICs owing to it treatment resistant properties, will survive. This failure of treatment to target tumor associated CICs could lead to cancer relapse.

The identification of CICs is based on the following unique properties:

1) Self-renewal- CICs supopulation can be serially transplanted for many generations.
2) Differentiation- pluripotent CICs not only form tumorigenic daughter CICs by symmetrical cell division but also generate repertoire of non-tumorigenic cells by asymmetrical cell division.
3) Tumorigenicity- CICs can initiate tumors when transplanted into animals.
4) Specific surface markers- CIC can be isolated from normal cells by specific surface markers.

Therefore, according to the definition and characteristics of CICs, hallmark features can be defined as self-renewal and lineage capacity. It is their ability for self-renewal that enables them to perpetuate in the tumor. Despite their capacity for self-renewal, CSCs have a relatively low proliferative rate. Infact, quiescent cancer initiating cells are known to stay for longer time in the resting phase of cell cycle than normal cells [131]. The conventional chemotherapies mostly targets rapidly proliferating cells with an aim of maximum cell removal, whereas CICs divide less frequently which makes them less susceptible to chemotherapy. CSC subpopulation is also demonstrated to show certain *in vitro* characteristics: (1) they can be separated using stem cell markers (2) they can grow in suspension cultures and form colonies (3) they are resistant to chemo and radiotherapy.

2.5.1 Colon stem cell markers

To date, there are many molecules known which are used to identify CSC subpopulation. The first putative colorectal CSC marker identified was CD133, a pentaspan transmembrane glycoprotein involve in the plasma membrane organization [132]. The cells found positive for CD133 were demonstrated to produce tumors in immunodeficient mice, while cells that did not express CD133 failed to show same [130, 133]. These findings were recapitulated by others [134-136]. Later, CD133 was used with other candidate stem cell markers to detect and separate CSCs, since use of single marker have several limitations [137-139]. Studies have shown that cells isolated on basis of CD133 also express CD44, epithelial specific antigen (EpCAM) and CD166 [16]. Likewise, another group has shown that CD44 separated cells also reflect peculiar stem cell properties under *in vitro* as well as *in vivo* conditions [140]. However there are limitations of using cell surface markers to isolate or identify CSCs. For example, they are not exclusively expressed by CSCs; same markers can also be expressed by non CICs. Moreover, surface markers used to isolate or identify stem cells from a specific tissue are environment dependent, meaning that the marker expression are subjected to change in context to stem cells environment [141]. Thus, the use of surface marker expression alone is insufficient to identify CSCs. Detection of surface markers must be associated with other functional assays, such as the sphere-forming assay in serum-free medium or soft agar medium, detection of enzymatic activity of ALDH1, and measurement of the expression of specific CSC genes to give additional evidences in support of CICs existence. Accordingly, the criteria to detect CSCs population were extended to molecules such as Lgr-5, Wnt activity/β-catenin, ALDH1 and many more (Table 2).

In recent studies, aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme is used to detect colon CICs [142, 143]. The main function of ALDH1 is to catalyze conversion of aldehydes into their corresponding carboxylic acids. Human colon cancer cells having high ALDH levels are demonstrated to initiate tumors in xenograft mice. It was also found that as few as 25 $ALDH^+$ colon cancer cells have an ability to generate tumors in NOD/SCID mice [143].

Table 2. list of candidate cancer inititating cell markers

Marker	Gene	Function
CD133	*PROML1*	Transmembrane glycoprotein Associated with primitive cell
CD24	*CD24*	Cell adhesion molecule p-selectin ligand on tumor cells linked to invitro invasiveness
CD44	*CD44*	Cell surface glycoprotein mediating cell adhesion and migration
CD166	*ALCAM*	Cell adhesion molecule Involved in neuronal extension, embryonic hemopoiesis, embryonic angiogenesis
EpCAM	*EPCAM*	Cell adhesion molecule Linked to Cadherin-Catenin pathway and Wnt pathway
ALDH1A1	*ALDH1A1*	Detoxifying enzyme involved in aldehydes oxidation stem cells differentiation Involved in resistance to chemotherapy (alkylating agents)
ALDH1B1	*ALDH1B1*	Detoxifying enzyme involved in aldehydes oxidation Early differentiation of stem cells
Lgr5	*LGR5*	Associated with intestinal stem cells Downstream target of Wnt pathway
β-catenin	*CTNNB1*	Regulation of cell cycle and proliferation

Furthermore, stem and progenitor cells of various origins are demonstrated to have elevated enzymatic activity of ALDH1. Moreover, high ALDH1 expression was detected in the areas of breast and colon where epithelial progenitor cells reside [144]. Also high ALDH1 expression in breast, pancreas, lung, prostate and bladder cancer patients were found to be positively correlated with overall reduced survival [144]. Similar association of high ALDH level with reduced survival was noticed in ovarian cancer patients. It was further seen that elevated ALDH1 correlates well with higher tumor malignancy [145]. Metastatic colon cancer also contains increased ALDH1B1 expression [146]. Overall, studying the CSCs by identifying them represents a promising method which could pave a path to understand the cancer pathogenesis. In this way, CSCs could emerge as a persuasive target for new therapies.

2.5.2 CICs and drug resistance

CSCs are demonstrated to be relatively resistant to radiation and cytotoxic systemic therapies in many studies [15, 147, 148]. Utilizing CD133 as a CSC marker, it was shown that $CD133^+$ cells are comparatively resistant to 5-FU or oxaliplatin treatments than $CD133^-$ cells [148]. Likewise, CSCs detected as $ESA^+CD44^+CD166^+$ subpopulation were found comparatively insensitive to irinotecan or cyclophosphamide treatments [149]. The resistance to cyclophosphamide was demonstrated to be originated from high ALDH levels in these cells. Accordingly, knockdown of ALDH1 by shRNA or inhibition of its activity by diethylaminobenzaldehyde was shown to increase the sensitivity of CICs to cyclophosphamide treatment, which results in tumor growth inhibition in mice [149]. However irinotecan resistance was not reversed by inhibiting ALDH activity in similar way, which reflects that mechanism of drug resistance in CICs is not common for all drugs. This was the first evidence of the mechanism utilized by CICs to avert drug cytotoxicity and support their survival under unfavourable conditions. Later studies identified that CICs display alterations of DNA repair, due to the presence of cytoprotective properties (including telomerase activation and high expression of antiapoptotic factors) and express high levels of proteins belonging to the ATP-binding cassette (ABC) membrane transporters family, involved in chemotherapeutic resistance [150]. Thus, many tumors may progress because CICs are not sensitive to the treatment. Taken together, it could be inferred that targeting CICs is of great importance in developing any anti-tumor therapy.

2.5.3 CSC-related signaling pathways

The anti-cancer therapies used till date, mostly targets intermediates of signaling pathways involved in cell proliferation, cell death and vascularization of tumors. Considering the prominent role of CICs in cancer particularly relapses, understanding the signaling pathways of CICs could lead to the effective management of cancer. Studying the signaling pathways of CICs could also offer big gains in designing new therapeutic strategies for CRC prevention. In this regard some of the important signaling pathways involved in regulaing CICs functions are Wnt/β-catenin, Notch and Hedgehog (Figure 9). Mutations leading to the constitutive activation of one or more of these pathways are observed in the most aggressive cancers.

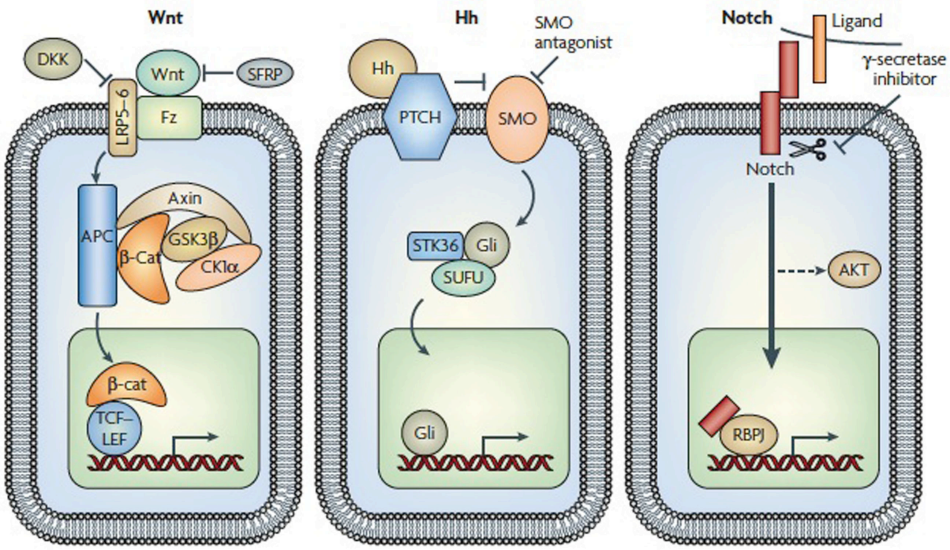

Figure 9. The self renewal pathways and their regulation during stem cell development and neoplastic alterations. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discovery 8, 806-823], copyright (2009)

The Wnt signaling pathway is know to play an important role in the self-renewal property of epithelial stem cells [151-153]. The impairment of this signaling is been shown in many cancer, particularly CRC [154, 155]. It was also reported that Wnt/β-catenin signaling can upregulate the ABC transporter pumps which were implicated in the drug resistance mechanism of CICs to cisplatin and 5-FU [156, 157]. The ABC transporters present on the plasma membrane of cells, functions to protect them from harmful toxins by pumping the agents out of the cells. It was later demonstrated in c-kit^{+} ovarian CICs that β-catenin knockdown can reverse the upregulation of ABCG2 transporter and render the cells susceptible to both cisplatin and paclitaxel, which further substantiates the role of β-catenin in CICs related chemoresistance [158].

Another signaling pathway involved in self-renewal and chemoresistance in CSCs is Notch signaling. Notch activation reportedly regulates many processes of cancer progression and metastasis. Notch signaling is involved in tumor generation, vascularization, mesenchymal transistion of epithelial cells and self renewal of CSCs [159]. Activated Notch signaling is reported in the mice with adenomas [160]. Notch was also linked to the inhibition of Kruppel-like factor 4 (KLF4) which reportedly prevent the colon cancer cells proliferation [161]. KLF4 expression was found to be negatively correlated to adenomatous tumors and carcinomas [162]. The CICs were reported to have 10 to 30 fold higher Notch

signaling than normal colon cancer cells [163]. Notch signaling is also found to be important for CICs survival as knockdown of Notch or its chemical inhibition induces apoptosis [163].

Sonic Hedgehog (Shh) signaling is also involved in the maintenance of CICs functions. Studies have shown that Hh signaling can regulate the self renewal properties of CSCs in different human cancers [164]. Hh signaling also controls the metastatic processes of tumors mostly by regulating CSC functions [165]. Hh and downstream activation of GLI is known to participate in the survival and proliferation of human colon carcinoma. Active Hh-GLI is also shown to be essential for tumor growth and mentainence of $CD133^+$ cells derived from colon epithelial cells [166].

2.6 Seed and soil: Interaction of CSCs and their microenvironment

The stem cell microenvironment or niche is defined as the neighbouring tissue of cancer or normal stem cells where they reside and grow. The signals from microenvironment either to promote or to inhibit the key biological functions such as proliferation and differentiation are crucial for sustaining normal differentiation of cells [167]. Stem cells within a niche usually stay dormant and undergo cell division at much slower pace than normal cells [129]. This is an adaptive mechanism, which ensures the genome stability of stem cells and protects it from harmful mutations. The stem cell niche is hypothesized to provide signals for the maintenance of this dormancy. Quiescent state protects the stem cells from the changes in their genetic material, which would otherwise be harmful for the tissue in which they reside and can lead to tumorous outgrowth. Indeed, changes in the stem cell microenvironment can drive the formation of CICs from stem cell or progenitor cells [168, 169]. Thus fine tuning of proliferative signals was essential to maintain the stem cells in their quiescent state, which permits their self-renewal and tissue regeneration. It is well known that the tumor microenvironment consists of cells of immune system, normal and cancer cells of tissue and stroma containing mesenchymal and endothelial cells [170]. Inflammatory microenvironment populated by immune cells are added as an new trait in the "hallmarks of cancer" [171]. Intriguingly, various inflammatory infiltrates and their products within the tumor microenvironment could also impact the CICs (Figure 10). Therefore, in general the properties of CICs like survival, and maintenance of their self renewal capacity largely depend on the signaling events occurring in their microenvironment. Certainly, the functions and profiles of immune cells in the tumor microenvironment have been reported to influence

tumor progression and clinical outcome in human CRC [172, 173]. Moreover inflammatory lipid mediators like prostaglandins and leukotrienes are demonstrated to stimulate tumor growth through establishing an inflammatory microenvironment [64]. However, researchers have not fully uncovered the pathways and signals, and more particularly to what extent the signals initiated by lipid derived inflammatory mediators could influence CICs properties. Hence, in depth understanding of CICs in context to the remaining tumor cells or other normal tissue-resident stem cells may shed some light for better management of cancer in future.

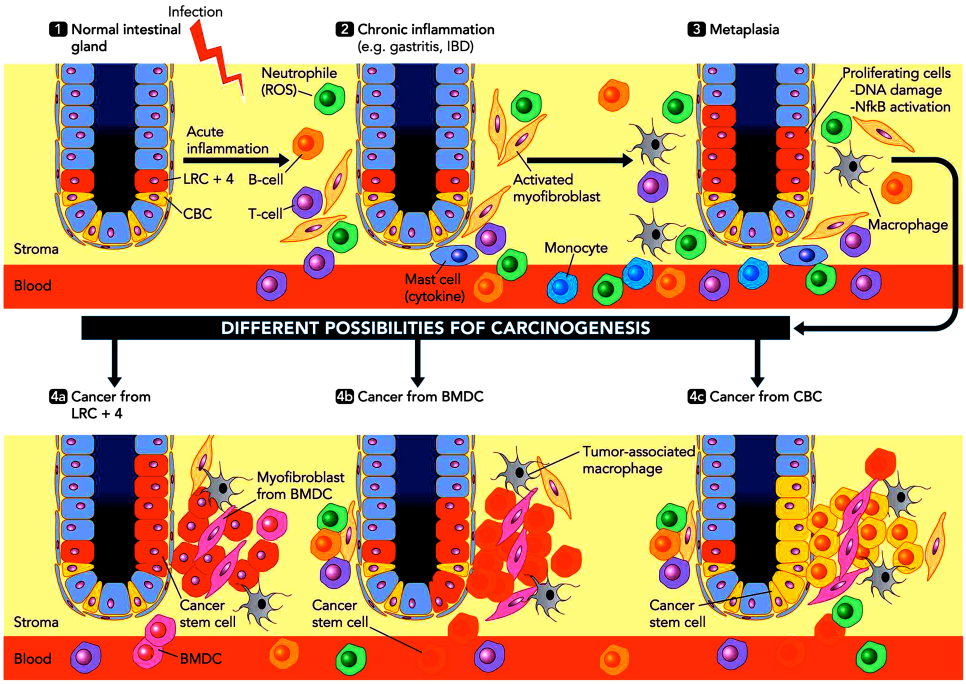

Figure 10. Schematic illustration of the interactions between chronic inflammation and related cancer development. Inflammatory microenvironment consists of variety of inflammatory cells and related mediators. Taken from Quante M and Wang TC, 2009. Nat Rev Gastroenterol Hepatol. 2009;6:724.

3. Present investigations

3.1 Aim

- The main aim of this thesis is to investigate how CICs are regulated by the tumor microenvironment; do inflammatory lipid mediators such as LTD_4 and PGE_2 play a role in this regulation?

- Investigation of CICs role on tumor growth and sensitivity against $CysLT_1R$ antagonist, montelukast.

3.2 Materials and methods

Drugs and antibodies

LTD_4, PGE_2 and monteleukast were purchased from Cayman Chemical Co. Aldefluor (ALDH) kit was from Stem Cell Technologies. Anti human CD326 (EpCAM) MicroBeads was from Miltenyi Biotec. The antibodies used were: Rabbit anti human COX-2 and 5-LOX polyclonal antibodies from Cayman Chemical; anti-mouse F4/80 antibody from AbD serotec; rabbit monoclonal anti-human Ki67 antibody from Thermo Fisher Scientific; rabbit anti human β-catenin, anti-human ALDH antibody and mouse anti-human allophycocyanin (APC)-coupled CD44 antibodies from BD Biosciences; anti-human BCL-2 from Santa Cruz Biotechnology; Mouse anti-human phycoerythrin (PE)- coupled CD133 antibody from Miltenyi Biotec. The Human IgG serum and 5-FU (5-fluorouracil) were procured from Sigma Chemical Co. All other chemicals were of analytical grade and obtained from Chemicon International or Sigma Chemical Co. unless otherwise stated.

Colon cancer cell lines

The colon cancer cell lines HCT-116 (ATCC# CCL- 247), Caco-2 (ATCC# HTB-37), SW-620 (ATCC# CCL- 227), SW-480 (ATCC# CCL-228), and HT-29 (ATCC# HTB-38) were obtained from the American Type Culture Collection (ATCC). HCT-116 and HT-29 were cultured in McCoy's 5A media; Caco-2 in Eagle's minimum essential medium; SW-620 cells in L-15 (Leibovitz); and SW-480 in RPMI-1640, supplemented with 10% fetal bovine serum (v/v), 55 IU/ml of

penicillin, and 55 μg/ml of streptomycin. The cells were grown until 5 d to 70–80% confluence at 37^0C in a humidified atmosphere of 5% CO_2 and regularly tested for mycoplasma.

Flow cytometry cell sorting

ALDH, CD133, and CD44 positive and negative subpopulations were analyzed in HCT- 116, Caco-2, SW-620, SW-480, and HT-29 colon cancer cell lines. $ALDH^+$ cells were sorted using Aldefluor kit whereas CD133 and CD44 were sorted using specific antibodies conjugated to phycoerythrine (PE) or allophycocyanin (APC) respectively. For analysis of CD45, single-cell suspensions of tumors were obtained using gentleMACS dissociator and stained with anti-mouse CD45-FITC antibody. LTD_4 (80 nM) or PGE_2 (100 nM) was used to stimulate the FACs sorted $ALDH^+$ and parental cells from HTC-116 and Caco-2 cell lines. 5-FU was used at 5 or 10 μg/ml for 5 days with change of media every three days. Live and dead cells were counted using a cell counter. All flow cytometric measurement was performed using the FACS Calibur. The analysis was performed using the Summit v4.6.

Soft agar colony formation assay

Soft agar assay was performed using conventional protocol, on the FACS-sorted HCT-116 and Caco-2-derived $ALDH^+$ cells seeded at the density of 5 x 10^3 cells/well and incubated with different drugs. The drugs used were: LTD_4 (80 nM); PGE_2 (100 nM), Montelukast (10 μM) and AH6809 (10 μM). In separate experiments, 1 x 10^4 HT-29 and SW-480 cells were seeded/ well to examine their colony formation capacity. The colonies were observed on 21 d under an inverted light microscope using 20X objective. The numbers of colonies larger than 50 μm were counted in each well.

Quantitative real time polymerase chain reaction (qPCR)

RNeasy Plus Mini kit (Qiagen) was used to extract RNA that was subsequently used for cDNA synthesis using RevertAid H Minus M-MuLV Reverse Transcriptase (Thermo Scientific). Maxima probe/ROX qPCR master mix and TaqMan gene expression assays were used for amplification in Mx3005P thermocycler. The comparative Ct method was used to determine the relative quantification of the gene expression levels. The expression levels of the different genes of interest were normalized to the housekeeping gene HPRT1 and analyzed with MxPro software (Invitrogen).

Radiation

A total of 3 x 10^5 HCT-116 and Caco-2 cells were grown per 60 mm dish for 3 days until they reached 80 % confluence, and then exposed to gamma radiation

using Gammacell 40 Exactor at 4 Gy and 8 Gy. The irradiated cell media was collected at 0 and 24 hours time points for PGE_2 measurement. In separate experiments, cells irradiated with same intensities were further processed for ALDH sorting for implantation in nude mice for tumor growth assessment.

Xenograft model

5–6 weeks old female nude mice (BalbC nu/nu) used in this study were purchased from Taconic Europe A/S. The Regional Ethical Committee for Animal Research at Lund University, Sweden approved all animal experiments. To induce subcutaneous human colon cancer xenografts, 1 x 10^4 $ALDH^+$ HCT-116 cells were inoculated subcutaneously into the flanks of recipient mice. Tumor development was monitored by palpation. Time to onset of a palpable tumor was recorded, and the tumor size was measured every three days using digital vernier caliper. Once palpable tumors were established, the mice were randomly divided into groups treated with vehicle, LTD_4 or PGE_2. The mice received daily subcutaneous injections of ethanol as vehicle, LTD_4 or PGE_2 at the dose of 10 µM. Tumor growth and tumor volume were monitored and estimated every third day. Tumor volumes were calculated according to the formula (length x $width^2$)/2. After 48 d, all mice were sacrificed, and the tumors removed, measured, weighed, and photographed. Tumor tissues were fixed in 10% buffered formalin, embedded in paraffin for immunohistochemistry analysis and/or processed further for tissue dissociation immediately for FACS analysis. For radiation study, 1 x 10^4 $ALDH^+$ HCT-116 cells irradiated at 4 or 8 Gy were injected subcutaneously into both the flanks of mice and monitored for tumor growth as described above. The mice in this group were studied for 60 days due to late onset of tumors in these groups. In paper III, 2.5 x 10^6 SW-480 or HT-29 cells were used to generate xenograft in 6- to 8-week-old athymic nude mice (BalbC nu/nu) following same procedure as described above. However in these experiments the mice received daily subcutaneous injections of DMSO as vehicle or montelukast (5mg/kg). After 21 days, mice were sacrificed, and tumors removed, measured, weighed, and photographed. Tumor tissues were fixed in 10% buffered formalin, embedded in paraffin for immunohistochemistry analysis and/or snap frozen in liquid nitrogen, and stored at -80^0C for qPCR.

Immunohistochemistry

Immunohistochemical staining was performed on 5 µm paraffin-embedded tumor sections using a dako automatic slide strainer, according to the manufacturer's instructions. The sections were stained with specific antibodies against protein of interest and developed using DAB solution. Tissues were counterstained with hematoxylin. The slides were photographed with a Nikon Eclipse 800 microscope and evaluated in a blinded fashion by two observers independently. The

immunoreactivity of β-catenin, COX-2, Ki67, 5-LOX, BCL-2, F4/80 and ALDH proteins in the tumor cells was determined based on following procedure. Briefly, staining intensity was scored as 0 (negative), 1 (very weak), 2 (week), 4 (medium) and 6 (strong). Extent of staining was scored as 0 (0%), 0.5 (1-5), 1 (6%-10%), 2 (11%-20%), 3 (21%-30%), 4 (31%-40%), 5 (41%-50%), 6 (51%-60%), 7 (61%-70%), 8 (71%-80%), 9 (81%-90%) and 10 (91%-100%) according to the percentage of positive staining area in relation to the whole carcinoma area. Then, the sum of intensity and extent score was calculated as the final staining scores for COX-2, Ki67, 5-LOX, F4/80 and ALDH proteins whereas β-catenin scores reflect only percent staining extent.

Cysteinyl leukotrienes (CysLTs) and PGE_2 by ELISA

CysLTs and PGE_2 levels were measured in cell media for *in vitro* experiments, whereas plasma samples were used for *in vivo* study with xenograft mice. Enzyme immunoassay (EIA) was performed according to the manufacturer's specifications. In both cases, the samples were purified prior to measurement by solid phase extraction through Sep-Pak Vac RC (C-18) cartridges (Water Corporation) applying manufacturer's guidelines.

Statistical analyses

Statistical analyses were performed with Prism 5 (GraphPad Software, Inc., San Diego, CA). Results are expressed as the mean ± SEM. All comparisons between mean values were performed by use of either one-way analysis of variance (ANOVA) with Newman-keuls post hoc test or two-way ANOVA or with student's t test wherever applied. P values of <0.05 were considered significant.

3.3 Results and discussion

3.3.1 The impact of inflammatory lipid mediators on colon cancer initiating cells (Paper I)

CRC is the leading cause of cancer and cancer related death worldwide [24]. Various factors have been suggested to promote colon carcinogenesis, inflammation being one of them. The presence of some inflammatory conditions near to the primary tumor site is indicated to initiate or encourage colorectal cancer [4, 26]. IBD is the best example of such relation where longstanding inflammation inflicts a high risk of CRC development [36, 37]. Moreover NSAIDs mediated long-term survival of cancer patients further strengthens the importance of inflammation in cancer progression [13].

Current therapies are able to reduce the tumor size but fail to cure the advance cancer stages and often lagged by tumor relapses. In this context, CICs concept seems relevant since these cells are highly chemo and radioresistant [174-176]. Considering the cancer stem cell paradigm, we hypothesize that cancer stem cell concept is also applicable to CRC and initiation of cancer stem cells are guided by its surrounding inflammatory microenvironment.

The long-standing problem to study this is to identify and isolate colonic stem cells due to lack of specific marker. In our study we clearly showed that ALDH is more specific marker for stemness compared to CD133 and CD44. The $ALDH^+$ cells apparently exhibit higher efficiency to produce colonies. Interestingly, we found that LTD_4 or PGE_2 stimulations further enriched the $ALDH^+$ populations, signifying their implication in CICs maturation. In addition, the colony assay data showed that inflammatory lipid mediators support the stem cells growth, as $ALDH^+$ cells produced more number of colonies in their presence. Furthermore, treatment with montelukast ($CysLT_1R$ antagonist) or AH6809 (PGE_2 receptor EP1, 2, 3 antagonist) significantly blocked the LTD_4 or PGE_2 induced colony formation from $ALDH^+$ cells. The data from xenograft mice supported our above results by demonstrating an increased tumor growth in LTD_4 or PGE_2 treated mice. Further we found that $ALDH^+$ cells were significantly resistant to 5-FU drug treatment compared to $ALDH^-$ cells, ascertaining our previous data that ALDH sorted cells efficiently presents the CICs with their characteristic self-renewing and chemoresistant ability. Moreover the impact of inflammatory lipid mediators on the self-renewal mechanism of CICs was evident by increased expression of genes that confer survival and self-renewal to CICs such as *GLI1, KLF4, BCL-2* and *ALDH1B1*. Consistent with reported radioresistance property of CICs, we found that $ALDH^+$ cells were highly resistant to radiation exposure compared to $ALDH^-$

cells, concurrent with increased PGE_2 release. Conclusively, our study reports ALDH marked cells represent effectively the CICs population in colon cancer cell lines and can be exploited to study their functional role in tumor generation and progression in CRC. We also established here that signals derived from inflammatory lipid mediators can augment the stemness of $ALDH^+$ cells, supporting the concepts that inflammatory microenvironment of tumor site can initiate signals that could change the behavior of stem cells to acquire cancer properties.

3.3.2 Eicosanoids leukotriene D_4 and prostaglandin E_2 promote tumorigenicity of colon cancer initiating cells in a xenograft mouse model (Paper II)

In our earlier study, we showed that inflammatory lipid mediators, which are abundantly present in tumor microenvironment, could stimulate CICs properties. However through which mechanism CICs contributes to cancer initiation and progression remain to be elucidated. In this study we illustrate that inflammatory mediators LTD_4 and PGE_2 promotes aberrant tumor growth in mice injected with ALDH marked CICs. CICs isolated on basis of high ALDH levels are demonstrated to improve colony forming capacity of colon cancer cells, as well as tumor growth in mice [177]. Prostaglandins and leukotrienes affect stem cell characteristics by multiple mechanisms. For example, PGE_2 improves mouse embryonic stem cells survival by inhibiting apoptosis via EP2–PI3K–Akt pathway [178]. It is also known to stimulate embryonic haematopoietic stem cell growth and development [179]. Similarly, LTB_4 and LTD_4 could induce stem and progenitor cells proliferation [180, 181]. Accordingly, we observed moderately increased percentage of Ki67 stained cells within tumors treated with LTD_4 or PGE_2 indicative of improved proliferation. Further we detected significant increase in COX-2 protein levels within tumors treated with LTD_4 or PGE_2. Functionally COX-2 overexpression is linked to phenotypic changes in cells such as resistance to apoptosis and increased proliferation, factors which could enhance the tumorigenisis [182, 183]. Our finding of high COX-2 expression paralleled with increased Ki67 stained cells in tumors after LTD_4 or PGE_2 treatment are consistent to these reports and highlights the involvement of COX-2 in the tumorigenic potential of CICs.

Moreover, we observed high percentage of β-catenin staining in cytoplasm and nuclear fractions of LTD_4 or PGE_2 treated tumors. The increased β-catenin levels in cytoplasm can cause increased nuclear translocation of this protein where it activates transcriptional factors like TCF/LEF [30]. These factors can amplify the transcription of genes that are involved in the maintenance of cell cycle and

proliferation like *cyclin D1, c-myc, and COX-2* [111, 113]. Taken together, our data suggest that inflammatory mediators encourage CICs evoked tumor growth possibly by stimulating β-catenin signaling, and concurrent upregulation of COX-2 and proliferation.

Leukotrienes also plays an importance role in inflammatory processes such as leukocyte chemoattraction, particularly of granulocytes and T cells, induction of rapid invasion and recruitment of these cells to the plasma membrane of endothelial cells [69, 184]. In agreement to this, we have noted that LTD_4 or PGE_2 treatment intensified the $CD45^+$ inflammatory cell accumulation in the tumors, with concurrent enrichment of $ALDH^+$ cell fractions. The tumors are also associated with high levels of macrophages, detected with F4/80 staining. Macrophages are demonstarted to release wide range of cytokines and eicosanoids like prostaglandins and leukotrienes [185]. We have also found that LTD_4 or PGE_2 treated mice have high circulating levels of CysLTs and PGE_2 and certain cytokines like IL-6, IL-1β, IL-2, TNF-α and CXCL1/KC/GRO which probably relate to intense macrophage accumulation in tumors. Further we noted that injection of $ALDH^+$ cells isolated from irradiated colon cells have a high potential to initiate tumor compared to equal number of irradiated parental cells, which were unable to produce tumors. Our result provides a strong support to the old notion that CICs subset possesses an inherent ability to withstand radiation therapy and can initiate tumor growth.

In conclusion, our study proposed the important role of LTD_4 and PGE_2 in regulation of CICs in colon cancer. This requires proper attention, as future studies must be designed to target not only CICs, but also associated inflammatory lipid mediators for development of effective therapies for colon cancer.

3.3.3 Role of colon cancer initiating cells in tumor malignancy and insensitivity against montelukast in xenograft model (Paper III)

Chemotherapy resistance is one of the major problems faced with currently used cancer treatment strategies. There is a great need to find the possible mechanism of drug resistance in tumors, an area that has recently attracted much attention. In earlier study, we have explored the potential of $CysLT_1R$ antagonist, montelukast as a potential alternative therapy against colon cancer [186]. However in this study, we observed some tumors with increased tumor size than others which did not respond to montelukast treatment. We take this opportunity to investigate the possible factors that might contribute to tumor insensitivity to montelukast. Keeping in view the proposed role of CICs in chemoresistance [15, 187], we focused mainly on the factors related to CICs in these tumors.

In this study, we found that big tumors had markedly enhanced expression of genes related to stemness in both DMSO and montelukast administered groups. The genes that were found upregulated consist of detoxifying enzyme aldehyde dehydrogenase, *ALDH1A1 and ALDH1B1;* transcription factors like *GLI1* and *KLF4*. These genes are typically associates with stem cell related hedgehog and Notch signaling and could be considered as readouts for CICs content in tumors. In addition we observed significantly increased BCL-2 protein levels in DMSO and montelukast treated big tumors compared to small tumors. Studies on CICs suggest that BCL-2 can modulate their chemoresistance property by inducing signaling pathways involved in the CIC survival. For instance, IL-4 inhibition in $CD133^+$ colon CICs demonstrated lowering of BCL-XL, coupled to the increased oxaliplatin and 5-FU sensitivity [188]. Further, we found increased nuclear β-catenin accumulation in big tumors from both DMSO and montelukast treated groups from SW-480 colon cancer cells, which reflects activated Wnt signaling in these tumors. Wnt/β-catenin signaling pathway is demonstrated earlier to confer chemoresistance against 5-FU [156, 157]. Interstingly, β-catenin siRNA knockdown is demonstrated to reverse chemoresistance to both cisplatin and paclitaxel [158]. While the mechanism through which Wnt mediates chemoresistance is not completely clear, one possibility is upregulated ABC membrane transporters.

We also found increased number of macrophages in the tumors that were bigger in tumor size than others. Macrophages, which are important component of stroma, are known to affect CICs properties in various manners. In addition, we noticed enhanced ALDH protein expression in big tumors compared to small, which supported to our gene expression findings.

Taken together, we observed that tumor sensitivity to given treatment is impacted majorly by increased CICs ALDH level, activation of BCL-2 and β-catenine pathway, which may confer the drug resistance. Thus it could be inferred here that variation in CICs biology in tumors could largely affect their growth pattern under given circumstances. Hence CICs inhibition may prove to be instrumental in optimizing drug failures and related relapses.

4. Summary

- Our study proposed the role of LTD_4 and PGE_2 in the regulation of CICs in colon cancer progression by creating a change in tumor environment, which eventually supports cell survival, proliferation and stemness.
- The variation in CICs content and its related signaling can largely affect the tumor growth and sensitivity to given drug.

5. Popularized summary

Colorectal cancer (CRC) is referred to as the cancer of colon and rectum. Millions of people develop CRC per year worldwide; however the risk is more in the patients with inflammatory bowel disease (IBD), contributing significantly to total CRC cases. The fact that IBD could lead to CRC progression indicates that inflammation could be an important parameter in deciding colon carcinogenesis. Many studies have also shown that tumors are infiltrated by host immune cells that enforce a massive inflammatory response with a motive to inhibit any abnormal growth. However, due to natural selection process, certain clones develop which can endure inflammation. Thus, inflammation actually provides signals for the development of the cancer cells, with better survivability. The cancer theories, however, suggest that, within the heterogeneous population of tumor cells, certain cells are present at apex in hierarchy and are accountable for tumor initiation. These cells, termed as cancer initiating cells (CICs), can self-renew and give rise to a whole repertoire of cells just like any other stem cells. However unlike normal stem cells they can give rise to new tumors. The tumorigenicity in these cells is presumed to be stemmed from accumulated mutations over the extended period. Further these cells are found to be resistannt to chemo and radiotherapy.

In our study we focused on the cancer initiating cells with an aim to know the processes that can regulate them. To this end, we examined whether eicosanoids, LTD_4 or PGE_2, presumably present in tumor microenvironment, have any role. We report that ALDH, a detoxifying enzyme present in high level in CICs offers a relatively better tool to isolate CICs. CICs isolated on this basis represented all *in vitro* CICs characteristics i.e; can give rise to colonies and are chemo and radioresistant. Further, we found that LTD_4 or PGE_2 improved their colony forming ability and survivability against chemo or radiotherapy. The CICs tumorigenicity, tested in immunodeficient mice also increased manifold in LTD_4 or PGE_2 presence. We detected noticeable increase in the inflammatory cells numbers in tumors treated with LTD_4 or PGE_2. We also found high levels of macrophages in tumors treated with LTD_4 and PGE_2. In addition we showed that CICs-evoked tumor contain more proliferative cells, coupled with high COX-2 levels and activated β-catenin signaling when treated with LTD_4 or PGE_2. The plasma levels of CysLTs, PGE_2 and cytokines like IL-6, IL-1β, IL-2, TNF-α and CXCL1/KC/GRO were also significantly increased upon LTD_4 or PGE_2 treatments in mice. These results reflect that eicosanoids play an important role in regulation of CICs, perhaps by allowing the changes in tumor microenvironment

that supports their growth. COX-2 and β-catenin pathways apparently contribute to LTD_4 or PGE_2 induced effects on CICs.

The chemoresistance mechanism of CICs ensures their survivability against challenging environment and makes them accountable for the failure of current therapies to cure tumor in advanced stages. $CysLT_1R$ is related to the colon carcinogenesis and represents a therapeutic target. Inhibition of $CysLT_1R$ through its antagonist is shown to prevent tumor development in xenograft mice, even though some tumors were apparently insensitive to given treatment. In this context we studied the tumors that did not respond to montelukast treatment. We observed that unresponsive tumors grow bigger compared to others and differ significantly in their CICs content and related signaling pathways compared to other small tumors. Bigger tumors expressed high mRNA and protein levels of ALDH, BCL-2 protein and had activated Wnt signaling. In addition, there was an increase in macrophage numbers in big tumors compared to small tumors. All these factors could support the tumor growth and resistance against drug-mediated cytotoxicity under given circumstances. Therefore, tumor growth and responsiveness to given drugs could vary with the CICs content. Thus targeting the CICs could be useful in designing new therapies for better management of cancer relapses and related drug failures.

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