CysLT1 receptor signaling and the tumor microenvironment in colon cancer models

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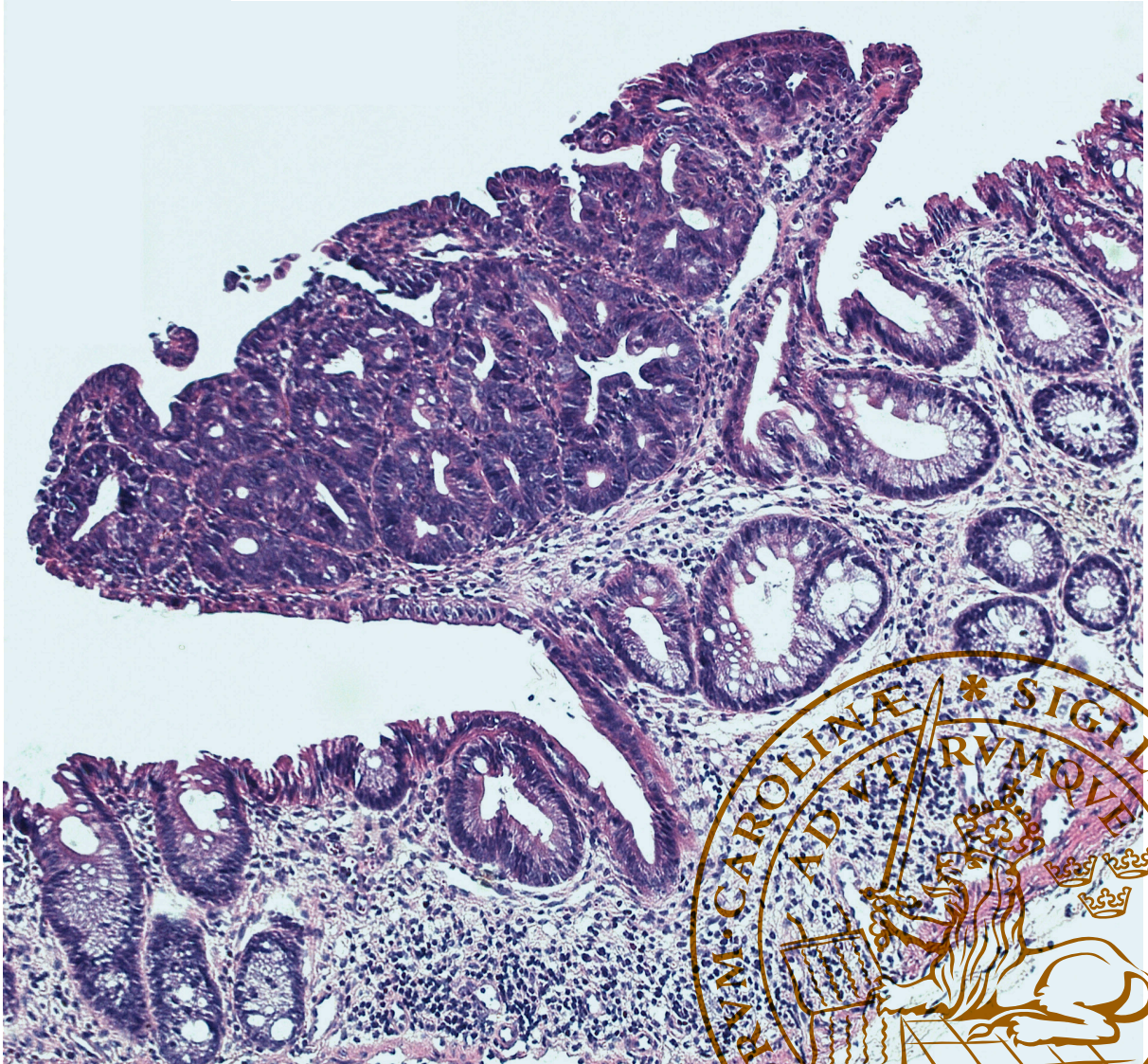

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About the thesis and the author

Janina Osman has a BSc in Pharmacology from University College London and an MSc in Biomedicine from Lund University. The focus of her doctoral thesis is to investigate the role of the cysteinyl leukotriene receptor 1, immune cells and cancer stem cells in the tumor microenvironment of colorectal cancer models, with the aim to prevent and treat colorectal cancer.

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Cysteinyl leukotriene receptor 1 signaling and the tumor microenvironment in colon cancer models

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LUND
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Abstract

Colorectal cancer (CRC) is one of the most common types of cancers in the world with increasing number of deaths, despite new therapies and the main cause of CRC death being due to distant metastasis. Inflammatory lipid mediators, such as prostaglandins and leukotrienes have been implicated in CRC development. Higher tumor expression of the cysteinyl leukotriene D4 receptor ($CysLT_1R$) has been associated with poorer prognosis in CRC patients. Cancer stem(-like) cells (CSCs) have the ability to self-renew and form tumors. These cells are believed to be resistant to therapy and responsible for cancer relapse. Foxy5, the wingless-type mammary tumor virus integration site 5A (WNT5A) agonist, has been shown to be involved in regulating colon cancer stem cells (CSCs).

The present thesis investigated the role of $CysLT_1R$ signaling and the tumor microenvironment in models of colon cancer tumorigenesis also considering CICs and CSCs in xenograft mouse models.

In the spontaneous colon cancer model, $Apc^{Min/+}$ mice without a functional CysLT1R ($Apc^{Min/+}$ $CysLT1R^{-/-}$) exhibited a reduced tumor burden in the small intestine of female but not male mice compared to $Apc^{Min/+}$ with functional CysLT1R. In a colitis-associated colon cancer model, female mice without a functional $CysLT_1R$ exhibited reduced tumor burden in the colon. Absence of CysLT1R expression resulted in tumors expressing decreased nuclear β-catenin and increased membranous β-catenin, with decreased COX-2 expression. Furthermore, in a xenograft mouse model, HCT-116 cells expressing high enzymatic activity of aldehyde dehydrogenase ($ALDH^+$) resulted in increased tumor expression of CSCs markers (ALDH and DCLK1) and increased nuclear β-catenin and COX-2, followed by increased tumor infiltration of M2-type macrophages (CD206+). In another xenograft model, Foxy5 (a WNT5A mimicking peptide) treatment was administered to HT29 and Caco2 tumors, which exhibited a decreased expression of CSC markers (ALDH and DCLK1). Foxy5 also reduced the expression of nuclear β-catenin and its downstream target Achaete Scute complex homolog 2, a transcription factor preserving CSCs. Finally, in a metastatic mouse model of colon cancer, mice without a functional CysLT1R exhibited the lowest incidence of liver metastasis followed by wildtype mice treated with montelukast and untreated wildtype mice exhibited the highest metastatic incidence. In a zebrafish model of metastasis LTD_4 CysLT1R signaling increased HT29 metastasis, which was inhibited by Montelukast treatment or in HT29 transfected cells (*CRISPR-Cas9 CYSLTR1).*

To conclude, LTD_4-CysLT1R signaling and downstream targets promote tumor progression in colon cancer models, while lack of CysLT1R signaling by gene knockout or montelukast inhibition decreases liver metastatic incidence. Furthermore, CSC expression was promoted by LTD_4 and PGE_2 but inhibited by Foxy5. These results indicate promising therapeutic opportunities by inhibiting CysLT1R signaling and inducing WNT5A signaling by Foxy5.

Key words: Colorectal cancer, Cysteinyl leukotriene receptor 1, Leukotriene D_4, Prostagalndin E_2, β-catenin, Cyclooxygenase-2, Cancer stem-like cells, Aldehyde dehydrogenase, DCLK1, WNT5A, Foxy5

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Neoplastic precursor aberrant crypt foci in the colon.

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To my family

"One, remember to look up at the stars and not down at your feet. Two, never give up work. It gives you purpose and meaning and life is empty without it. Three, if you are lucky enough to find love, remember it is there and don't throw it away."

Stephen Hawking

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List of papers included in this thesis

I. Savari S, Chandrashekar NK*, **Osman J***, Douglas D, Bellamkonda K, Jönsson G, Juhas M, Greicius G, Pettersson S, Sjölander A. 'Cysteinyl leukotriene 1 receptor influences intestinal polyp incidence in a gender-specific manner in the Apc Min/+ mouse model', *Carcinogenesis*, 2016;37(5): 491–499. doi: 10.1093/carcin/bgw031

II. **Osman J***, Savari S*, Chandrashekar NK, Bellamkonda K, Douglas D, Sjölander A. 'Cysteinyl leukotriene receptor 1 facilitates tumorigenesis in a mouse model of colitis-associated colon cancer'. *Oncotarget*. 2017;8(21):34773–34786. doi:10.18632/oncotarget.16718

III. Bellamkonda K, Chandrashekar NK, **Osman J**, Selvanesan BC, Savari S, Sjölander A. 'The eicosanoids leukotriene D4 and prostaglandin E2 promote the tumorigenicity of colon cancer-initiating cells in a xenograft mouse model'. *BMC Cancer*. 2016 July;16:425. doi:10.1186/s12885-016-2466-z

IV. **Osman J**, Bellamkonda K, Liu Q, Andersson T, Sjölander A. 'The WNT5A Agonist Foxy5 Reduces the Number of Colonic Cancer Stem Cells in a Xenograft Mouse Model of Human Colonic Cancer'. *Anticancer research*. 2019;39(4):1719-1728. https://doi.org/10.21873/anticanres.13278

V. **Osman J**, Satapati SR*, Topi G*, Sjölander A. 'The role of CysLT1R signaling in colon cancer metastasis' *(Manuscript)*

*these authors contributed equally

Abbreviations

ACS	American Cancer Society
ALDH	Aldehyde dehydrogenase
AMPs	Antimicrobial peptides
AOM	Azoxymethane
APC	Adenomatous polyposis coli
ASCL2	Achaete Scute homolog 2
BrdU	Bromodeoxyuridine
CAC	Colitis-associated colon cancer
cAMP	cyclic adenosine 3', 5'-monophosphate
CD	Crohn's disease
CIC	Cancer initiating cell
CK1	casein kinase 1
CMS	Consensus molecular subtypes
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
CRC	Colorectal cancer
CSC	Cancer stem cell
CysLT	Cysteinyl leukotriene
CysLT1R	Cysteinyl leukotriene receptor 1
CysLT2R	Cysteinyl leukotriene receptor 2
DCC	Deleted in colon cancer
DCLK1	Doublecortin-like kinase 1
DPC4	Deleted in pancreatic cancer 4
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulphate (salt)
EP2	Prostaglandin E receptor 2
EP4	Prostaglandin E receptor 4

FAP	Familial adenomatous polyposis
FLAP	Five lipoxygenase activating protein
GPCRs	G-protein coupled receptors
GSK3β	glycogen synthase kinase-3
HNPCC	Hereditary nonpolyposis colorectal cancer
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocytes
IL	Interleukin
KRAS	Kirsten rat sarcoma virus
LOX	Lipoxygenase
LTD_4	Leukotriene D4
Min	Murine intestinal neoplasia
MK	Montelukast
MMR	Mismatch repair
MSH	DNA mismatch repair protein
MSI	Microsatellite instability
MUC2	Mucin 2
NK cell	Natural killer cell
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PMS2	PMS1 homolog 2, mismatch repair system component
TAM	Tumor associated macrophages
Th1	T helper 1
TME	Tumor microenvironment
TNM	Tumor, lymph node, metastasis
TP53	Tumor protein 53
TSG	Tumor suppressor gene
UC	Ulcerative colitis
WHO	World Health Organization
Wnt	Wingless-type mammary tumor virus integration site

Populärvetenskaplig sammanfattning

Cancer i tjocktarmen, också känd som koloncancer, är den tredje vanligaste cancerformen och den fjärde vanligaste orsaken till cancerrelaterade dödsfall i världen. Patienter med ulcerativ kolit eller Crohns sjukdom, som präglas av kronisk inflammation i tarmen, har en ökad risk för att utveckla koloncancer. Personer med en viss nedärvd mutation i genen *APC* har en större risk för att utveckla koloncancer. *APC* genen är också inaktiverad i många fall av sporadisk koloncancer. Tumörens mikromiljö består av immunceller som frisätter viktiga inflammatoriska molekyler däribland leukotriener (LTD_4) och prostaglandiner (PGE_2). Inflammation orsakad av leukotriener (LTD_4) kan stimulera tillväxt samt migration av epitelceller vilket kan leda till tumörutveckling. Koloncancer patienter med högt uttryck av den pro-inflammatoriska cysteinyl leukotrien 1 receptorn, $CysLT_1R$, hade en sämre prognos. Montelukast, ett läkemedel som blockerar $CysLT_1R$, har visat sig hindra tumörtillväxt i en musmodell av koloncancer. I nuläget är det viktigt att hitta säkrare behandlingsmetoder för cancerpatienter.

Vi har tidigare visat att tumörer minskar i tillväxten när $CysLT_1R$ hämmas i immunförsvagade möss med humana koloncancerceller. Tumörens förmåga att växa och sprida sig beror på tumörmikromiljön och signalerna mellan tumörcellerna och närliggande immunceller, cancerstamceller och andra komponenter. Molekylen Foxy5 har visats vara inblandad i regleringen av cancerstamceller. I detta arbete valde vi att studera hur $CysLT_1R$ påverkar tumörbildning och spridning i olika modeller för koloncancer. Vi undersökte även hur markörer för olika cancerstamceller påverkas av LTD_4, PGE_2 samt Foxy5.

Arbete I, beskriver hur vi använde oss av möss med en mutation i Apc-genen, som spontant bildar tumörer i framförallt tunntarmen. Vi kunde se minskad tumörbildning hos honor men inte hos hanar som fattades $CysLT_1R$. Avsaknaden av $CysLT_1R$ resulterade i minskat aktivt β-catenin och förhöjt inaktivt β-catenin, med minskat uttryck för enzymen som producerar prostaglandiner, COX-2. Dessutom kunde vi fastställa en minskad generell inflammation.

Arbete II, beskriver våra studier av en kemiskt inducerad modell för kolit-associerad tjocktarmscancer i möss. Vi kunde observera att möss som saknade $CysLT_1R$ hade en minskad tumörbörda. Som i paper I, kunde vi se att avsaknaden

av $CysLT_1R$ resulterade i minskat aktivt β-catenin, förhöjt inaktivt β-catenin, med minskat COX-2.

Arbete III, beskriver möss med implanterade koloncancerceller som har högt uttryck av enzymen ALDH, som ses som en markör för cancerstamceller. Möss behandlade med LTD_4 eller PGE_2, utvecklade store tumörer med högre uttryck av markörerna för cancerstamceller (ALDH and DCLK1). Tumörer behandlade med LTD_4 eller PGE_2 hade högre aktivt β-catenin och COX-2, följd av en ökad närvaro av immunceller (CD206+ makrofager) som är associerade med att hjälpa tumörer.

Arbete IV, beskriver möss med implanterade koloncancerceller som behandlades med Foxy5 (en WNT5A molekyl), vilken resulterade I ett minskat uttryck av markörer för cancerstamcellerna (ALDH and DCLK1). Foxy5 minskade även uttrycket av aktiv β-catenin samt proteinet ASCL2 som bevarar cancerstamceller.

Arbete V, beskriver modeller av koloncancermetastaser. I en metastasmodell i möss observerade vi färre möss med metastaser om de saknade $CysLT_1R$, som även var mindre i storlek. I en metastasmodell med zebrafiskar observerade vi att LTD_4 via $CysLT_1R$ ökade metastaser och att montelukast behandling eller $CysLT_1R$ inaktivering visade på minskade metastaser. Hos patienter med koloncancer, var högt uttryck av $CysLT_1R$ i tumörer associerat med metastaser.

Sammanfattningsvis, LTD_4-$CysLT_1R$ signaler hjälper tumörtillväxt i modeller av koloncancer, medan avsaknad av $CysLT_1R$ signaler genom genetisk inaktivering eller montelukast behandling minskade incidenser av levermetastaser. Uttryck av cancerstamceller ökade i samband med LTD_4 eller PGE_2 behandling eller minskade med Foxy5 behandling. Dessa resultat tyder på nya lovande behandlingar för koloncancerpatienter genom att hindra $CysLT_1R$ signaler och att framkalla WNT5A signaler med Foxy5. Dessa fem arbeten ökar vår kunskap inom hur olika komponenter i tumörmiljön påverkar utvecklingen av koloncancer.

Popular scientific summary

Cancer is a tumor that is malignant and consists of abnormal cells that grow without limit and can spread to other parts of the body. Cancer in the large intestine is called colorectal cancer and this type of cancer is one of the most common in the world with increasing number of deaths in recent years. The risk of developing colorectal cancer is due to hereditary mutations, early diagnosis of inflammatory bowel disease, older age and an unhealthy lifestyle, which includes obesity, lack of physical activity, excessive smoking and drinking with a diet lacking in fiber. Despite new therapies, it is still one of the most common causes of death due to cancer. The main cause of death due to colorectal cancer is distant metastasis, which is cancer spreading to other organs and tissues. Patients with inflammatory bowel disease have a higher risk of developing colorectal cancer due to episodes of chronic inflammation in the digestive tract. Also, people with a certain hereditary mutation to a protective gene termed *adenomatous polyposis coli* (abbreviated with *APC*) also have a higher risk of developing colorectal cancer. The *APC* gene is also mutated in many cases of non-inherited, i.e. sporadic, colorectal cancer. When this protective gene is lost in cells, it allows cells to grow in an unlimited fashion by activating other favorable genes via the protein, β-catenin.

Tumors consist of other components besides tumor cells, such as immune cells and blood vessels. All of these components are termed the tumor microenvironment and can aid in tumor growth and spreading. A minor component of the tumor microenvironment is cells that are responsible for initiating tumors with properties of stem cells, sometimes referred to as cancer initiating cells (CICs) or cancer stem cells (CSCs). These cells can renew themselves and are believed to be responsible for cancer recurrence, as they are believed to be resistant to cancer therapy. A molecule Foxy5, the wingless-type mammary tumor virus integration site 5A (WNT5A) agonist, has been shown to be involved in regulating colon cancer stem cells.

Inflammatory molecules such as prostaglandins and leukotrienes are mainly released by immune cells, and have been linked with progression of colorectal cancer. In patients with colorectal cancer, a worse outcome, in terms of survival, was observed if the tumor strongly expressed a specific protein, termed cysteinyl leukotriene receptor 1 ($CysLT_1R$). When activated by the molecule leukotriene D_4

(LTD_4), $CysLT_1R$ signals into the tumor cell and can activate genes that enable tumor survival and growth. We have previously observed that mice treated with montelukast, a blocker of $CysLT_1R$, had smaller tumors.

The present thesis investigated the role of $CysLT_1R$ signaling and the tumor microenvironment in models of colon cancer. We also investigated the effect of LTD_4, PGE_2 and Foxy5 on CSC markers. It is necessary to find improved and safer therapies for treating colorectal cancer patients.

Paper I, describes the use of a spontaneous colon cancer model in mice due to a mutation in the *Apc* gene. We observed that female, but not male mice with an *Apc* mutation develop fewer and smaller tumors if they also completely lacked the $CysLT_1R$. Absence of $CysLT_1R$ resulted in tumors expressing decreased active β-catenin and increased inactive β-catenin, with decreased expression of the enzyme responsible for prostaglandin production, cyclooxygenase 2 (COX-2). We also observed an overall decrease in inflammation.

Paper II, describes how we induced a colitis-associated colon cancer model in female mice. We observed that female mice without the $CysLT_1R$ had a reduced tumor burden in the colon. As in paper II, decreased active β-catenin was seen with decreased COX-2 and reduced inflammation.

Paper III, describes mice implanted with human colon cancer cells that express high levels of the enzyme aldehyde dehydrogenase ($ALDH^+$), which is seen as a stem cell marker. The mice treated with the cysteinyl leukotriene LTD_4 or the prostaglandin PGE_2, had larger tumors and increased tumor expression of the CSC markers (ALDH and DCLK1). Tumors treated with LTD_4 or PGE_2 had increased active β-catenin and COX-2, followed by increased tumor presence of immune cells (CD206+ macrophages) associated with aiding tumors.

Paper IV, describes mice implanted with human colon cancer cells and treated with Foxy5 (WNT5A mimicking molecule), which resulted in decreased expression of CSC markers (ALDH and DCLK1). Foxy5 also reduced the expression of active β-catenin and a protein that preserves CSCs, ASCL2.

Paper V, describes models of colon cancer metastasis. In a mouse model of metastasis, we observed that fewer mice developed liver metastasis if they lacked the functional $CysLT_1R$, which also were smaller in size. In a zebrafish model of metastasis LTD_4–$CysLT_1R$ signaling increased metastasis and montelukast treatment or inactivation of the gene for $CysLT_1R$ showed decreased metastasis. In colorectal cancer patients, high expression of $CysLT_1R$ was seen in patients with metastasis.

To conclude, LTD_4-$CysLT_1R$ signaling promotes tumor progression in colon cancer models, while lack of $CysLT_1R$ signaling by gene inactivation or montelukast treatment decreased incidence of liver metastasis. Furthermore, CSC

expression was increased by LTD_4 or PGE_2 but inhibited by Foxy5. These results indicate promising therapeutic opportunities by inhibiting $CysLT_1R$ signaling and inducing WNT5A signaling by Foxy5 in colorectal cancer. These five papers attempt to broaden our knowledge about the role of different tumor microenvironment components on colon cancer progression.

Résumé de vulgarisation scientifique

Le cancer du gros intestin, également appelé cancer du colon, figure parmi les trois types de cancer les plus communs au monde et c'est la quatrième des causes de décès liées au cancer. Les patients souffrant de colite ulcérative ou maladie de Crohn, qui se caractérise par une inflammation chronique de l'intestin, courent un risque plus élevé de développer le cancer du colon. Les personnes ayant une certaine mutation héréditaire du gène *APC* courent elles aussi un plus grand risque de développer le cancer du colon. Le gène APC est aussi inactivé dans beaucoup de cas de cancer du colon sporadique. Le microenvironnement de la tumeur se compose de cellules immunitaires qui libèrent d'importantes molécules inflammatoires parmi lesquelles se trouvent les leucotriènes (LTD_4) et les prostaglandines (PGE_2). Une inflammation causée par les leucotriènes (LTD_4) peut stimuler la croissance ainsi que la migration des cellules épithéliales ce qui peut entrainer le développement d'une tumeur. Les patients souffrant d'un cancer du colon ayant une expression élevée du récepteur pro-inflammatoire cystéinyl leuctotriène 1 ($CysLT_1R$), avaient un pronostic moins favorable, mais le Montelukast, un médicament qui bloque le $CysLT_1R$, semble empêcher la croissance de la tumeur dans un modèle de cancer du colon des souris. Actuellement, le plus important c'est de trouver des traitements plus sûrs pour les patients souffrant du cancer.

Nous avons démontré auparavant que la croissance des tumeurs diminue lorsqu'une inhibition du $CysLT_1R$ a lieu chez des souris immunodéprimées avec des cellules de cancer du colon humain. La capacité qu'a une tumeur à croître et à se répandre dépend de son microenvironnement ainsi que des signaux entre les cellules tumorales et des cellules immunitaires proches, des cellules-souche du cancer et d'autres composantes. Il apparaît que la molécule Foxy5 est impliquée dans la régulation des cellules souche du cancer. Dans ce travail nous avons choisi d'étudier comment le $CysLT_1R$ influence le développement du cancer ainsi que sa propagation dans différents modèles du cancer du colon. Nous avons également étudié la manière dont les indicateurs de divers types de cellules souche du cancer sont influencés par le LTD_4, le PGE_2 ainsi que le Foxy5.

L' «Etude I » décrit comment nous avons utilisé des souris avec une mutation du gène APC, qui développe des tumeurs, principalement dans l'intestin grêle, de façon spontanée. Nous avons pu observer une diminution du développement des

tumeurs parmi les femelles mais pas parmi les mâles qui manquaient de $CysLT_1R$ étant absent chez les deux. Le manque de $CysLT_1R$ eut pour résultat moins de β-caténine active et une augmentation de la β-caténine inactive avec une moindre expression de l'enzyme qui produit les prostaglandines, le COX-2. Nous avons aussi pu constater une diminution de l'inflammation générale.

L' « Etude II » décrit nos recherches sur un modèle chimiquement induit de cancer du gros intestin lié à la colite chez la souris. Nous avons pu observer que les souris manquant de $CysLT_1R$ développaient moins le cancer. Comme dans l'Etude I, nous avons put constater que l'absence de $CysLT_1R$ donnait une diminution de la β-caténine active, une augmentation de la β-caténine inactive avec une diminution du COX-2.

L' « Etude III » décrit des souris ayant des cellules de cancer du colon implantées avec une forte teneur de l'enzyme ALDH, qui est considéré comme un marqueur des cellules souche du cancer. Les souris traitées avec le LTD_4 ou le PGE_2 ont développé des tumeurs plus importantes avec une teneur plus élevée des indicateurs des cellules souche du cancer (ALDH et DCLK1). Les tumeurs traitées avec le LTD_4 et le PGE_2 avaient plus de β-caténine active et de COX-2, ainsi qu'une augmentation de la présence de cellules immunitaires (macrophages CD206+) qui sont connues pour favoriser les tumeurs.

L' « Etude IV » décri des souris ayant subi une implantation de cellules de cancer du colon et qui ont été traitées avec le Foxy5 (une molécule WNT5A), ce qui résultat en une diminution des indicateurs des cellules souche du cancer (ALDH et DCLK1). Le Foxy5 a également diminué la teneur de β-caténine active ainsi que de la protéine ASCL2 qui préserve les cellules souche du cancer.

L' « Etude V » décrit des modèles de métastases du cancer du colon. Dans un modèle de métastase chez les souris nous avons observé qu'il avait moins de souris ayant des métastases lorsque celles-ci manquaient de $CysLT_1R$. En outre, les métastases observées étaient de moindre taille. Dans un modèle de métastase utilisant des poissons-zèbres nous avons observé que le LTD_4 augmentait le nombre de métastases par le biais du $CysLT_1R$ et qu'un traitement au Montelukast ou une désactivation du $CysLT_1R$ montrait des signes d'une diminution des métastases. Parmi les patients souffrant du cancer du colon, une teneur élevée de $CysLT_1R$ était associée à l'apparition de métastases.

En résumé, les signaux LTD_4-$CysLT_1R$ favorisent la croissance des tumeurs dans les modèles du cancer du colon tandis que l'absence de signaux $CysLT_1R$, par l'inactivation génétique ou par le traitement au Montelukast, diminue l'apparition de métastases au foie. La teneur de cellules souche du cancer augmentait avec le traitement au LTD_4 ou au PGE_2 et diminuait avec le traitement au Foxy5. Ces résultats augurent de nouveaux traitements prometteurs pour les patients souffrant

du cancer du colon car ils arrêtent les signaux CystLT$_1$R et fomentent des signaux WNT5A avec le Foxy5. Ces cinq travaux augmentent nos connaissances sur la manière dont les différentes composantes dans l'environnement de la tumeur influencent le développement du cancer du colon.

Introduction

Tumors can be benign or malignant and have been described as wounds that never heal (Dvorak, 2015). A malignant tumor or cancer is considered to be a group of diseases that consist of abnormal cells that undergo uncontrolled growth and have the ability to spread to other parts of the body. Cancer that originates in the epithelium of the large intestine is termed colorectal cancer (CRC) and is one of the most frequent types of cancer, for both women and men, in terms of incidence and mortality. For the year of 2018, CRC was the 4th most frequent type of cancer in Sweden and the 2nd most frequent cause of cancer-related death according to The Swedish Cancer Society (Cancerfonden, 2020). CRC is considered to be a cancer more common in the older population (Haggar & Boushey, 2009), with a median age of 66 and 69, in males and females, respectively. The spread of CRC to distant organs (metastasis) is the main cause of death in CRC patients (Zhu et al, 2012). Due to early detection and therapy, CRC incidence has dropped in those over the age of 50. However, during recent years the patients that are diagnosed are becoming younger, with increasing incidences in those under the age of 50 (ACS, 2020; García-Aranda et al, 2019). The number of deaths from CRC is still rising in numbers (García-Aranda et al, 2019) and it is therefore imperative to explore new treatment options.

Tumor formation is promoted by oncogenes and tumor suppressor genes (TSG). Gain-of-function mutations in proto-oncogenes, and inactivation of both alleles of tumor suppressor genes (TSG) results in stimulated tumor growth. Such a TSG is the adenomatous polyposis coli gene, *APC*, which normally suppresses cell growth and proliferation and if lost, promotes malignant transformation (Gold, 2017). During tumor development, several traits – termed the hallmarks of cancer – are acquired for tumor growth and metastasis to occur. These hallmarks encompass tumor cell survival by maintained proliferation, inactivation of growth suppression by TSGs, resisting cell death, allowing endless replication and promotion of angiogenesis, and activating local invasion and distant metastasis. Additional hallmarks now include reprograming energy metabolism and evading destruction by immune cells. A function of the immune system is to target cancer cells and destroy them. However, some cancers can evade immune detection and eventually spread to other organs (Hanahan & Weinberg, 2011). The chronic inflammation seen in the gastrointestinal epithelium of patients with inflammatory bowel disease (IBD) is believed to be the basis for the increased risk of CRC development

(Ullman & Itzkowitz, 2011). Pro-inflammatory lipid mediators such as cysteinyl leukotrienes (CysLTs) and prostaglandin E_2 (PGE_2) have been implicated in colorectal cancer development (Nielsen et al, 2003; Rigas et al, 1993). Furthermore, blocking these pro-inflammatory signals with cysteinyl leukotriene receptor blockers, such as montelukast, or non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, has shown to reduce the risk of developing colorectal cancer (Tsai et al, 2016; Rothwell et al, 2010). A minority of the tumor microenvironment consists of cancer stem cells (CSCs), which are believed to renew themselves and form tumors (Chen & Huang, 2014). Additionally, in CRC patients, better survival was observed in tumor tissue with high expression of wingless-type mammary tumor virus integration site 5A, abbreviated WNT5A (Mehdawi et al, 2016b).

In this thesis we investigated the role of the tumor microenvironment in the form of inflammatory signaling by $CysLT_1R$ and cancer stem-like cells such as CSCs and CICs.

Background

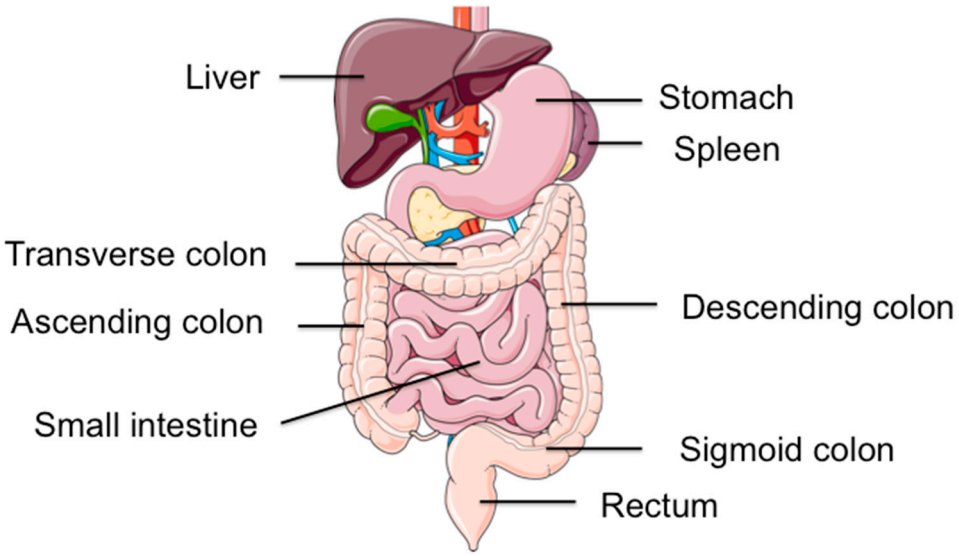

Figure 1. The location of the small and large intestine (colon and rectum) in the human body. Also, location of the liver, spleen and stomach.

The intestine

The intestine is part of the digestive tract and consists of the small intestine and the large intestine, which is also termed colon (Figure 1). The intestine has a tubular shape made up of three layers: a single-cell lining of epithelial cells facing the lumen and an outer muscle layer with the stroma separating them. The small intestine consists of the duodenum, jejunum and ileum and its epithelium consists of villi and crypts. Specialized epithelial cells called goblet cells are responsible for secreting and lining the intestinal epithelium with mucus (mainly Mucin-2) that acts as a barrier against bacteria (Johansson et al, 2011). When food exits the stomach and enters the small intestine, bile and enzymes that are secreted from the liver, pancreas and gallbladder further digest it. Intestinal absorptive cells (enterocytes) in the small intestine have microvilli on the luminal part, which

increases their surface area for nutrients absorption. The large intestine consists of colon and rectum with its epithelium consisting of crypts. The main function of the colon is to extract water and nutrients from food that is consumed and finally excreted. The intestinal lumen is lined with intestinal epithelial cells that have differentiated into cells with specific functions: enterocytes, goblet cells, hormone-secreting enteroendocrine cells and anti-parasitic tuft cells. Intestinal epithelial cells originate from dividing stem cells in the crypt base, migrate upward and become differentiated due to decreasing availability of stemness factors, before reaching the top and being shed into the lumen. This process takes approximately 4-5 days (Burgueño et al, 2020) (Figure 2).

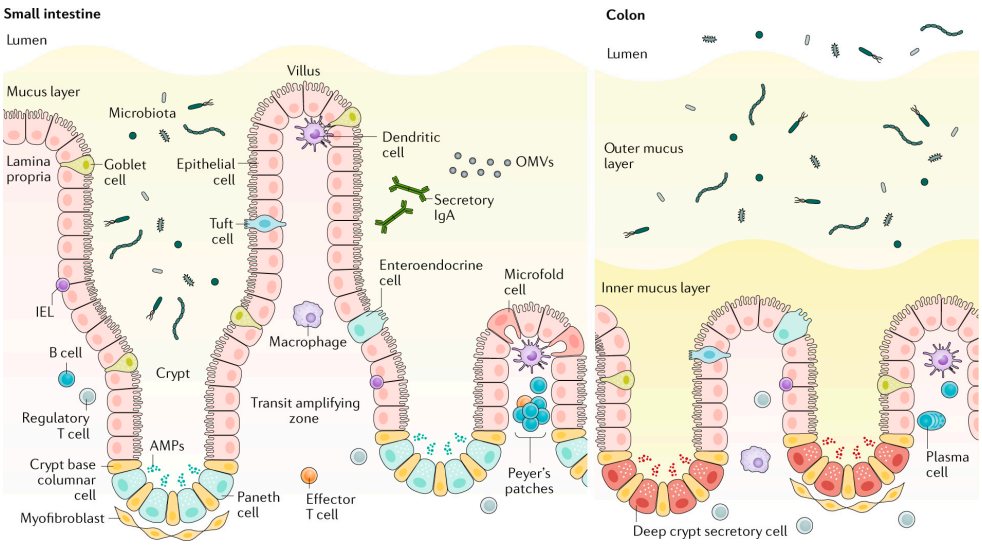

Figure 2. The immune cells and myofibroblasts present in the mucosa of the small intestine and colon. AMPs: antimicrobial peptides; OMVs: outer membrane vehicles; IEL: intraepithelial lymphocytes. IgA: immunoglobulin A. Reprinted by permission from Springer Nature Copyright © 2020 [Nature Reviews Gastroenterology & Hepatology, p. 265]

The intestinal microenvironment

A homeostasis exists in the intestinal microenvironment between epithelial cells, immune cells and commensal bacteria (Brown et al, 2013). Mucus production from goblet cells forms a protective barrier from mechanical damage and that caused by chemicals, enzymes and microbes (Linden et al, 2008). In the colon, the commensal bacteria aid digestion and synthesis of cobalamin (vitamin K) and (riboflavin) vitamin B_{12}, which are involved in blood coagulation and cellular metabolism, respectively (LeBlanc et al, 2013). The epithelial cells and immune cells have an immune-tolerance toward the co-existing gut microbiota. In the small intestine, Paneth cells are located near the stem cells and provide them with

growth factors, while in the colon, deep secretory crypt cells have a similar function (Burgueño et al, 2020). Peyer's patches are lymph-like nodules in the ileum and have an immune role responsible for maintaining the bacterial population and preventing pathogenic bacterial growth. The area below the epithelial cells in the intestine, is termed the lamina propria and consists of stromal myofibroblasts, endothelial cells that form blood vessels and different immune cells that guard the epithelial barrier, including macrophages, dendritic cells and B and T lymphocytes. Intraepithelial lymphocytes (IEL) also reside between epithelial cells and secrete antimicrobial peptides (AMPs) into the lumen. The epithelium and the lamina propria form the mucosa (Burgueño et al, 2020). (Figure 2)

Colorectal cancer

Colorectal cancer was the third most common cancer in 2018, accounting for 1.80 million cases. In terms of mortality, colorectal cancer was also the second cancer responsible for cancer-related deaths, with 860 000 cases (Bray et al, 2018). Risk factors include age, family history of colorectal cancer and a western lifestyle, which entails obesity, low physical activity, tobacco smoke, high alcohol consumption and intake of unhealthy foods (ACS, 2020). In CRC, both genomic and epigenetic changes are frequent in promoting tumorigenesis (Armaghany et al, 2012).

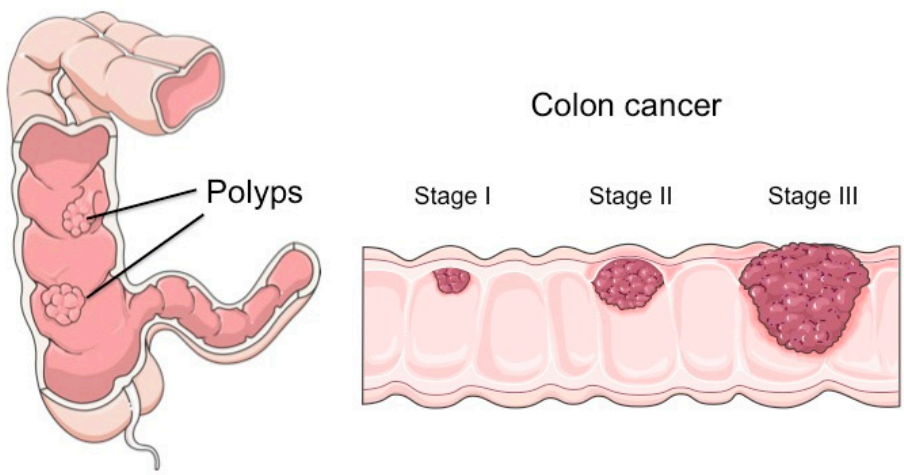

Figure 3. Colon cancer progression. The image to the left represents polyps originating from the mucosal epithelial cells in the colon. The image to the right represents the different stages of colon cancer, except stage IV which is metastasis.

Colorectal cancer classification and staging

The most commonly used system for staging cancers is the one recommended by the American Joint Committee on Cancer. Colorectal cancer stage is categorized according to the primary tumor size and extent (T), the number of regional lymph nodes with metastasis (N) and the presence of distant metastases (M), as seen in Table 1 (American Joint Committee on Cancer). The TNM staging system by AJCC helps to determine the best treatment options based on prognostic evidence. Stage I or II refers to primary CRC tumors without metastasis to the lymph nodes or other organs. Stage I tumor involves invasion of submucosa (T1) or muscularis propria (T2). Stage II tumors involve invasion of muscularis propria into the nearby colorectal tissue or through the visceral peritoneum or directly invades or adheres to adjacent organs or structures. Stage III tumors are characterized by lymph node involvement. Stage IV tumors are characterized by distant metastasis (AJCC 8th edition; Figure 3).

Table 1: TNM staging of colorectal carcinoma according to the American Joint Committee on Cancer (American Joint Committee on Cancer, 8th ed.)

	Primary tumor	Regional lymph nodes	Distant metastasis
Stage 0	Tis	N0	M0
Stage I	T1-T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage IIIA	T1-T2	N1/N1c	M0
	T1	N2a	M0
Stage IIIB	T3-T4a	N1/N1c	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
Stage IIIC	T4	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b
Stage IVC	Any T	Any N	M1c

The TNM staging of colorectal cancer refers to adenocarcinoma, high grade neuroendocrine carcinoma and squamous carcinoma of the colon and rectum. © 2017-2019, PathologyOutlines.com, Inc.

Hereditary and sporadic colorectal cancer

Hereditary colorectal cancer entails an inherited germline mutation increasing the risk of developing colorectal cancer due to increased risk of an additional somatic mutation. Sporadic colorectal cancer entails that mutations occur during one's lifetime, which result in cancer formation. In CRC, the two most commonly mutated suppressor genes are *adenomatous polyposis coli* (*APC)* and *tumor*

protein p53 (*TP53)*. The *APC* gene is mutated in 76% of CRCs - in both hereditary and sporadic colorectal cancer (Gold, 2017; Stoffel & Kastrinos, 2014; Ullman & Itzkowitz, 2011). *APC* inactivating mutations lead to WNT signaling and β-catenin activation, which promotes proliferation. *TP53* regulates proliferation as it can halt cell cycle progression or initiate apoptosis (Hanahan & Weinberg, 2011).

The two most common forms of hereditary CRC are the Lynch syndrome and familial adenomatous polyposis (FAP). The majority of Lynch syndrome tumors exhibit high DNA microsatellite instability with an accelerated adenoma to carcinoma progression, and a deficiency in DNA mismatch repair (MMR) proteins MSH2, MSH6, mutL homolog 1 (MLH1), or PMS1 homolog 2, mismatch repair system component (PMS2). FAP is characterized by hundreds to thousands of colonic adenoma-polyps, due to germline mutations in *APC*. Untreated FAP would lead to CRC in more than 90% of cases (Stoffel & Kastrinos, 2014).

The APC protein aggregates and forms a complex with Axin, glycogen synthase kinase-3 (GSK3β) and casein kinase 1 (CK1) to phosphorylate β-catenin for destruction (Schatoff et al, 2017), which hinders β-catenin from entering the nucleus to act as a transcription factor and to initiate gene transcription of oncogenes such as *cyclin D1, MYC* and *COX* (Shtutman et al, 1999; Salim et al, 2014; Bienz & Clevers, 2000).

In sporadic colon cancer, it is believed that the normal mucosa undergoes an early APC mutation resulting in the progression of early to late adenoma before developing to carcinoma. In colitis-associated colorectal cancer, colitis without dysplasia undergoes p53 loss of heterozygosity, COX-2 activation and MSI, resulting in indefinite dysplasia, which progresses to high-grade dysplasia, finally resulting in carcinoma. While, for sporadic colon cancer to occur, several mutations need to take place. An early event in adenoma formation is mutation of *APC* followed by microsatellite instability, activation of KRAS and COX-2. Progression into the late adenoma harbors mutations in the genes: deleted in colon cancer (DCC), deleted in pancreatic cancer 4 (DPC4), and a final mutation in the protein p53, is needed to develop into carcinoma (Ullman & Itzkowitz, 2011).

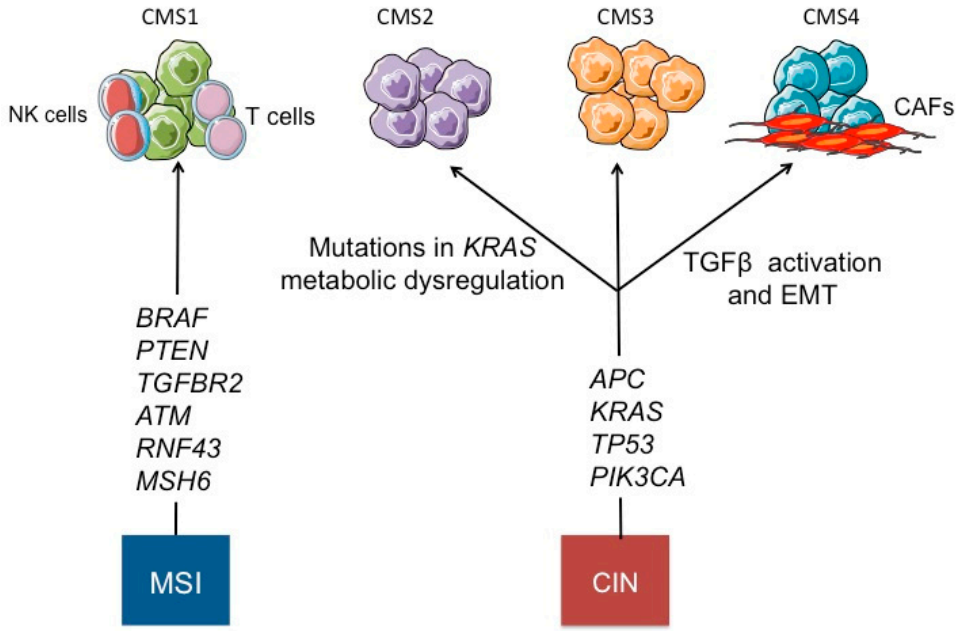

Figure 4. Colorectal cancer Consensus Molecular Consortium. CMS1 has microsatellite instability (MSI) and a high infiltration of immune cells. CMS2,3 and 4 all have chromosomal instability and mainly *KRAS* mutations. CMS2 also has metabolic dyregulation and CMS4 has a high infiltration of cancer associated fibroblasts (CAFs).

The CRC Consensus Consortium decided on separating CRC into four subtypes (CMS1, 2, 3, 4) to better correlate cancer cell characteristics based on gene expression with clinical relationship (Figure 4). These subtypes were referred to as Consensus Molecular Subtypes (CMS) and can be roughly separated into CRCs with microsatellite instability (MSI) and CRCs with chromosomal instability (CIN). The CMS1 subgroup has MSI with high immune infiltration (CD8+ cytotoxic T cells, CD4+ activated Th1 cells and NK cells). The CMS1 tumors develop from serrated polyps located in the proximal colon (ascending and transverse colon). CMS1 patients without metastasis are considered to have a favorable prognosis probably due to immune cell infiltration. The other three groups (CMS2, 3, 4) are considered to have chromosomal instability and have genetic mutations in *APC*, *Kirsten rat sarcoma virus* (*KRAS), TP53 and PIK3CA*. The tumors of the CMS2 subtype develop in the distal colon (descending and sigmoid part) and they possess distinct WNT/β-catenin and MYC signaling activation with little immune infiltration. The CMS3 subgroup has metabolic dysregulation with *KRAS* mutation. The tumors of the CMS4 subgroup develop from serrated polyps and possess transforming growth factor β (TGFβ) activation, increased inflammation with increased cancer-associated fibroblasts (CAFs),

angiogenesis and epithelial-to-mesenchymal transition, which is associated with metastasis. A possible fifth subgroup might exist with mixed CMS features indicating a transitional state or intratumoral heterogeneity (Thanki et al, 2017).

WNT signaling

The WNT signaling pathway is important in embryonic development, adult tissue homeostasis and repair. In the normal intestine and other tissues, WNT molecules act locally on stem cells in crypts to maintain their ability of self-renewal while decreased expression of WNT results in cell differentiation (Zhou et al, 2017; Reya et al, 2001). Two main WNT pathways exist, those acting on β-catenin (canonical) and those that do not (non-canonical pathway). For the WNT/β-catenin pathway, WNTs bind to and activate the receptor complex of the transmembrane Frizzled receptor (FZD) and lipopolysaccharide like receptor protein 5/6 receptor (LRP5/6). This initiates enlistment of cytosolic partner proteins Disheveled (Dvl) and Axin to the membrane, which disrupts the β-catenin destruction complex. This freeing of β-catenin allows it to accumulate in the cytosol and move into the nucleus where it associates with TCF/LEF transcription factors to drive transcription of WNT responsive genes (Schatoff et al, 2017; MacDonald et al, 2012) (Figure 5).

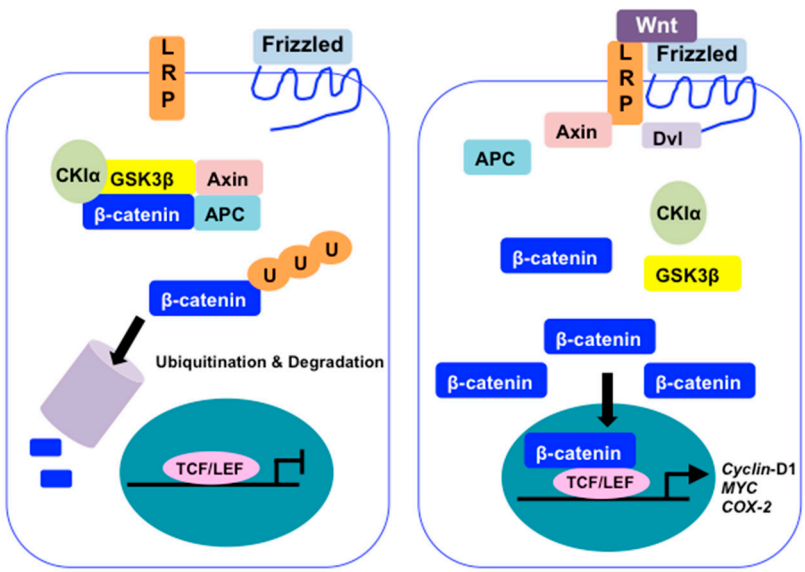

Figure 5. Canonical WNT/β-catenin signaling. Left panel: In the absence of Wnt, β-catenin is bound by the destruction complex and tagged for ubiquitination. and degradation by the proteosome. Right panel: Wnt binds to Frizzled and LRP receptor complex resulting in the disruption of th edestruction complex, allowing β-catenin to accumulate in the cytosol and enter the nucleus to turn on transcription of target genes *cyclin-D1, MYC, COX-2*.

A majority of CRCs exhibit hyperactivation of the WNT signaling pathway (Schatoff et al, 2017). The non-canonical WNTs, WNT5A, WNT4 and WNT11, do not activate the WNT/β-catenin pathway and are considered to be non-transforming (McDonald & Silver, 2009; Rao & Kuhl, 2010; Veeman et al, 2003). WNT5A has an important role in regulating planar cell polarity (PCP) and during embryonic development (Asem et al, 2016). WNT5A has been shown to antagonize canonical WNT/β-catenin upregulated gene targets (McDonald & Silver, 2009). WNT5A has contrasting functions in different cancers, either as an oncogene in e.g. melanoma and pancreatic cancer or as a tumor suppressor gene in e.g. colorectal and breast cancer, this is most likely due to the specific type of cancer and the availability of target receptors (Zhou et al, 2017).

Inflammation and colorectal cancer

Tumors are described as wounds that never heal, due to their inflammatory component and their common features to normal wound healing (Dvorak, 2015). Inflammation is now considered to be one of the hallmarks of cancer, where cancer cells escape the lethal attack directed by innate immune cells (Hanahan & Weinberg, 2011). On the other hand, patients with chronic inflammatory bowel disease have a higher risk of developing CRC. It is therefore evident that the type of immune cell present in the tumor microenvironment is critical for determining patient outcome.

Inflammatory bowel disease

Around 10-15% of patients with inflammatory bowel disease (IBD) die from CRC (Stidham & Higgins, 2018). IBD is divided into ulcerative colitis (UC) and Crohn's disease (CD), where chronic inflammation affects the colon in UC patients or the whole gastrointestinal tract in CD (Molodecky et al, 2012). The relative risk of developing CRC increases drastically if IBD is diagnosed at an early age. Furthermore, CRC in IBD develops from low- to high-grade dysplasia, eventually culminating in adenocarcinoma. Therefore, the main treatment of IBD-dysplasia is preventive colonic surgery (Stidham & Higgins, 2018). It has been shown in mouse studies that intestinal bacteria are necessary for inducing inflammation and for the development of colitis and neoplasia (Ullman & Itzkowitz, 2011)

Inflammatory lipid mediators and colorectal cancer

Increased levels of inflammatory lipid mediators have been implicated in several inflammatory-related diseases such as asthma, rhinitis, ulcerative colitis and Crohn's disease (Kanaoka & Boyce, 2014). Inflammatory lipid mediators, also known as eicosanoids, mainly exert their pro-inflammatory function as prostaglandins or leukotrienes, which are produced from membrane-bound arachidonic acid. Arachidonic acid is released from the membrane by cytosolic phospholipase A_2 and can eventually be converted by cyclooxygenase enzymes to prostaglandins or thromboxanes, by lipoxygenases to leukotrienes or hydroxy eicosatetraenoic acids, and by the cytochrome P-450 enzymes to epoxides (Kanaoka & Boyce, 2014; Park et al, 2006).

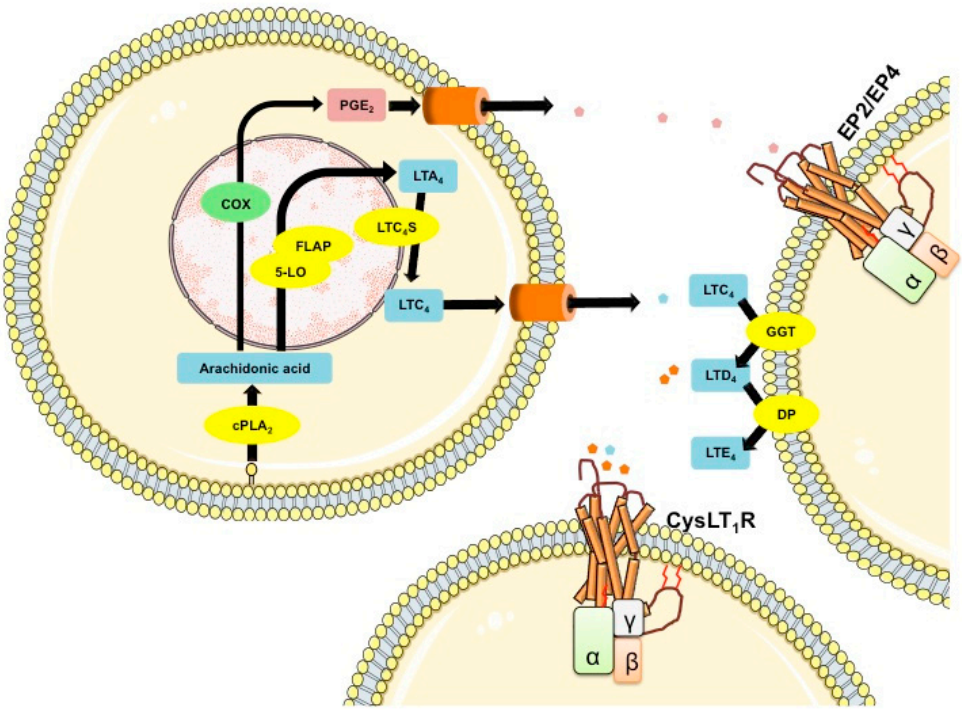

Figure 6. Synthesis of pro-inflammatory mediators: cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) by 5-lipoxygenase and PGE_2 by cyclooxygenase from arachidonic acid. $cPLA_2$:cytosolic phospholipase A_2; 5-LO: 5-lipoxygenase; FLAP: 5-lipoxygenase activating protein; LTC_4S: leukotriene C_4 synthase; GGT: gamma-glutamyl transpeptidase; DP: dipeptidase. COX: cyclooxygenase; PGE_2: prostaglandin E_2; EP2/4: prostaglandin E receptor 2 and 4.

Prostaglandins

Arachidonic acid can be converted into prostaglandins by the two enzymes cyclooxygenase 1 and 2 (COX-1 and COX-2). COX-1 is constitutively expressed in cells while the enzymes COX-2 and microsomal prostaglandin E synthase 1 (mPGES-1) are induced by stress and cytokines in inflammation and cancer. Prostaglandin E_2 is produced by the conversion of prostaglandin H_2 (PGH_2) by cytosolic prostaglandin E synthases (cPGES/COX-1) pairing or microsomal PGES/COX-2 pairing. PGE_2 can diffuse out of cells or be actively transported via the multidrug resistance associated protein 4 (Smyth et al, 2009; Park et al, 2006). PGE2 can bind to and activate four prostaglandin E receptors (EP1-4) and activation of EP2 and EP4 results in increased intracellular cyclic adenosine 3', 5'-monophosphate (cAMP) while PGE_2-activation of EP3 results in decreased cAMP (Takafuji et al, 2000; Hull et al, 2004). The pro-inflammatory effects of PGE_2 increases blood flow, fluid retention and immune cell infiltration. PGE_2-EP2 activation results in an increased vascular permeability and leukocyte infiltration. (Smyth et al, 2009). The degradation of PGE_2 is performed by the enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which is considered to be a tumor suppressor that is downregulated early in colorectal tumorigenesis. In colorectal cancer cells, knockdown of β-catenin was shown to induce increased expression of 15-PGDH (Smartt et al. 2012)

Cyclooxygenase pathway in cancer

COX-2 is mainly implicated in inflammatory diseases and cancer occurrence and associated with poor cancer prognosis (Smyth et al, 2009). The lack of mPGES-1 in mice with colon cancer resulted in reduced tumors. A decrease in nuclear β-catenin was seen in carcinogen induced aberrant crypt foci of *mPGES-1*$^{-/-}$ mice while no change was observed in *Apc* mutated *mPGES-1*$^{-/-}$ mice (Nakanishi et al, 2008). In the immune context, PGE2 has been demonstrated to block the activity and expansion of $CD3^+CD8^+$ T cells and thereby contributing to the evasion of tumor cells from the immune surveillance (Kalinski, 2012).

Leukotrienes

The leukotrienes were first discovered in leukocytes and have been implicated in asthma of the bronchi. Activation of the cysteinyl leukotriene receptor 1 (CysLT1R) induces bronchi smooth muscle constriction and proliferation, fluid retention and eosinophil migration (Lynch et al, 1999). The $CysLT_1R$ antagonists, montelukast, pranlukast and zafirlukast are used in the clinic to treat asthma. These antagonists inhibit inflammation and constriction of the bronchi smooth muscles (Bäck et al, 2011).

The leukotrienes exert their function by binding to and activating specific seven transmembrane domain receptors known as G-protein coupled receptors (GPCRs): $CysLT_1R$, $CysLT_2R$, BLT1, BLT2. With the help of 5-lipoxygenase activating protein (FLAP), membrane bound arachidonic acid is converted by 5-lipoxygenase (5-LOX) into 5-hydroperoxyeicosatetraenoic acid (HPETE) and further to the unstable leukotriene A4 (LTA_4). For synthesis of cysteinyl leukotriene C_4 (LTC_4), glutathione is added to LTA_4 by the membranous enzyme, LTC_4 synthase (LTC_4S), which is located in specific immune cells, such as macrophages, mast cells, eosinophils and basophils. LTC_4 is then transported out of the cell by the ATP-binding cassette (ABC) transporters 1 and 4 to exert its receptor action or be further converted by the surface enzymes gamma-glutamyl transpeptidase (GGT) to LTD_4 and a serum dipeptidase (DP) to the stable LTE_4. Among the CysLTs, LTE_4 has the longest half-life and can be detected in the urine, while LTC_4 and LTD_4 have a shorter half-life (Kanaoka & Boyce, 2014). Neutrophils harbor the cytosolic enzyme LTA_4 hydrolase, which can convert LTA_4 to the effective chemoattractant LTB_4 (Liu et al, 2015).

The cysteinyl leukotrienes bind to the cysteinyl leukotriene receptors 1 and 2 with different affinity. LTD_4 has the highest affinity for the $CysLT_1R$, while LTC_4 has equal affinity for $CysLT_1R$ and $CysLT_2R$. The quite stable, LTE_4 has the lowest affinity for both receptors (Singh et al, 2010). Both CysLTRs are expressed on immune and smooth muscle cells (Kanaoka & Boyce, 2014). $CysLT_1R$ is expressed on immune cells (macrophages, mast cells, eosinophils, neutrophils and B lymphocytes) and in lung smooth muscle, spleen and smooth muscle, while $CysLT_2R$ is expressed in the adrenal gland, heart, spleen, brain and spinal cord. The human $CysLT_1R$ gene is located on the long arms of the X chromosome (Xq13-Xq21). The human $CysLT_2R$ gene is located on chromosome 13 (13q14) in proximity to an atopic asthma marker (Bäck et al, 2011). The expression of $CysLT_1R$ has been observed in the small intestine and colon (Singh et al, 2010).

Cysteinyl leukotriene signaling in cancer

Asthma patients that have been treated with $CysLT_1R$ blockers, montelukast or zafirlukast, exhibited a lower incidence of cancer (Tsai et al, 2017). An increase in pro-inflammatory mediators was also seen in colon cancer patients (Rigas et al, 1993). Increased expression of the $CysLT_1R$ and the 5-LOX enzyme, which is responsible for LT production, has been observed in colorectal cancer (Nielsen et al, 2003) and increased $CysLT_1R$ expression in CRC has been associated with worse outcome (Magnusson et al, 2010). LTD_4 stimulation of colon cancer cells induced proliferation and migration and could be blocked by inhibiting $CysLT_1R$. Furthermore, LTD_4 stimulation induced phosphorylation of the glycogen synthase kinase 3 (GSK-3β), which resulted in free β-catenin (Salim et al, 2014).

Tumor microenvironment

The tumor microenvironment (TME) consists of tumor stroma, minority of cancer stem-like cells (CSCs or CICs) and other non-tumor cells, such as tumor-infiltrating immune cells, cancer-associated fibroblasts (CAFs) and vascular endothelial cells (Chen & Huang, 2014). The overall metabolic profile of the TME depends on the interplay between malignant cells and non-malignant components (Rybstein et al, 2018). To avoid recognition and death by the programmed death-1 (PD-1) receptors on T cells, tumor cells express programmed death ligand-1 (PDL-1; Thanki et al, 2017).

Tumor infiltrating leukocytes in cancer

Tumors are often infiltrated by leukocytes (white blood cells) and the specific subset of lymphocytes has been associated with patient survival (Gooden et al, 2011). Immune cells are mainly categorized into innate or adaptive immunity, where innate immune cells are fast responding while adaptive immune cells are slower to respond. The innate immunity comprises of complement proteins and specific immune cells which include macrophages, dendritic cells (DC), mast cells, natural killer (NK) cells and granulocytes (neutrophils, eosinophils, basophils). The adaptive immunity comprises of specific lymphocytes, which include B cells, $CD4^+$ T cells and $CD8^+$ T cells. Furthermore, natural killer T cells and γδ T cells are considered to belong to both the innate and adaptive immunity (Dranoff, 2004). Different T cells have different functions; $CD8^+$ T cells are cytotoxic to tumor cells, $CD4^+$ T helper cells secrete cytokine proteins, $CD4^+$ T regulatory cell ($Foxp3^+$) suppress T cells (Gooden et al, 2011).

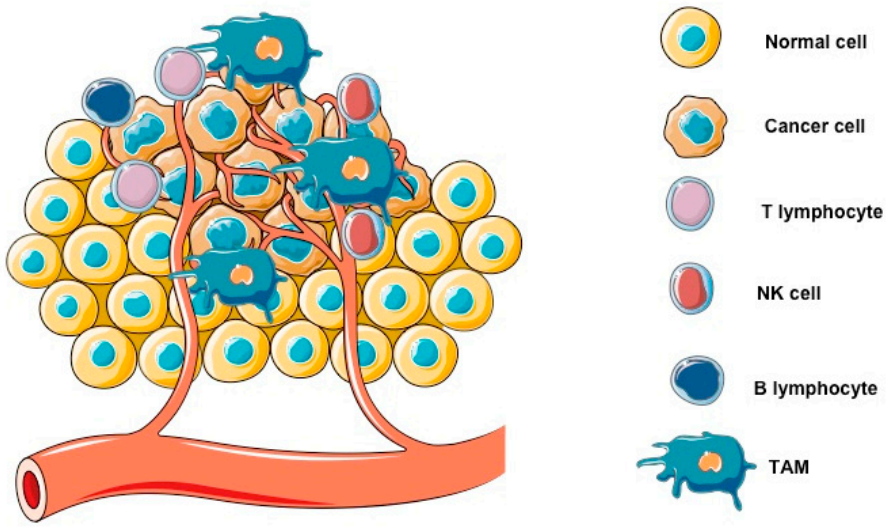

Figure 7. Immune infiltrating cells in the tumor microenvironment. TAM: tumor-associated macrophages; NK cell: natural killer cell.

Different immune cells are found to be present in the tumor microenvironment. The main types of immune cells that can destroy cancer cells are natural killer cells, and two T cell subtypes ($CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells) (Bantug et al, 2018; O'Neill et al, 2016). In addition to these cells, other subtypes of T cells have been observed in the TME, such as aβ TCR–expressing T cells, γδ T cells and natural killer T cells, and tumor associated macrophages (TAMs) (Gooden et al, 2011; Mantovani et al, 2017). Macrophages are classically divided into M1 and M2 phenotypes. M1 macrophages are cytotoxic to tumors and are activated by interferon γ (IFNγ) while M2 macrophages that are involved in tissue repair and remodeling are activated by specific interleukins (IL-4 or IL-13). Tumor promoting TAMs are immunosuppressive and of the M2-like phenotype. They can promote tumor growth and migration and have the ability to promote the formation of blood vessels, which makes an environment that can support cancer stem cells (Mantovani et al, 2017).

Tumor development and growth can be inhibited by immune cells, e.g. by the recognition of tumor antigens by $CD8^+$ T cells (Gajewski et al, 2013). An increase in tumor infiltrating $CD3^+$ T cells or $CD8^+$ T cells has been associated with a better prognosis in solid tumors (Gooden et al, 2011). TAMs can promote tumor growth and migration as well as invasion and metastasis. TAMs are known to protect the tumor from cytotoxic immune responses by releasing immunosuppressive substances (Mantovani et al, 2017). Furthermore, immunodeficient mice with disrupted development or function of specific immune cells ($CD8^+$ T cells, $CD4^+$ Th1 helper T cells, or natural killer cells) exhibited increased tumor incidence.

Disrupted function in both T and natural killer cells was also observed to increase tumor occurrence (Teng et al, 2008; Kim et al, 2007). In CRC, increased tumor infiltration of cytotoxic T lymphocytes, especially $CD8^+$ lymphocytes, were associated with a better patient survival (Naito et al, 1998).

Colorectal cancer metastasis

Metastasis is a process with many steps, where cancer cells from the primary site disseminate and enter lymph or blood vessels, travel to distant sites, exit vessels and colonize other organs and grow (Atashzar et al, 2020). The main sites of colon cancer metastasis is primarily the liver, secondly the lungs and thirdly the brain. About half of CRC patients develop metastasis and these have a 5-year survival rate of between 12-13% for the year 2018 (Zhu et al, 2012). The "seed and soil" model of metastasis proposes that a hospitable environment (soil) is needed for cancer cells (seed) to develop (Chen & Huang, 2014). This might explain that the liver is the most frequent site of CRC metastasis. Furthermore, the activation of the phosphoinositide 3-kinase (PI3K) has been associated with CRC metastasis (Zhu et al, 2012). (Figure 8).

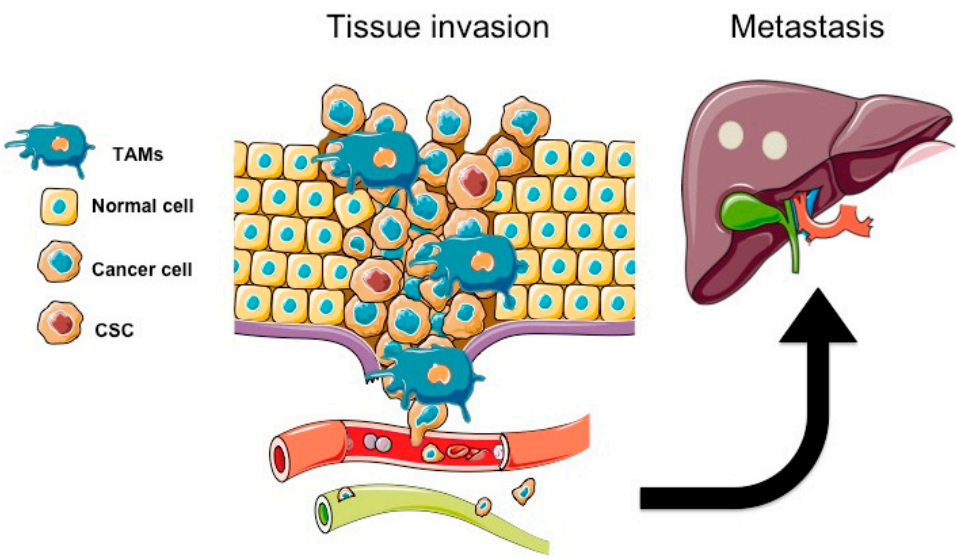

Figure 8. Cancer cell invasion through the basement membrane into blood and lymph vessels leading to distant metastasis in the liver. CSC: cancer stem cells; TAMs: tumor associated macrophages.

Cancer stem cells

Cancer stem cells (CSCs) are believed to be different from normal stem cells and have been referred to as either cancer stem-like cells, cancer initiating cells (CICs) or tumor initiating cells (TICs). It has been proposed that when cancer therapy fails due to recurrence or relapse that this is due to the survival of CSCs. Classical cancer therapies target fast proliferating and differentiating cells and CSCs are more quiescent, which can escape their proposed role in recurrence (Boman & Wicha, 2008). It is believed that CSCs can originate by three possible mechanisms: genomic instability in normal cells (cancer initiation), horizontal gene transfer (of fragmented DNA by tumor cells) or by microenvironmental changes (such as IL-6). It is probably factors in the microenvironment that contribute to the differentiation and transformation of CSCs (Atashzar et al, 2020). It is believed that cancer arises in stem cells or in progenitor cells that acquire stem cell-like properties (Reya et al, 2001).

Colon cancer stem cell markers

In colon cancer, different markers exist to denote cancer stem cells (CSCs). These cells exhibit stem cell-like properties with the ability to renew themselves (Reya T et al, 2001). CSCs are believed to be responsible for cancer resistance towards therapy and cancer recurrence (Atashzar MR et al, 2020). There are several markers that are used as colon CSC markers: $CD133^+$, $CD44^+$, $CD26^+$ and ALDH1 (Wakamatsu et al, 2012; Atashzar et al, 2020). These overlap somewhat with representative cell-surface markers of CSCs: CD133, CD44, EPCAM and LGR5 (Katoh M, 2017). Additionally, increased levels of aldehyde dehydrogenase 1 (ALDH1) were observed in gastric lymph node metastasis (Wakamatsu et al, 2012). Furthermore, increased levels of doublecortin-like kinase 1 (DCLK1) are considered to be a specific CSC marker in CRC when compared to normal colonic mucosa (Mohammadi Y et al, 2018). It is believed that the development and evolution of CSCs involves abnormal WNT signaling - both canonical, through β-catenin, and non-canonical. It is further believed that this WNT signaling is part of a larger network that includes Notch and Hedgehog signaling, which controls the expression of functional CSC markers (Katoh, 2017).

Colorectal cancer treatment

Colorectal cancer treatment depends on the diagnosed TNM stage. Stage I and early stage II CRC with no spread to the lymph nodes, are treated by surgical excision of polyps or parts of the colon. Treatment of high-grade stage II CRC or stage III CRC that has spread to lymph nodes includes surgery and sometimes adjuvant chemotherapy. Stage IV metastatic CRC – if localized and small in the

liver or lungs – is treated by surgery, and neo-adjuvant therapy to shrink the tumor and/or adjuvant chemotherapy to kill the cancer cells. Since the 1990's, the standard adjuvant chemotherapy for patients with high-risk stage II or stage III CRC has been 5-fluorouracil and leucovorin (folinic acid). Today, oxaliplatin has become the new standard chemotherapy and combination treatments with fluoropyrimidine and oxaliplatin are considered as standard treatment (Carrato, 2008). Metastatic CRC patients that have an increased incidence of *KRAS* mutations do not have good effects of treatments targeting the epidermal growth factor receptor (EGFR) by the monoclonal antibody cetuximab (Thanki et al, 2017). New therapies include targeting the vascular endothelial growth factor (VEGF) and angiogenesis, with bevacizumab.

Present investigations

Aims

The general aim of the thesis was to investigate the role of cysteinyl leukotriene receptor 1 ($CysLT_1R$)–signaling and the tumor microenvironment, such as immune cell infiltration and expression of cancer stem-like cells (such as cancer initiating cells and cancer stem cells) in different colon cancer models.

The specific aims of this thesis were:

Paper I

To investigate the role of the $CysLT_1R$ and the immune cells in the tumor microenvironment in a spontaneous model of colon cancer in male and female $Apc^{Min/+}$ mice.

Paper II

To investigate the role of the $CysLT_1R$ and the immune cells in the tumor microenvironment in an induced colitis-associated colon cancer model in female mice.

Paper III

To investigate the effect of $CysLT_1R$ signaling via LTD_4 and PGE_2 on colon cancer cells and immune cells in the tumor microenvironment in a xenograft model of colon cancer.

Paper IV

To investigate the role of Foxy5, a WNT5A mimicking peptide, on cancer stem cells and $CysLT_1R$ downstream targets, COX-2 and β-catenin, in a xenograft model of colon cancer.

Paper V

To investigate the role of $CysLT_1R$ signaling in colon cancer metastasis models, by LTD_4 stimulation, montelukast inhibition or lack of functional $CysLT_1R$.

Materials and methods

Chemicals

The $CysLT_1R$ antagonist montelukast, the $CysLT_1R$ agonist LTD_4 and the agonist PGE2 were purchased from Cayman Chemicals Co. (Ann Arbor, MI, USA). Azoxymethane (AOM) and dextran sodium sulphate salt (DSS) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and MP Biomedicals (Santa Ana, CA, USA), respectively. The WNT5A mimicking peptide, Foxy5, was manufactured in Bachem (Bubendorf, Switzerland) and provided courtesy of WntResearch AB (Malmoe, Skane, Sweden).

Antibodies

The ALDEFLOUR (ALDH) kit was procured from Stem Cell Technologies (Grenoble, France). The anti-human CD326 (EpCAM) MicroBeads were procured from Miltenyi Biotec (Gladbach, Germany). The fluorescent tagged antibodies were the following: anti-mouse CD45-FITC antibody was procured from Santa Cruz Biotechnology (Santa Cruz, CA), anti-mouse CD206-Alexaflour 647 antibody was procured from AbD Serotech (Dusseldorf, Germany), anti-mouse LY6G-PE, CD4-PE, and F4/80-PE conjugated antibodies were procured from BD Biosciences (Franklin Lakes, NJ, USA), rat anti-mouse CD3-Alexa Fluor® 647 antibody, rat anti-mouse CD4-PerCP-Cy™ 5.5 antibody, rat anti-mouse CD8-FITC antibody and istotype controls for each fluorochrome were procured from (BD Pharmingen, Franklin Lakes, NJ). For immunohistochemistry analysis, rat anti-mouse F4/80 antibody were procured from AbD serotec (Raleigh, NC, USA), the rabbit anti-human 5-LOX and COX-2 polyclonal antibodies were acquired from Cayman Chemical, mouse anti-human ALDH, rabbit anti-human β-catenin antibody (1:500), and matrigel matrix were acquired from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit anti-human Dclk1 antibody and mouse monoclonal anti-Achaete Scute homolog2 (ASCL2; clone 7E2) were obtained from Merck Millipore, Burlington, MA, USA), Millipore (Temecula, CA, USA). Rabbit monoclonal antibody to double cortin-like kinase 1 (DCLK1; clone EPR6085, 1:600 or 1:800; Abcam, Cambridge, UK), rabbit anti-15-PGDH (1:500; Novus Biologicals, Denver, CO, USA), rabbit anti-COX2 (1:400; Abcam, Cambridge, UK), and rabbit monoclonal anti-non-phospho (active) β-catenin (Ser45) (D2U8Y)(1:500; Cell Signaling, Leiden, the Netherlands). Anti-human CD45 polyclonal antibody and mouse anti-BrdU monoclonal antibody, goat anti-human Mucin 2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). All other reagents were of analytical grade and were procured from Sigma Chemical Co. (St. Louis, MO) or Chemicon International (Temecula, CA) unless specified otherwise.

Colon cancer cell lines

All the human colon cancer cell lines were procured from the American Type Culture Collection (ATCC): HT-29 (ATCC# HTB-38™), HCT-116 (ATCC# CCL- 247™) and Caco-2 (ATCC# HTB-37™). The mouse colon cancer cell line MC38 (Kerafast, CVCL_B288), was acquired from Kerafast. The human colon cancer cell lines HT-29 and HCT-116 were cultured in McCoy's 5A media with glutamine (HyClone™, GE Healthcare Life Sciences) and the Caco-2 cell line was cultured in Dulbecco's modified eagle medium (DMEM), and all were supplemented with 10% fetal bovine serum with 100 µg/ml penicillin/streptomycin. The mouse colon cancer cell line was cultured in DMEM-high glucose supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 4mM glutamine, 1 mM sodium pyruvate, 55mg/ml streptomycin, 55 IU/ml penicillin, 50ug/ml gentamycin sulfate. All cell lines were grown until 70–80% confluent for experimental use in an incubator with a temperature of 37°C in a humidified atmosphere of 5% CO_2. All cells were regularly tested for mycoplasma contamination.

Flow cytometry cell sorting

To obtain $ALDH^+$ HCT-116 cells, for the xenograft study, the ALDEFLUOR™ kit (Stem Cell Technologies, Durham, USA) was used to identify cells with high ALDH enzymatic activity ($ALDH^+$), according to manufacturers instructions. Briefly, the adherent HCT-116 cells were detached by 0.25 % trypsin and 0.02 % EDTA, resuspended in serum-free culture media, rinsed with 0.5 % bovine serum albumin in PBS and stained with ALDEFLUOR™ reagent to identify $ALDH^+$ cells or in combination with the ALDH inhibitor, diethylaminobenzaldehyde (DEAB), as a control for background fluorescence (Stem Cell Technologies, Durham, USA). The activated ALDEFLUOR™ reagent was added directly to 1 × 106 cells/ml ALDEFLUOR™ Assay Buffer and then incubated at 37 °C for 40 min. Viable ALDH bright fluorescent HCT-116 cells (HCT-116 ALDH+) were sorted by fluorescent-activated cell sorting (FACS) using a FACSCalibur or FACSAria flow cytometer (BD Biosciences). $ALDH^+$ HCT-116 cells (1x104 cells) were injected subcutaneously into BalbC nu/nu mouse flanks in a 1:1 ratio of PBS and Matrigel™. The data were analyzed with Summit v4.6. At the experimental end point, tumors were excised, rinsed and re-suspended in PBS, and dissociated into single cells.

Magnetic cell sorting

The excised xenograft tumors ($ALDH^+$ HCT-116) were dissociation by gentleMACS™ Dissociator and filtered through a 70-µm mesh, rinsed with PBS

and then the number of cells were calculated by the Countess automated cell counter (ThermoFisher). The single-cell suspension was labeled with magnetic microbeads for the epithelial marker CD326 (EpCAM) that separated epithelial tumor cells from mouse cells by magnetic associated cell sorting. Further analysis by FACS was performed for the separated cells. The mouse cells were stained for immune markers CD45, CD4, LY6G, F4/80, CD206 and the EpCAM positive tumor cells were stained for ALDH.

Flow cytometry

For single cell analysis by flow cytometry, xenograft tumors and polyp areas of mouse small intestine were dissected out, rinsed with ice-cold phosphate-buffered saline (PBS) and cut into smaller pieces and re-suspended in 10%FBS in RPMI medium with 0.5mg/ml Collagenase P (Roche Diagnostics, Basel, Switzerland). Further dissociation was performed by gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA). For analysis of different T cell markers, the single-cell suspension from the colon or small intestine was fixated with 4% buffered formalin. Non-specific staining was blocked by Mouse BD Fc Block™ and incubated with different T cell markers: a combination of conjugated CD3-Alexa Fluor™647 and CD4-PerCP-Cy™ 5.5 antibody or CD3-Alexa Fluor™647 and CD8-FITC antibody. Conjugated isotype controls for each antibody were used to determine non-specific background. The utilized flow cytometry antibodies were purchased from BD Pharmingen, Franklin Lakes, NJ. The cells were detected on the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Results were analyzed on the Summit v4.3 Software (Dako, Fort Collins, CO).

For analysis of CD45+ leukocytes by flow cytometry, HCT-116 xenograft tumors were dissociated into single-cell suspensions, which were portioned into 1 × 106 cell-aliquots in PBS and re-suspended in 100 µl of PBS with 1 % mouse serum. Cells were incubated with Mouse BD Fc Block™ for 45 minutes followed by 45-minutes incubation of CD45-FITC antibody (5 µl) and then washed. Cells were re-suspended in 100 µl of PBS with 0.1 % µg/µl of 7-Amino-Actinomycin D (BD Pharmingen) to exclude nonviable cells, and incubated for 10 min. A final volume of 500 µl was obtained by the addition of PBS for analysis by flow cytometry. The analysis was performed using the Summit v4.6 Software (Dako, Fort Collins, CO).

Animal models

$Apc^{Min/+}$ mouse model

Breeding was initiated between C57BL/6J-$Apc^{Min/+}$ mice from the Jackson Laboratory (Bar. Harbor, ME) and C57BL/6N mice with a *Cysltr1* gene disruption (*$Cysltr1^{-/-}$*) were gifted to us from Professor Frank Austen from the Harvard Medical School at Brigham and Women's Hospital in Boston, MA (Maekawa et al, 2002). Male and female offspring between 6 and 8-weeks old carrying an *$Apc^{Min/+}$* mutation were included in the study. The mice were divided into different groups based on their *Cysltr1* genotype. Since the $CysLT_1R$ is located on the X-chromosome, female mice were divided into three groups (*$Apc^{Min/+}$, $Cysltr1^{+/-}Apc^{Min/+}$ and $Cysltr1^{-/-}Apc^{Min/+}$*) and males were divided into two groups (*$Apc^{Min/+}$* and *$Cysltr1^{-/o}Apc^{Min/+}$*). At the time of sacrificed, when the animals were 14-weeks-old, the intestine was removed and tumor number and size was evaluated and either by a dissection microscope (2X). Half of the females per group had the entire colon and/or distal part of the small intestine fixed in 10% buffered formalin and embedded in paraffin or frozen in liquid nitrogen and stored at -80°C. Animal breeding was established and maintained at the Malmö-based animal facility of Lund University, in accordance with ethical permit M263-12, which was approved by the Swedish Regional Ethical Committee for Animal Research at Lund University.

AOM/DSS model

Female mice between 6- and 8-weeks old were included in the study. Wildtype and *$Cysltr1^{-/-}$* mice were given an initial intraperitoneal injection of 10 mg/kg azoxymethane (AOM; Sigma-Aldrich Corporation, St. Louis, MO, USA) in 0.9% NaCl, and then followed by a 5-day administration of 2% dextran sodium sulfate (DSS) in the drinking water *ad libitum* at day 7 and 26 (MP Biomedicals, Santa Ana, CA, USA). Control mice only received an intraperitoneal saline injection (vehicle: 0.9% NaCl). The weight of each mouse was documented every third day. Euthanasia was performed by CO_2 asphyxiation either at day 31 (colitis sacrifice) or at 60 days (CAC sacrifice) after the initial injection. The entire colon was removed and the majority of fat appendages were excised without damaging it. Subsequently, the colon was rinsed with cold PBS to remove feces, and cut from anus to the cecum border, and then length and weight was documented. Circa 50% of female mice per genotype had their colons placed flat in 10% formalin fixation buffer overnight and then kept in 70% ethanol. A dissection microscope (20x) was utilized to count and measure the size and number of colonic polyps. The colon was then cut into smaller pieces (around 2 cm in size) and embedded in paraffin for further immunohistochemical analysis. The remaining mice had their colon

tissue cut to 1-or 2-cm sizes and quickly frozen in liquid nitrogen for further storage at–80°C for later RNA and protein analysis.

Xenograft mouse models

Female nude mice (BalbC nu/nu) between the ages of 5 to 6 weeks were utilized in these models and procured from Taconic Europe A/S (Ry, Denmark). The animal experiments were approved by the Swedish Regional Ethical Committee for Animal Research at Lund University (M401-12; M163-15). Colon cancer xenografts were induced in all mice by subcutaneous injection of human colon cancer cell lines into both mouse flanks. Tumor development was detected by palpation. The time required for tangible tumors to develop was documented to be 7 days for HT29 and Caco2 cells and 10–14 days for $ALDH^+$ HCT-116. The mice were then randomly split into groups and a caliper was utilized to measure the tumor size every three days. For the $ALDH^+$ HCT-116 study, FACS-sorted 1×10^4 HCT-116 cells expressing high ALDH were re-suspended in a cold Matrigel:PBS mixture with a ratio of 1:1 (BD Biosciences), and 100 µl was finally injected subcutaneously into each flank. Three groups were utilized for each treatment (vehicle, LTD_4 or PGE_2). Daily subcutaneous injections were administered to mice of either ethanol (5 %) as vehicle, 24.8 µg/kg/day of LTD_4 or 17.6 µg/kg/day of PGE_2. Tumor growth was monitored, and the tumor volume was estimated every third day. The mice were sacrificed 48 days after the start of the experiments. The subcutaneous tumors were excised, weighed and measured, and photographically documented. Subcutaneous tumors were either processed for tissue dissociation and analysis by FACS or placed in 10 % buffered formalin for fixation and later paraffin embedding for analysis of proteins by immunohistochemistry. Tumor volumes were estimated according to the formula $(\text{length} \times \text{width}^2)/2$.

For the Foxy5 study, 2.5×10^6 low-passage HT-29 or Caco-2 cells were injected subcutaneously into mice (n=10 for HT-29 and n=10 for Caco-2). Different treatments were given to each of the two groups (saline vehicle alone or Foxy5). The mice received a total of nine intraperitoneal injections of either vehicle or Foxy5 (40 µg per dose, ~2 µg/g) every second day from day 7 to day 23. The dose of Foxy5 was selected on the basis of published data (Säfholm et al, 2008; Canesin et al, 2017). All mice were sacrificed on day 24. Tumor diameters (d1 and d2) were measured with a caliper on day 23, to calculate tumor volumes according to the formula, $V=(\pi/6)(d1\times d2)3/2$. At the time of sacrificed, mouse tumors were removed. One tumor from each animal was frozen at −80°C, and the other tumor was fixed in 4% paraformaldehyde at 4°C for 48 hours, washed with water, dehydrated with increasing concentrations of ethanol, immersed in xylene and finally embedded in paraffin. The embedded tumor tissues were then investigated by immunohistochemical (IHC) staining.

Metastasis mouse model

This study utilized a mouse model of liver metastasis. Female mice (8 to 12 weeks old) on a C57BL/6N background were chosen for this study and divided into three groups: wildtype females (n=15), Montelukast (MK) treated wildtype (n=8) and *Cysltr1*$^{-/-}$ females (n=15). Liver metastasis was induced by surgical intervention and dividing the spleen in half in accordance with the protocol published by Soares (Soares et al, 2014). MC38 cells (3x10^5 re-suspended in 50µl PBS) were aspirated to a syringe with 100µl PBS, to be injected into half of the spleen (a suspension of MC38 cells in 150µl PBS). Five minutes after the injection, the splenic half was excised. Montelukast (10mg/kg body weight) suspended in 100µl PBS was intraperitoneally injected to the specific group every second day with start on the day after inoculation until the mice were sacrificed. The mice were sacrificed 21 days after inoculation; at that time mice were weighed and livers were removed, weighed, documented photographically and appraised for metastatic foci. At the time of sacrifice, cardiac puncture was performed and blood was collected. Sodium citrate (100 µl) was added to blood and spun down (10 min, 4°C, 1500 *g*) to obtain plasma, which was quickly frozen in liquid nitrogen. The liver of each mouse was either frozen in liquid nitrogen or stored at -80°C or placed in 10% buffered formalin for additional investigation.

Zebrafish model

A metastatic model in zebrafish embryo was utilized for this study. Transgenic green fluorescent zebrafish (*Tg(fli1:eGFP*) were procured from SciLife Lab in Uppsala, Sweden. The fish were upheld and experiments were executed according to the standard protocols at the BMC facility in Lund, according to Swedish European animal welfare regulations (Ek et al, 2016).

A HT29 xenograft model in zebrafish embryos was utilized (Zhao et al, 2011) to investigate the $CysLT_1R$ signaling by a 2-day-stimulation of LTD4, inhibition by Montelukast or the addition of DMSO control. Additionally, HT29 cells underwent transfection with *CRISPR-Cas9 CYSLTR1* targeting the $CYSLT_1R$ or a control (*shCTRL)*. The HT29 cells were then labeled by vibrant-DiI (red colour). By way of microinjection, the DiI-labeled cells were injected into the perivitelline space of two-day post-fertilization zebrafish embryos. Colon cancer metastatic spread in embryos was investigated after a 2-day-incubation period at 28°C. A Nikon fluorescence microscope (Nikon eclipse 80i, USA) was used to photograph embryos and the ImageJ software (NIH, Bethesda, USA) was used to evaluate metastatic spread.

Patient data

CRC Malmö cohort

Colorectal cancer tumor material was collected in retrospect from patients who had undergone cancer surgery at Malmö University Hospital during the year 1990 and had been diagnosed with adenocarcinoma (Mehdawi et al, 2016a). Due to the available tumor samples on paraffin-embedded blocks, 72 out of 120 patients could be studied. The CRC TNM staging system was used to categorize tumors (Greene et al., 2002) and tumor grade was determined as either high, medium, or low. Vital status and the reason for death were acquired from the Swedish Cause of Death Registry until 31st of December 2000. The study was sanctioned by the Ethical Committee at Lund University (LU 52-99 and 367/2005). Retrospective immunohistochemical staining of nuclear $CysLT_1R$ expression were utilized (Öhd et al, 2003). We investigated the proportions of patients with high nuclear $CysLT_1R$ expression and association with TNM stage categories including distant metastasis (M1), cancer spread to the lymph nodes (N1/2), tumor extent (T) or TNM stage (I, II, III, IV). The proportion of patients within different categories were statistically analyzed by the χ-square test.

TCGA-COAD database

The correlation between mRNA levels of $CysLT_1R$ *(CysLTR1)* and β-catenin (*CTNNB1*) was evaluated from a public database, The Cancer Genome Atlas Colon Adenocarcinoma colon cancer (TCGA-COAD) data collection with 286 samples. (The TCGA results are in whole based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/).

Genotyping

The mouse-breeding colony was genotyped for the presence of *Cysltr1* alleles alone or for the presence of $Apc^{Min/+}$ and *Cysltr1* alleles by polymerase chain reaction (PCR) assays. Genomic DNA was extracted from ear punch biopsies, by first incubating samples overnight at 55°C with 180 µl Digestion solution and 20 µl Proteinase K followed by the use of the GeneJET Genomic DNA Purification Kit (K0721) according to the manufacturer's instructions. The DNA was measured on Thermo Scientific™ NanoDrop spectrophotometer (Thermofisher) and either 25 or 50 ng was used in the following PCR reaction. The Fermentas Taq DNA Polymerase (# EP0401) was used with a reaction volume of 20 µl. The following six primers were used for genotyping (see Table 2 below):

Table 2: Primers used for genotyping ear-punch biopsies from mice.

Primer type	Primer sequence
***Apc*-wild-type (IMR0033)**	5′-GCCATCCCTTCACGTTAG-3′
***Apc*-common (IMR0034)**	5′-TTCCACTTTGGCATAAGGC-3′
***Apc*-mutant (IMR0758)**	5′-TTCTGAGAAAGACAGAAGTTA-3′
mCysLT1R sense	5'-AAA ACA ATG ACG TGC ACT ATC ATA AAG-3'
mCysLT1R antisense	5'-AAT CAT GTA TAC TTC GAA GGC TGA-3'
Neo antisense	5'-ATC TTg TTC AAT ggC CgA TCC CAT-3'

After PCR recation, the primers in Table 2, generated an *Apc* product of 600bp for the wild-type and 340bp for the $Apc^{Min/+}$ allele, and generating a product of 284bp for the *Cysltr1* wild-type allele and 333bp for the null *Cysltr1* allele.

Gel electrophoresis was performed with 2.5% agarose gel with GelRed (Biotum) in either TAE (Tris-acetate-EDTA) or TBE buffer (Tris-borate-EDTA). The Orange Loading Dye Solution (Fermentas # R0631) was added to each sample. The O'GeneRuler 100 bp DNA Ladder (# SM1143) and O'GeneRuler 50 bp (# SM0613) DNA Ladders from Fermentas were used. Positive controls together with the samples were run on constant voltage 100 V for 1 to 2.5 hours until a clear separation was seen. The images were taken using BioRad's Molecular Imager Chemi Doc XRS for Nucleic Acid Gels.

Immunohistochemistry

Formalin-fixed tissues were embedded in paraffin and sectioned to 4 or 5 μm thickness and mounted on glass slides for immunohistochemical staining. All procedures for tissue sectioning and staining were performed by utilizing the Dako automatic slide stainer (Agilent Technologies, Santa Clara, CA) in accordance with the manufacturer's instructions, if not stated otherwise. All tissues were counterstained with hematoxylin. The slides were scanned with Aperio ScanScope CS (Aperio Technologies, Vista, CA), and images were evaluated independently in a blinded fashion, by two observers. Paraffin-embedded sections of small intestine and colon were sectioned to 5 μm and immunostaining was performed by utilizing the Dako automatic slide stainer in accordance with the manufacturer's instructions.

For the dysplastic lesions, the estimation of the percentage of 5-LOX positive epithelial cells per polyp was utilized. COX-2 protein expression was identified by the image-analyzing software HALO v2.0 (Indica Labs; Albuquerque, NM, USA), and determined the total percentage of COX-2 positive cells within the annotated epithelial polyps based on the pixel algorithms. Epithelial cells were identified with the aid of the Classifier module, and the COX-2 positive staining was detected using the AreaQuantification algorithm, with a manually adjusted positive-staining threshold. β-catenin subcellular localization for positive stained

epithelial cells within polyps was determined. Goblet cells were identified as the number of Muc-2 positive cells per crypt, where ten crypts adjacent to polyps were assessed. The villi (n = 10) selected for cell quantification were similar in size and in near proximity of polyps within the distal part of the small intestine. Proliferating cells (anti-BrdU) within polyps were identified. Infiltrating CD45 and F4/80 immunostaining was evaluated as the number of positive cells per polyp area. IgG controls (negative controls) were stained for by using rabbit-HRP, Flex mouse/rabbit-HRP and rabbit anti-rat with subsequent rabbit-HRP staining (all from Dako).

For HCT-116 xenograft tumors, the immunoreactivity of β-catenin, COX-2, 5-LOX and F4/80 proteins was determined based on the following immunoreactive score. Briefly, staining intensity was scored as 0 (negative), 1 (very weak), 2 (weak), 4 (medium) or 6 (strong). The 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 the positive stained area in comparison with the complete tumor area. Furthermore, the sum of the intensity and extent score was regarded as the concluding staining scores for 5-LOX, COX-2, F4/80 and β-catenin proteins.

HT29 and Caco2 xenograft tumors were sectioned (4 μm) for immunohistochemical staining. Cut sections of tumors were treated with hydrogen peroxide (1–3 %), blocked, and incubated to determine ALDH, COX-2 or F4/80 positivity at a dilution of 1:100, or 5-LOX and Dclk1 positivity at a dilution of 1:200 dilution, or β-catenin positivity at 1:300. Biotinylated secondary staining was followed by ABC reagent (Vector Laboratories Inc., Burlingame, CA). Signals were detected using DAB solution (Vector Laboratories). The slides were scanned using the Aperio ScanScope CS system (Aperio Technologies Inc, Vista, CA, USA), and images were evaluated in a blinded fashion by two independent observers.

All incubations with a primary antibody were performed overnight in a humidity chamber at 4°C. After washing, the sectioned tumor samples were incubated for 30 min at room temperature with a secondary antibody and developed using either an EnVision+ System-HRP labelled polymer anti-mouse or anti-rabbit antibody (K4000 and K4002 respectively; Dako), visualized by addition of 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories Inc., Burlingame, CA, USA) for 2-5 min and then finally counterstained with haematoxylin.

Serum and plasma separation from blood

At the time of mouse euthanasia, cardiac puncture was performed to collect blood and immediate addition of a nonselective COX inhibitor, indomethacin, (Sigma–Aldrich, St Louis, MO) at a final concentration of 10 μg/ml. Serum was obtained

by allowing the blood to clot for 30 min on ice followed by centrifugation serum separator tubes from Becton Dickinson (6000 *g*, 2 min, 4°C) and plasma was obtained by addition of the anticoagulant sodium citrate and followed by centrifugation (5000 *g*, 4 min, 4°C). Blood plasma was obtained by centrifugation (1500 *g*, 10 min, 4°C).

Immunoassay of CysLTs and PGE_2

Serum samples were pooled from five mice per group and individual plasma samples were considered for separation of CysLTs and prostaglandins by solid-phase extraction Sep-Pak (C18-500mg) cartridges purchased from Water Corporation (Milford, MA). The levels of CysLTs and prostaglandin E2 (PGE2) in serum or plasma were measured using a competitive enzyme-linked immunosorbent assay purchased from Enzo Life Sciences. Measurements were according to manufacturer's instructions.

Multiplex immunoassays

Plasma, serum, colon tissue with polyps and liver metastasis samples were utilized to measure cytokine levels using a multiplex sandwich immunoassay format and the electro-chemiluminescence MSD proinflammatory multiplex kit (Meso-Scale Discovery, Gaithersburg, MD). Ten cytokines were quantified from 2-fold-diluted, 4-fold-diluted or undiluted samples run in duplicate or triplicate. The MSD multispot array was run according to the manufacturer's protocol. Briefly, 96-well plates pre-coated with ten capture antibodies for TNFα, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFNγ and CXCL1 (KC/GRO) were incubated for 2 hours with samples. Successively, detection antibodies were added to the plate and incubated for extra 2 hours. Read buffer was added after washing and the plate was read using an MS2400 imager (Meso Scale Discovery).

For AOM/DSS treated mice, serum and lysed colon tissue with polyps were used. The frozen colon tissue samples with identified polyps (around 100 mg) were cut into small pieces and placed in 600µl lysis buffer for homogenization with a sonicator on ice. For the liver metastatic model, the appropriate volume of lysis buffer was adjusted based on the weight of available liver metastasis tissue. The frozen metastatic tissue samples were cut into small pieces and placed in appropriate lysis buffer (100 mg frozen tissue per 600µl lysis buffer) for homogenization with a sonicator on ice. The sonicated samples were then incubated while mixing at 4°C for 20 min and then centrifuged at 20,000 *g* for 15 min at 4°C for supernatant collection. The plasma or liver metastatic tissue were 2-fold diluted and run in duplicate. For the HCT-116 xenograft mice, undiluted plasma cytokines were analyzed.

Western blot

For AOM/DSS treated mice, colon lysates from wild type (+/+) or Cysltr1$^{-/-}$ (–/–) female mice and corresponding vehicle were lysed as described for the multiplex immunoassay. The samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride membrane, followed by blocking with 5% bovine serum albumin in PBS for 1 hour, incubated with primary antibody in 0.05% Tween-20 in PBS for 1 hour at room temperature, washed and incubated with secondary antibody conjugated with horseradish peroxidase in 0.05% Tween-20 in PBS for 1 hour at room temperature and washed before addition of the enhanced chemiluminescence reagent and detected proteins with Molecular Imager (ChemiDoc XRS + ImageLab software from Bio-Rad). Colon lysate samples were incubated with the $CysLT_2R$ (F-15) antibody (1:500) from Santa Cruz, and after stripping, re-incubated with an actin (I-19) antibody (1:3000), also from Santa Cruz, to ensure equal loading controls.

Western blot analysis was performed as above on the MC38 cell line to evaluate $CysLT_1R$ expression by whole-cell lysis in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Igepal, 5 mM EDTA and 0.1% SDS) and. The primary antibody for the CysLT1 Receptor (C-16) (1:500) was procured from Santa Cruz.

Gene expression analysis

For the Apc$^{Min/+}$ mice, total RNA was extracted from five sections of the small intestine, S1–S5 (S1 being the most proximal and S5 the most distal section), and from the colon of male and female Apc$^{Min/+}$ mice (n = 2 for each gender). The RNA was reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). QPCR was performed using Maxima Probe/Rox qPCR (Thermo Scientific). Expression of *Cysltr1*, estrogen receptor α and β mRNA and also PGE_2 receptors EP2 and EP4 mRNA were analyzed in males and females for each section of the intestinal tract using the ΔΔCt method and normalizing against *glyceraldehyde-3-phosphate dehydrogenase (Gapdh*).

Total RNA was extracted from frozen colon tissue in accordance with the manufacturer's instructions. The RNeasy Plus Mini kit from Qiagen was utilized (Hilden, Germany). cDNA was synthesized from 1.0 μg RNA by RevertAid H Minus M-MuLV Reverse Transcriptase (Thermo Scientific, Waltham, MA). QPCR was performed using Maxima probe/ROX qPCR master mix and 0.9 μM TaqMan probes. The TaqMan probes used for *ctnnb1 (β-catenin), ptgs2* (*COX-2)*, *hpgd (15-PGDH)*, *ccnd1 (cyclin D1)*, *alox5* (*5-LOX), ptprc (CD45), CD4, CD8α, IL-12α, arginase-1 (Arg-1), IFN-*γ, *IL-4* and *IL-10* gene expression were Mm00483039_m1, Mm00478374_m1, Mm00515121_m1, Mm01182747_m1, Mm00432359_m1, Mm01293577_m1, Mm00442754_m1, Mm01182108_m1, Mm00434169_m1, Mm00475988_m1, Mm01168134_m1, Mm00445259_m1 and

Mm01288386_m1, respectively (Applied Biosystem, Life Technologies, Waltham, MA). The Mx3005P Thermocycler was utilized for the amplification (Agilent Technologies, Inc., Santa Clara, CA, USA) using MxPro software (Invitrogen Corp, Carlsbad, CA, USA), and a comparative 2-(ΔΔCt) method was used to evaluate the data. All data were normalized against the housekeeping gene *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase; Mm99999915_g1) and vehicle control wild type mice.

QPCR reactions employing TaqMan gene expression assays were used to measure tumor tissue expression of CysLT1R (Hs00272624_s1), PTGER2 (Hs00168754_m1), PTGRR4 (Hs00168761_m1), Arginase-1 (Mm00475988_m1), IL-1β (Mm00434228_m1; Hs00174097_m1), IL-6 (Mm00446190_m1; Hs00985639_m1) and IL-10 (Mm01288386_m1) genes (Applied Biosystems, Cambridge, United Kingdom) as described above.

Statistical analysis

GraphPad Prism version 5.0a (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analyses. Paired or unpaired t test (Student's t test) was performed for comparison between two groups, while one-way or two-way ANOVA was used to compare more than two groups. P value ≤ 0.05 was considered significant. All data are presented as mean ± standard error of the mean (SEM).

Results and discussion

Paper I

Cysteinyl leukotriene 1 receptor influences intestinal polyp incidence in a gender-specific manner in the Apc Min/+ mouse model

Introduction

Inflammation is considered to play a role in cancer development. In CRC, the most common genetic mutation relates to the *APC* gene. Overexpression of the pro-inflammatory cysteinyl leukotriene D_4-receptor (CysLT1R) in colorectal cancers has been associated with poor prognosis (Magnusson et al, 2010). LTD_4-CysLT1R signaling has been shown to induce accumulation of nuclear β-catenin and proliferation colon cancer cells (Salim et al, 2014).

Results and discussion

This study utilized the $Apc^{Min/+}$ spontaneous colon cancer model, which develops polyps mainly in the small intestine and to a lesser degree in the colon. $Apc^{Min/+}$ mutated male and female mice were considered for this study with additional mutations in the *Cysltr1* (wildtype, heterozygous and homozygous knockout). Only females were heterozygous as the *Cysltr1* is located on the X-chromosome (Lynch et al, 1999). *Cysltr1*-disrupted female mice ($Cysltr1^{-/-}Apc^{Min/+}$, $Cysltr1^{+/-}Apc^{Min/+}$) developed significant fewer polyps in the small intestine compared to *Cysltr1*-wildtype females ($Apc^{Min/+}$), while no difference was seen in males with different *Cysltr1* genotypes. Female $Apc^{Min/+}$ mice developed almost three times as many small intestinal polyps compared to male $Apc^{Min/+}$ mice. No difference was seen in colonic polyps for all mice independent of *Cysltr1* genotype. In light of this, mRNA expression of intestinal *Cysltr1* was measured in male and female mice and an overall increased *Cysltr1* expression was seen in female mice. The protein expression in the small intestine of female mice was further investigated by IHC. Increased CysLT1R expression has been associated with increased COX-2 and 5-LOX expression in colorectal cancer tissue (Ohd et al, 2003). In the current study, expression of the 5-LOX enzyme, responsible for cysteinyl leukotriene synthesis, did not differ in $Apc^{Min/+}$ mice independent of *Cysltr1* genotype. However, expression of the COX-2 enzyme, responsible for prostaglandin synthesis, was decreased in *Cysltr1* knockout mice ($Cysltr1^{-/-}$

Apc$^{Min/+}$) compared to *Cysltr1* wildtype mice (*Apc*$^{Min/+}$). Increased COX-2 expression has been observed in a large number of colorectal adenomas and carcinomas (Eberhart et al, 1994). Furthermore, in *Apc*$^{\Delta 716}$ knockout mice, pharmacological inhibition of COX-2 was associated with decreased incidence and size of intestinal polyps (Oshima et al, 2001). Serum levels of PGE2 and CysLTs (LTC4, LTD4, LTE4) were significantly reduced in *Cysltr1*-disrupted female mice (*Cysltr1*$^{-/-}$*Apc*$^{Min/+}$, *Cysltr1*$^{+/-}$*Apc*$^{Min/+}$) compared to female *Apc*$^{Min/+}$ mice. *Cysltr1* disruption did not affect Mucin-2 expression in villi adjacent to polyps. Proliferation was measured by counting BrdU positive cells, however, no difference was observed between *Apc*$^{Min/+}$ female mice with different *Cysltr1* genotypes. In CRC, nuclear β-catenin is believed to be involved in progression of adenoma to carcinoma (Phelps et al, 2009). Female *Cysltr1* knockout mice (*Cysltr1*$^{-/-}$*Apc*$^{Min/+}$) displayed low expression of nuclear β-catenin and high expression of membranous β-catenin when compared to *Apc*$^{Min/+}$mice, which displayed high nuclear and low membranous β-catenin expression. A link between blocked PGE_2 synthesis and decreased nuclear β-catenin was seen in mouse aberrant crypt foci in a carcinogen-induced colon cancer model (Nakanishi et al, 2008). Further analysis of the immune infiltrate was conducted. Leukocyte polyp infiltration in the small intestine, measured by $CD45^+$ cells, did not differ between the *Cysltr1* genotypes. Further leukocyte analysis of small intestinal polyp areas revealed a tendency for increased $CD3^+CD8^+$ lymphocyte infiltration in *Cysltr1* knockout mice (*Cysltr1*$^{-/-}$*Apc*$^{Min/+}$).

Paper II

Cysteinyl leukotriene receptor 1 facilitates tumorigenesis in a mouse model of colitis-associated colon cancer

Introduction

A risk factor for developing CRC is chronic intestinal inflammation, as seen in ulcerative colitis patients (Guagnozzi & Lucendo, 2012). Nuclear accumulation of β-catenin together with KRAS activation promotes progression of colon adenoma to carcinoma (Phelps et al, 2009). We have previously reported that LTD_4-$CysLT_1R$ signaling in colon cancer cells induced nuclear accumulation of β-catenin, which induced migration (Salim et al, 2014).

Results and discussion

This study utilized the colitis-associated colon cancer model to investigate the role of $CysLT_1R$ in female wildtype and *Cysltr1* knockout (*Cysltr1*$^{-/-}$) mice. The AOM/DSS model induced colitis and colonic polyps in wildtype and *Cysltr1*$^{-/-}$ female mice. No polyps were seen in the vehicle control groups. In AOM/DSS treated mice a decreased colitis score, identified by decreased colonic weight: length ratio (mg/cm), was observed in *Cysltr1*$^{-/-}$ mice compared to wildtype mice. Furthermore, *Cysltr1*$^{-/-}$ mice exhibited colonic polyps of a smaller size (up to 1.5 mm in diameter) while wildtype mice exhibited polyps of a larger size (>1.5mm). Wildtype polyps were serrated adenomas while *Cysltr1*$^{-/-}$ polyps were low-grade dysplastic in nature, indicating a lower dysplasia in knockout mice. Additionally, *Cysltr1*$^{-/-}$ polyp epithelia exhibited a higher membranous β-catenin and lower nuclear β-catenin expression, with decreased 5-LOX and COX-2 expression compared to wildtype polyps. Further investigation confirmed a decreased mRNA *Cox-2* level with an increased mRNA level of the enzyme responsible for prostaglandin E_2 degradation, 15-hydroxyprostaglandin dehydrogenase (15-PGDH). In pre-neoplastic aberrant crypt foci, a reduction of Muc-2 positive cells is seen compared to the normal intestine (Otori et al, 1995; Siu et al, 1997; Pretlow et al, 2003). An increased expression of Muc-2 was seen in normal crypts adjacent to polyps of *Cysltr1*$^{-/-}$ mice compared to wildtype mice. A decreased tumor infiltration of $CD45^+$ leukocytes and $F4/80^+$ macrophages was seen in *Cysltr1*$^{-/-}$ mice, which, was supported by decreased mRNA levels of *CD45, CD4, Arg-1* and *IFN-γ* in *Cysltr1*$^{-/-}$ polyp areas. Inflammatory cytokines measured in both serum and colon tissue lysates, with identified polyps, exhibited decreased IL-1β, TNFα and CXCL1 levels in *Cysltr1*$^{-/-}$ mice. In *Cysltr1*$^{-/-}$ mice, low-dysplastic colonic polyps and decreased *CD4* and *Arg-1* levels could be connected as arginase expression in macrophages can be facilitated by $CD4^+$ Th2 cells to promote tumor growth and inhibition of tumor cytotoxicity (Munder, 2009). This together with decreased F4/80 tumor infiltration and decreased IFN-γ would indicate a decrease

in both type-1 and type-2 macrophages (Verbeke et al, 2011). In wildtype mice, LTD_4-CysLT1R signaling could account for the more dysplastic polyps, since LTD_4, as well as, M2 macrophage-medium and TNFα have been reported to induce colon cancer cell invasion via matrix metallopeptidase-9 (MMP-9) activation *in vitro* (Vinnakota et al, 2017). IL-1β and TNFα can further up-regulate CXCL1 levels, and in this study, all of these are increased in wildtype mice compared to knockout mice.

Paper III

The eicosanoids leukotriene D4 and prostaglandin E2 promote the tumorigenicity of colon cancer-initiating cells in a xenograft mouse model

Introduction

PGE_2 has been known to promote tumor growth in different mouse models of colon cancer (Wang & Dubois, 2010) and has been associated with poor prognosis in colon cancer patients (Rigas et al, 1993). The LTD_4-$CysLT_1$ receptor has been upregulated in colon cancer (Nielsen et al, 2003) and increased tumor expression of $CysLT_1R$ has been associated with poor prognosis in colon cancer patients (Ohd et al, 2003). High ALDH activity is considered to be a marker of CSCs (Wakamatsu et al, 2012). In previous research, LTD_4 and PGE_2 stimulation of $ALDH^+$ HCT-116 cells resulted in increased tumor growth and resistance to radiation and 5-fluorouracil treatment (Bellamkonda et al, 2015).

Results and discussion

The xenograft mouse model was utilized to study the effects of LTD_4 or PGE_2 administration on $ALDH^+$ HCT-116 colon cancer cells compared to a vehicle control group. Administration on LTD_4 or PGE_2 resulted in increased tumor weight and volume compared to the vehicle-$ALDH^+$ group. LTD_4 or PGE_2 stimulation resulted in increased protein expression of ALDH and the intestinal stem cell marker Dclk1 in xenografts. Additionally, increased expression of COX-2 and increased nuclear accumulation of β-catenin was seen in LTD_4 or PGE_2 treated xenografts, while no difference was seen in 5-LOX expression. Furthermore, analysis of xenografts by flow cytometry revealed that LTD_4 or PGE_2 treatment, compared to the vehicle control, resulted in a significant increase in the percentage of tumor infiltrating leukocytes ($CD45^+$), neutrophils ($CD45^+Ly6G^+$), $CD4^+$ cells ($CD45^+CD4^+$), macrophages ($CD45^+F4/80^+$) and specific M2-type macrophages ($F4/80^+CD206^+$). Further analysis revealed increased F4/80 immunoreactive scores by immunohistochemistry and increased mRNA expression of arginase-1 were observed for LTD_4 or PGE_2 treated xenograft tumors. PGE_2 has been found to have a part in M2-macrophages polarization towards the pro-tumorigenic type (Mantovani et al, 2017). The effect of LTD_4 or PGE_2 administration on xenograft tumors was evaluated by measuring plasma and mRNA levels of tumor cytokines. LTD_4 or PGE_2 treatment induced increased IL-1β and IL-6 plasma and mRNA levels, while only inducing increased plasma levels of IL-2 and CXCL1. Increased IL-10 plasma and mRNA levels were only induced by LTD_4 treatment, with no effect by PGE_2 treatment and increased TNFα plasma levels were only induced by PGE_2 treatment, with no effect by LTD_4 treatment. Moreover, increased plasma levels were seen for both CysLTs

and PGE_2 with either LTD_4 or PGE_2 administration. Further in vitro analyses were conducted where HCT-116 cells were FACS sorted into $ALDH^+$ or $ALDH^-$ cells and treated for 48 hours with LTD_4 or PGE_2. The mRNA expression of the receptors for LTD_4 (*CYSLT1R*) and PGE_2 (*PTGER2, PTGER4*) were then analyzed. The mRNA level of *CYSLT1R* was increased in ALDH+ cells compared to the $ALDH^-$ cells, and only LTD_4 stimulation increased *CYSLT1R* expression in $ALDH^+$ cells. The mRNA level of *PTGER2* was increased in ALDH+ cells compared to the $ALDH^-$ cells, and both LTD_4 and PGE_2 increased *PTGER2* relative to vehicle in $ALDH^+$. *IL-1β* was increased for each treatment group in $ALDH^+$ cells when compared to $ALDH^-$ cells, with no difference between treatment groups for $ALDH^+$ cells, as was seen *in vivo* probably due to tumor infiltrating immune cells. The mRNA level of *IL-6* was increased in both $ALDH^+$ and $ALDH^-$ cells due to PGE_2 treatment when compared to vehicle and due to LTD4 treatment when compared to vehicle in $ALDH^+$ cells. Together, these data indicate a role of macrophages in releasing inflammatory mediators that promote CSC in colon cancer.

Paper IV

The WNT5A Agonist Foxy5 Reduces the Number of Colonic Cancer Stem Cells in a Xenograft Mouse Model of Human Colonic Cancer

Introduction

Cancer stem cells are known to resist radiation therapy and chemotherapy, and are believed to be the cause of cancer treatment failure (Boman & Wicha, 2008). In CRC patients, increased tumor expression of WNT5A and 15-PGDH was associated with better survival, and *in vitro* administration of Foxy5 (a WNT5A mimicking peptide) resulted in increased 15-PGDH expression (Mehdawi et al, 2016b).

Results and discussion

The xenograft mouse model was utilized to study the effect of Foxy5 on the human colon cancer cells, HT-29 and Caco-2. Foxy5 treatment reduced the expression of the stem-cell marker ALDH and the specific colon CSC marker DCLK1 in both HT-29 and Caco-2 cells. In accordance with this, reduced *ALDH* and *DCLK1* mRNA levels were also seen in Foxy5 treated xenograft tumors. This supports the role of WNT5A as a tumor suppressor in colorectal (Zhou et al, 2017). In CRC patients, PGE_2 was reported to have a positive correlation with several CSC markers and promoting CSC numbers (Wang et al, 2015). In the xenograft study, Foxy5 treatment reduced tumor expression of COX-2 and increased tumor expression of 15-PGDH in HT-29 and Caco-2 xenografts. Both enzymes target PGE_2, and since 15-PGDH has been reported as a tumor suppressor (Mehdawi et al, 2016b), these results indicate a decrease in PGE_2 with decreased expression of colonic CSC markers (Wang et al, 2015). Nuclear β-catenin has been associated with poor CRC patient prognosis (Salim et al, 2013). Foxy5 treatment also reduced the expression of nuclear β-catenin and ASCL2, which have been reported to result in the promotion of stemness (Schuijers et al, 2015). Foxy5 has an inhibitory role regarding the decreased colon CSC numbers, probably due to inhibition of β-catenin and PGE_2 signaling.

Paper V

The role of CysLT$_1$R signaling in colon cancer metastasis

Introduction

In xenograft models of colon cancer a decreased tumor burden was seen due to treatment with the CysLT$_1$R inhibitor, montelukast (Savari et al, 2013). In previous thesis studies, we observed a decreased tumor burden with low-dysplastic tumors in mice lacking functional CysLT$_1$R (*Cysltr1*$^{-/-}$). Furthermore, in xenograft mice, LTD$_4$ and PGE$_2$ administration promoted tumor growth and tumor infiltration of immune cells, which together are believed to contribute to CSCs.

Results and discussion

This study utilized a syngeneic mouse model of colon cancer metastasis, xenograft zebrafish model of metastasis and patient data. MC38 colon cancer cells were confirmed to express the CysLT$_1$R by Western blot, and proliferation was measured in the presence of different montelukast concentrations and DMSO control. No increased cytotoxicity was observed for MC38 cells with increasing montelukast concentrations. Next, montelukast block of CysLT$_1$R signaling restricted trans-well migration of MC38 cells. In mice lacking the CysLT$_1$R (*Cysltr1*$^{-/-}$) and in mice treated with montelukast, a decreased incidence of liver metastatic foci was seen. In the zebrafish xenograft model of metastasis LTD$_4$ treatment caused an increase in metastatic spread while montelukast treatment or knockdown exhibited a decreased metastatic spread. Also, CRC patients with high CysLT$_1$R expression displayed an increase in tumor extent, TNM stage, lymph node involvement and metastatic spread.

Conclusions

I. The results indicate a decreased tumor burden in female, and not male *Apc*$^{Min/+}$ mice, which also lack functional $CysLT_1R$ by exhibiting decreased levels of COX-2 and PGE_2 together with a decreased nuclear accumulation of β-catenin. Our results highlight the therapeutic possibility that $CysLT_1R$ inhibition can have in colorectal cancer.

II. The results indicate that mice lacking with induced colitis associated colon cancer had more differentiated tumors exemplified by more membranous accumulation of β-catenin, increased mucin 2 expression and decreased immune infiltration.

III. The results indicate that the inflammatory lipid mediators, LTD_4 and PGE_2, cause CICs to promote tumor growth, which is crucial to target in cancer therapy.

IV. Foxy5 points to having tumor suppressor function on colon CSC. The results indicate that it could be plausible to co-administer Foxy5 with standard adjuvant chemotherapy to sensitize resistant CSCs responsible for recurrence.

V. Inhibition of $CysLT_1R$ signaling resulted in decreased metastasis, which indicates that inhibition of the $CysLT_1R$ could be beneficial for CRC patient outcome.

Conflict of interest

Tommy Andersson is a shareholder of WntResearch AB and is the part-time Chief Scientific Officer of WntResearch AB. However, this academic thesis and the corresponding paper published in Anticancer Research was sponsored by Lund University, and the author's adhered to all guidelines for publication.

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