Immune Cell Profiling of Colorectal Cancers: Unravelling the Connection to Treatment Responses

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Cover illustration: Immune cell infiltration in rectal cancer; blue: nuclei, red: tumor cells, green: MxA^+ cell, light blue: T cells, magenta: cytotoxic T cells, orange: γδ T cells. © Azar Rezapour 2023

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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie

تقدیم به خانواده محبوبم که همواره با عشق چراغ راه و پشتیبانم بودند.

To my beloved family

ABSTRACT

Understanding the interplay between tumor cells and the immune system holds the key to developing more effective treatments for cancer patients. Therefore, we have emphasized our efforts on deciphering the intricacies of these interactions to pave a way for more effective therapeutic strategies. We observed that different microenvironments in tumor and adjacent colonic tissues influence immune cell behavior differently. Notably, cells known as antigen-presenting cells within the tumor show different activation and functional status compared to those in the colon. Despite this, they react similarly when exposed to signals from activated T cells, immune cells responsible for attacking cancer cells.

The challenge remains in predicting how patients with rectal cancer will respond to treatments. Several studies have highlighted the potential of using tumor-infiltrating lymphocytes (TILs) as markers to predict treatment outcomes. An emerging predictive tool, the biopsy-adapted Immunoscore (IS_B), assesses TILs to forecast tumor regression. To enhance its accuracy, we used a method incorporating multiplex immunofluorescence. By focusing on certain T cell subsets and the expression of Myxovirus resistance protein A (MxA), a component indicative of anti-tumoral inflammation, this refined method provided a better predictive framework. Specifically, the presence of MxA^+ cells in the tumor stroma, combined with the density of $CD8^+$ T cells, offered improved predictions regarding patients with a complete response to neoadjuvant treatment i.e., no detectable rectal cancer after chemoradiotherapy.

However, while TIL density and the presence of MxA-expressing cells provided insights regarding complete responders, our approach did not distinguish non-responders from treatment responders. A more nuanced approach revealed that non-responders had a higher proportion of a particular T-cell subset, $CD8^+CD103^+39^-$, present in the tumor but not in paired rectal tissue prior to treatment. In addition, non-responders exhibited a lower expression of PD-1 on TILs compared to responders, indicating an inadequate tumor microenvironment for immune cell activation. However, when T cells were stimulated *in vitro*, their responses were overall similar, regardless of how the tumor responded to the neoadjuvant treatment in the patient.

In conclusion, while tools like the IS_B offer predictive insights, refining these with multiparametric immune activity screens of the pre-operative biopsy can provide a more accurate picture of the patient's ensuing responses to neoadjuvant treatment. This has significant implications for patient stratification and could possibly lead to the development of more personalized treatment regimens of rectal cancers.

Keywords: Rectal cancer, tumor infiltrating T cells, MxA, Interferon type I, CD39, CD103, PD-1, cytokines, response to the treatment.

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SAMMANFATTNING PÅ SVENSKA

Att bekämpa cancer är en av de största utmaningarna inom medicinen. Forskare runt om i världen arbetar outtröttligt för att förstå hur våra egna kroppars försvar kan spela en roll i denna kamp. I mitt arbete har jag djupdykt i detta spännande området där våra immunceller (vita blodkroppar) – som normalt bekämpar infektioner - möter och interagerar med cancerceller. Jag har fokuserat på hur immunceller infiltrerar änd-och tjocktarmstumörer. Genom att förstå detta möte hoppas vi hitta nyckeln till att skapa bättre och effektivare behandlingar för de som lider av cancer.

Vi kunde påvisa att fagocytiska celler, som flaggar på sin yta delar av tumörceller som de ätit upp för att leda immunförsvaret till attack mot tumören har inte samma förmåga inuti en tjocktarmstumör som om de kommer från närliggande tarmvävnad. Immuncellerna har alltså nedsatt funktion i tumören. Trots dessa skillnader, när fagocyterna ställs inför en grupp av andra immunceller - T-lymfocyter som kan avdöda cancercellerna – så reagerar de likvärdigt mot signaler utsöndrade från aktiverade T-lymfocyter.

Patienter med ändtarmscancer ges oftast cellgifts- och strålbehandling innan tumörområdet avlägsnas kirurgiskt. Hur väl patienter svarar på denna förbehandling skiljer sig dock avsevärt – allt från att tumören helt försvinner till att den oförändrat växer vidare. Ett särskilt intresse har riktats mot T-lymfocyter som kan tränga in i tumören och attackerar den. Kan närvaron av dessa i tumören innan behandling hjälpa oss att förutsäga hur en patient kommer att svara på behandling? Flera forskningsstudier har föreslagit att mätningar av tumörinfiltrerande T-lymfocyter (TILs) skulle kunna användas till just detta. Vi har därför utgått från en metod som kallas "Immunoscore" för att utvärdera om det kan hjälpa oss att förutse om en tumör kommer att försvinna i svar på behandling. Denna metod räknar antalet TILs och var de befinner sig i en preoperativ biopsi. För att göra denna förutsägelse ännu bättre, har vi använt utvecklat metoden till att mäta flera olika faktorer samtidigt. Vi har särskilt fokuserat på en grupp T-lymfocyter så kallade cytotoxiska T celler ($CD8^+$) och ett protein som kallas MxA, (Myxovirusresistensprotein A) som kan berätta för oss vilken typ av inflammation som pågår i tumören. Kombinationen av en kvantifiering av dessa faktorer gav oss en tydligare bild av vilka patienter som sannolikt kommer att ha ett komplett svar på behandlingen, det vill säga de fall där tumören försvinner helt efter strål- och cellgiftsbehandling.

Trots dessa framsteg är det dock fortfarande svårt att urskilja de patienter som *inte* kommer att svara väl på behandlingen. Genom att analysera det immunologiska landskapet djupare kunde vi visa att patienter vars cancer inte dragit sig tillbaka efter behandling hade ett högre antal av en särskild typ av

T-cell lymfocyter ($CD8^+CD103^+CD39^-$) i tumören före behandling, men inte i opåverkad ändtarmsvävnad från samma patient. Dessutom uppvisade denna grupp av patienter ett lägre uttryck av en molekyl PD-1 på ytan av TILs jämfört med de som svarar på behandlingen. PD-1 fungerar som en strömbrytare som kan dämpa immunsvaret när detta är aktiverats för kraftigt. När TILs från biopsierna aktiverades utanför kroppen, i laboratoriet, utsöndrades effektormolekyler dock likvärdigt oavsett hur tumören svarade på behandlingen. Detta avslöjar att tumörcellerna och/eller närmiljön skiljer sig mellan patienterna och att faktorer lokalt kan påverka T-lymfocyternas förmåga.

Sammanfattningsvis, tyder vår forskning på att medan verktyg som Immunoscore ger oss viktiga ledtrådar, kan vi genom ytterligare mätningar av biopsin få en ännu skarpare bild av hur patienter kommer att reagera på behandling. Detta kan i sin tur leda till bättre skräddarsydda behandlingar av ändtarmscancer – ett steg närmare individanpassad medicin.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Liang, F. Rezapour, A. Szeponik, L. Alsén, S. Wettergren, Y. Bexe Lindskog, E. Quiding-Järbrink, M. Yrlid, U. **Antigen Presenting Cells from Tumor and Colon of Colorectal Cancer Patients Are Distinct in Activation and Functional Status, but Comparably Responsive to Activated T Cells**. *Cancers* **2021**, *13*, 5247.

DOI:10.3390/ cancers13205247

II. Rezapour, A. Rydbeck, D. Byvald, F. Tasselius, V. Danielsson, G. Angenete, E. Yrlid, U. **A Type I Interferon Footprint in Pre-Operative Biopsies Is an Independent Biomarker That in Combination with $CD8^+$ T Cell Quantification Can Improve the Prediction of Response to Neoadjuvant Treatment of Rectal Adenocarcinoma.** *OncoImmunology* **2023**; May10;12(1):2209473

DOI:10.1080/2162402X.2023.2209473

III. Rezapour, A. Tasselius, V. Danielsson, G. Falk, P. Angenete, E. Yrlid, U. **PD-1 levels and CD103+CD39- proportions among CD8 T cells as predictors of non-responders to neoadjuvant treatment of rectal cancer.** (Manuscript)

CONTENT

ABBREVIATIONS

AJCC	American joint committee on cancer
APC	Adenomatous polyposis coli
APCs	Antigen presenting cells
ARG1	Arginase-1
ATP	Adenosine triphosphate
BMI	Body mass index
CAR	Chimeric antigen receptors
Caspase	Cysteine-aspartic proteases
CCL	CC chemokine ligand
CD	Cluster of differentiation
cGAS	Cyclic GMP-AMP synthase
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus molecular subtypes
CpG	5'—C—phosphate—G—3'
CRC	Colorectal cancer
CT	Computing tomography
CT	Tumor center
CTL	Cytotoxic T cells
CTLA-4	Cytotoxic T-lymphocyte–associated antigen 4
CTNNB1	β-catenin gene
CXCR	C-X-C Chemokine receptor
DAB	Diaminobenzidine
DAMP	Damage association molecular pattern
DC	Dendritic cells
DCC	Deleted in colorectal cancer gene
DFS	Disease-free survival
dMMR	Mismatch repair deficient
DMSO	Dimethyl sulfoxide
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin-embedded

FMO	Fluorescence minus one
FOXP3	Forkhead box P3
GATA-3	GATA binding protein 3
GI	Gastro intestinal
GrzB	Granzyme B
Gy	Gray
HBSS	Hanks' balanced salt solution
HLA	Human leukocyte antigen
HMGB-1	High mobility group box 1
HNPCC	Hereditary non-polyposis colorectal cancer
HRP	Horseradish peroxidase
ICI	Immune checkpoint inhibitor
IDO	Indoleamine 2,3-dioxygenase
IF	Immunofluorescence
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IM	Invasive margin
iNOS	Inducible nitric oxide synthase
IS	Immunoscore
ISB	Biopsy-adapted Immunoscore
KRAS	Kirsten rat sarcoma virus gene
LAG-3	Lymphocyte-activation gene 3 protein
LPS	Lipopolysaccharide
MAIT	Mucosal-associated invariant T cells
MCP-1	Monocyte chemoattractant protein-1
MCSF	Macrophage colony-stimulating factor
mDC	Myeloid dendritic cells
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MLH1	MutL homolog 1
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MSH	MutS homolog
MSI	Microsatellite instability
MSS	Microsatellite stability
MUTYH	mutY homolog
MxA	Myxovirus resistance protein A
NET	Neutrophil extracellular traps

NK	Natural killer
NKT	Natural killer T
NSAID	Non-steroidal anti-inflammatory drug
nT	Neoadjuvant treatment
pAPC	Professional antigen presenting cell
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
pDC	Plasmacytoid dendritic cells
PET	Positron emission tomography
PFA	Paraformaldehyde
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
pMMR	proficient mismatch repair
PMN	Polymorphonuclear neutrophils
PMS2	Post meiotic segregation increased 2 gene
RORgt	Retinoic acid-related Orphan Receptor γ
ROS	Reactive oxygen species
SMAD	Suppressor of mothers against decapentaplegic
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TAM	Tumor associated macrophages
TAN	Tumor associated neutrophils
TCF	T cell transcription factor
T_{CM}	Central memory T cells
TCR	T cells Receptors
T_{EM}	Effector memory T cells
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domain
TIL	Tumor infiltrating lymphocytes
TIM3	T cell immunoglobulin and mucin domain 3
TLR	Toll-like Receptor
TLS	Tertiary lymphoid structures
TME	Tumor microenvironment
TNF	Tumor necrosis factor

TP53	Tumor Protein 53
T_{PM}	Peripheral memory T cells
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory
TRG	Tumor Regression Grade
T_{RM}	Resident memory T cells
TSA	Tyramide signal amplification
UICC	Union for international cancer control
VEGF	Vascular endothelial growth factor
WHO	World health organization
Wnt	Wingless-related integration site

1 INTRODUCTION

1.1 Brief Introduction

Cancer is a generic term for uncontrolled cell division that originates from malignantly transformed healthy human cells, a transformation caused by an accumulation of mutations. These mutations are often involved in cell proliferation and survival [1], that can disrupt tissue homeostasis leading to invasive cancer growth [2]. Such transformed cells have a potential to invade or spread to the surrounding tissue or other distant parts of the body; a process known as metastasis. Without proper therapeutic and/or surgical interventions the prognosis of the disease is poor. Beyond the basic definition, cancer is an intricate and dynamic disease delineated by fundamental hallmarks introduced by Hanahan and Weinberg [3]. These encompass sustaining growth signalling, insensitivity to growth suppressors, enabling replicative immortality, resisting apoptosis, sustaining angiogenesis, tissue invasion and metastasis, evasion from immunosurveillance, and reprogramming of energy metabolism [4,5] (Figure 1).

Colorectal cancer (CRC) originates from epithelial cells in the colon or rectum and continues to be one of the largest leading causes of cancer-related mortality globally. The crucial role of the tumor microenvironment (TME), including the bystander effects caused by non-cancerous nearby cells such as stromal cells, and the interactions between tumor cells and immune cells has over the last decade been highlighted in various forms of cancer [6-8]. Several studies have found that in the landscape of oncology, the immune system is a double-edged sword as it protects against tumor formation but can also inadvertently facilitate tumor growth [9-11]. Thus, a deeper comprehension of what roles the different immune cells that infiltrate CRCs play during the initiation and pro- or regression following treatment is of great importance.

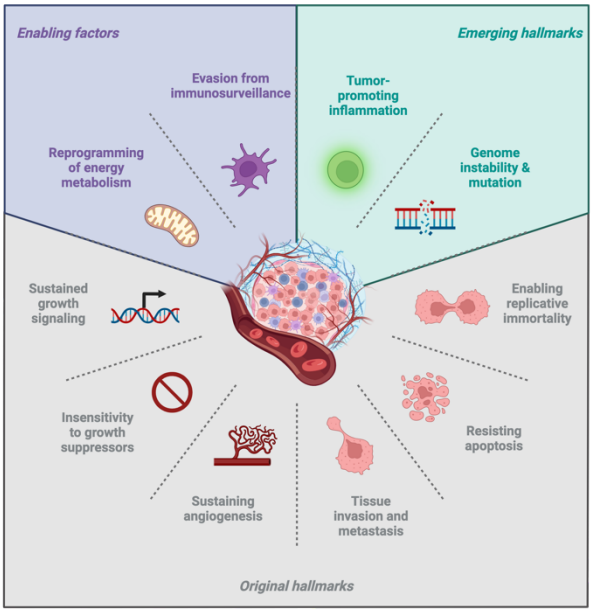

Figure 1: Conceptual Diagram Illustrating the Hallmarks of Cancer. The original hallmarks are depicted at the bottom. The enabling factors, are represented at the top left, and the emerging hallmarks, highlighted at the top right. Retrieved from https://app.biorender.com/biorender-templates.

1.2 Colorectal Cancer

The focus of this thesis is on colorectal adenocarcinoma, which comprises lesions originating from various parts of the colon (cecum, proximal, transverse, and distal) and the rectum. A special emphasis is placed on the latter. However, due to the similarities in fundamental characteristics between the colon and rectum, the cancers at these two locations are often grouped together as CRC in this section.

1.2.1 Epidemiology

CRC accounts 10% of all cancer cases worldwide. Out of these cases, approximately two-third are colon cancer, and the rest are rectal cancer [12]. Although a decrease in mortality rate of CRC over the past 20 years has been reported in many countries [13,14] and the life expectancy of patients has improved, CRC is still ranked as the second leading cause of total malignancy deaths worldwide [15]. Due to the common practice of recording cases of rectal cancer as colon cancer in mortality statistics at least in United States, there's potential for inaccuracy in cancer-specific mortality rates. As such, reporting the combined mortality rate for colon and rectal cancer is deemed more accurate for global discussions [16]. However, Swedish data differentiate between the two sites [17].

In fact, 1 out of 24 in the population will be diagnosed with CRC during their life [15] and this incidence is estimated to be even higher in northern Europe [18]. Today the median age of CRC patients is around 70, but due to an age-shift towards younger adults the median has dropped over the last two decades [19].

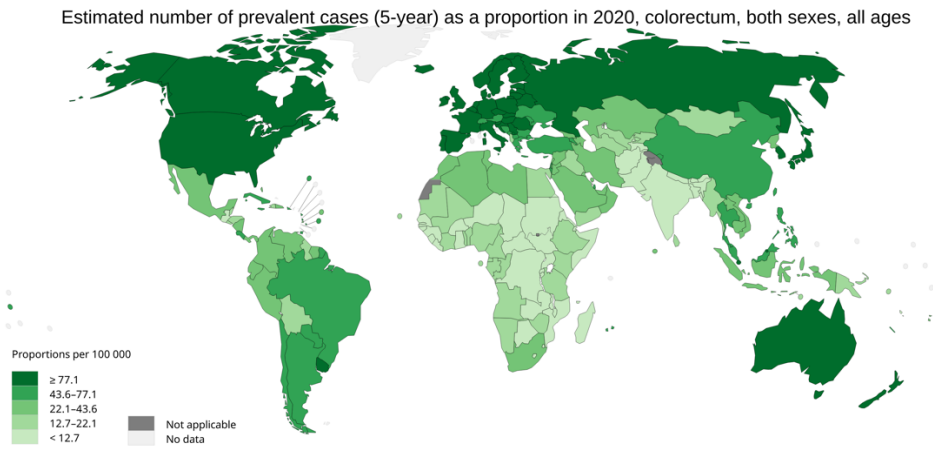

Figure 2: Estimated Number of Prevalent Cases (5-year) of Colorectal Cancer as a Proportion (both sexes and all ages) in 2020. (Data source, GLOBOCAN 2020; http://gco.iarc.fr/today) World Health Organization.

1.2.2 Etiology and risk factors

The incidence of CRC is influenced by a range of modifiable and unmodifiable factors. The Human Development Index (HDI), a metric that evaluates countries' average achievements in health, education, and income, provides insights into CRC risk [12,20]. As countries have progressed up the HDI scale, there has been a shift from infection-based to life-style oriented cancer causes [21]. Countries with a high HDI indicate a move towards cancers predominantly influenced by modifiable factors, i.e. western lifestyles like CRC [21]. The upswing in lifestyle-related CRC cases is alarming. Reports estimate that by 2040 there could be a rise in CRC incidence in developed and developing countries by 50% and 80%, respectively [22]. This rise is chiefly due to life style changes with primary contributors being western-style diets, habits like smoking and excessive alcohol consumption, as well as low physical activity [23,24]. Factors such as obesity [25] and type II diabetes mellitus (primarily due to its association with hyperinsulinemia [26]), are also contributing to an elevated risk of developing CRC [27]. In contrast, some evidence support that calcium, vitamin D and vitamins of group B, have protective effects against CRC [28].

Among unmodifiable factors, the impact of genetics is undeniable. Remarkably, only about 5% of all CRC cases can be traced back to specific genetic predispositions in the setting of Mendelian inheritance syndrome, notably familial adenomatoses polyposis and Lynch syndrome [29,30]. Cases that are not directly associated with known genetic conditions are often categorized based on family history. Namely, having first-degree relatives diagnosed with CRC, at any point in life, raises an individual's own susceptibility to the disease [31,32].

Chronic inflammatory conditions, like Ulcerative Colitis and Crohn's disease, are known to increase the risk of CRC development [26,33]. This increased risk is likely attributed to the amplified cell turn over resulting from chronic inflammation and subsequent rise in sporadic mutations[26]. Medications, like non-steroidal anti-inflammatory drugs (NSAIDs), are associated with reduced CRC risks, but their adverse side effects such as the risk of gastrointestinal (GI) bleeding make them unsuitable for routine prophylactic use over an extended time [26]. The increase in CRC incidence, along with other types of cancer, is partly due to the ongoing increase in life expectancy, since aging is also tied to CRC onset [26].

Certain demographic factors, such as gender, play a pivotal role. Men are found to have a 30% heightened rectal cancer risk compared to women [12]. Although the exact cause of this variation between males and females is not yet clear, it has been suggested that testosterone and estrogen levels might play a role [34]. Body mass index (BMI) has a significant association with rectal cancer in males but not in females [26]. Alcohol consumption increases rectal cancer risks in women and distal colon cancer in men [26]. Compared to non-smokers, smoking increases the risk of rectal cancer rather than colon cancer [26,35] with a greater risk for women [26,36]. Use of NSAIDs like Aspirin was significantly associated with reduced risk of rectal cancer rather than colon cancer [35]. Other risk factors were distributed similarly between colon cancer and rectal cancer [26].

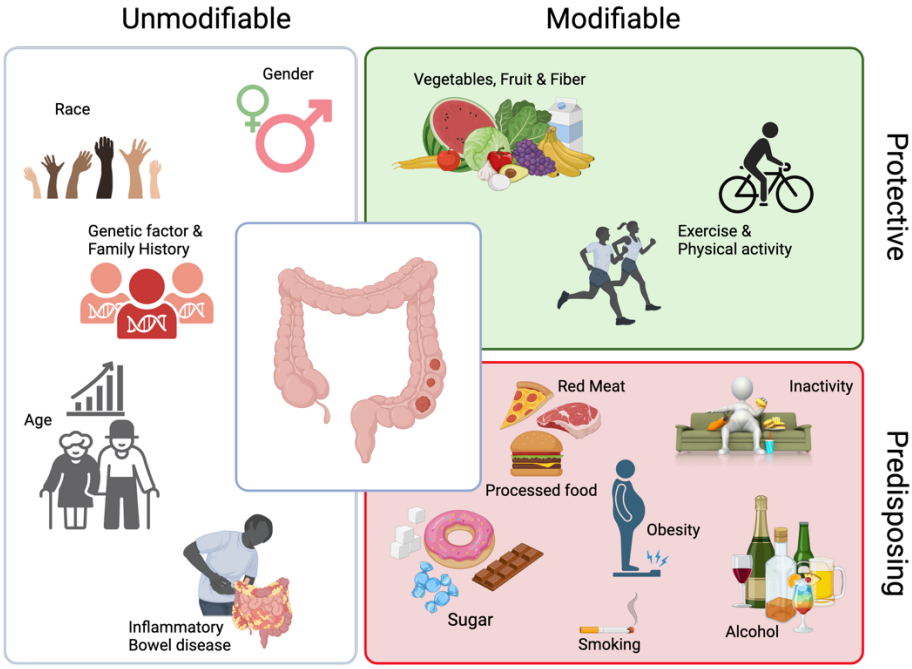

Figure 3: Factors Influencing the Risk of Colorectal Cancer (CRC). Unmodifiable factors include gender, age, family history of CRC, genetic predispositions, and chronic inflammatory states. Modifiable risk factors include obesity, smoking, alcohol consumption, high sugar intake, and diets rich in red meat and processed foods. Conversely, regular exercise and diets high in vegetables, fruits, and fiber offer protective benefits against CRC. Created with BioRender.com

1.2.3 Anatomical and histopathological features of CRC

The colon spans approximately 1.5 meters in length. It begins at the cecum and comprises different sections: The ascending colon, extending from cecum to right colic (hepatic) flexure; the transverse colon running from right colic flexure to left colic (splenic) flexures; the descending colon, stretching from splenic flexure to sigmoid colon [37]. The majority of polyps originate in the left-sided colon [38,39]. However, these protrusions can develop anywhere in the colon. The polyp formation and how it develops to cancer lesion will be discussed further in section 1.4.

The superior mesenteric artery supplies blood to the right side of the colon, while the inferior mesenteric artery caters to the left side and the

sigmoid colon [40]. Venous drainage in these areas follows similar divisions with the superior and inferior mesenteric vein [40]. Unlike the colon, which physiologically plays the primary role in the absorption of water, electrolytes and some vitamins, the rectum serves as a reservoir for feces until defecation [41]. The rectum spans approximately 12 to 15 cm extending from the rectosigmoid to the anorectal junction [42].The vascular supply of the rectum is distinct from that of the colon. The proximal, middle and lower sections are supplied by the superior middle, and inferior rectal arteries respectively [43]. Adequate blood flow to the colon and rectum is maintained by collateral circulation, an important consideration during cancer surgeries [40]. The rectum has a dual venous drainage system. The majority of the rectum drains into the portal vein. In contrast, the distal section drains into the systemic circulation. Lymph from the colon and upper rectum drains to the mesenteric nodes, while the lower rectum drains to the internal iliac nodes [40,42].

From a clinical perspective, CRC is traditionally subdivided based on its anatomical location. It can also be classified histologically, and further classification according to molecular subtype will be discussed in section 1.4. CRCs display significant heterogeneity and are characterized by three primary types [44,45]. The most prevalent type is classical colorectal adenocarcinoma developing from the epithelial layer of the colon/rectum and constituting more than 90% of the cases [46]. Sporadic occurrences are observed in two-thirds of CRC cases, while the remaining cases shows some patterns of hereditary predisposition [30]. Several recognized hereditary conditions lead to CRC and include familial adenomatous polyposis (FAP), hereditary nonpolyposis ccolorectal cancer (HNPCC; also known as lynch syndrome), Peutz-jeghers syndrome, MUTYH-associated polyposis, juveline polyposis syndrome, serrated polyposis syndrome, and hereditary mixed polyposis syndrome [30,47]. While FAP is associated with mutation in the tumor suppressor gene APC and usually develops in distal colon and rectum, HNPCC results from mutation in the DNA mismatch repair (MMR) genes and with 15% of the cases found in the rectum [48]. Mutation in various MMR genes regulate cell growth and survival. Crypts are epithelial invaginations that encircle the villus within the gut. The crypt base acts as the hub for actively proliferating progenitor cells, which is vital for the ongoing self-renewal of the gut epithelium [49]. The direct

descendants of these stem cells proliferate a finite number of times leading to the formation of a group of rapidly multiplying cells located just above the base stem cells. Since differentiated epithelial cells of colonic crypts structure have a short half-life, a significant amount of colon epithelial cells need to be continuously regenerated throughout life [49]. If any mutation affects the life cycle of these cells, it could initiate the development of a polyp lesion. Further mutation can then drive malignant transformation. The polyp formation and how it develops into a cancer lesion will be discussed further in section 1.4. Mucinous adenocarcinoma (10-20% of CRC patients [50]), characterized by abundant pools of extracellular mucin [51], and signet cell carcinoma (<1% [52]) are two less common variants of cancer in the colon and rectum [44,45]. These are typically located in the ascending colon [48] and they are more frequently seen in males and younger patients [44]. The risk of metastasis to regional lymph nodes and the peritoneum at the time of diagnosis is higher in these tumors [45,53]. Lastly, squamous cell, neuroendocrine, adenosquamous, spindle cell, medullary carcinoma and undifferentiated tumors are all rare subtypes of cancer in the colon and rectum [44]. The prevalence of none of these subsets account for < 0.5% of all cancers in colon and rectum [54-58].

Epithelial-mesenchymal transition (EMT) is a biological process wherein epithelial cells gradually lose characteristics and transform into mesenchymal cells, acquiring migratory and invasive capabilities [59]. CRC can undergo EMT, depart from the primary tumor site, utilize the circulatory system to migrate and subsequently form secondary lesions elsewhere in the body [60]. In addition to hematogenous spread, CRC can also metastasize locally through adjacent tissue [60]. Even though metastasis can occur in any tissue or organ including brain [48,60], typically, due to colon's venous drainage via the portal system, the liver is the primary site of CRC hematogenous spread, followed by lungs and bones [48]. However, tumors originating in distal rectum, appear to primarily spread to the lungs. This behavior is attributed to the drainage of the inferior rectal vein into the inferior vena cava, instead of the portal venous system [48,61]. Moreover, rectal cancers located in lower parts of the rectum demonstrate increased rates of loco-regional recurrence [49].

1.2.4 Clinical parameters and diagnosis

Chronic abdominal pain, altered bowel habits, rectal bleeding, and anemia, are the most common clinical manifestations in CRC [62]. While fecal occult blood test and colonoscopy stand out as the most effective screening methods [26,63] for preventing CRC and decreasing CRC-associated mortality [26,64], specifically in hereditary and colitis-associated types, the conclusive diagnosis relies on pathological evaluation of colonoscopy-guided biopsies. This analysis determines the lesion types and grade of differentiation.

The TNM system set by the American Joint Committee on Cancer / Union for International Cancer Control (AJCC/UICC), is an anatomical based tumor staging classification, that clarifies tumor progression [65]. Moreover, it guides clinicians in subsequent treatment of malignancies (Table 1). However, it cannot anticipate post-operative outcome [66]. In this system, the focus is on tumor size (T), the involvement of regional and draining lymph nodes (N), and evidence for distant metastasis (M) [67]. Together these facts determine the overall TNM-stage of the cancer (Table 2). Importantly, the immune system has the potential to eliminate tumor cells and whether this can be unleashed is not considered by the TNM classification.

Table 1. TNM classification of colorectal cancer

T: Primary tumor size	
TX	Tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ:* Involvement of lamina propria
T1	Invasion to submucosa
	Subclassification of T1
	Sm1: Invasion to 1/3 submucosa
	Sm2: Invasion to 2/3 submucosa
	Sm3: Invasion involved entire submucosa
T2	Invasion to the muscularis propria
T3	Invasion to the muscularis propria and pericolorectal tissue
	Subclassification of T3
T3a	T3a: Minimal invasion, <1 mm outside the muscle propria boundary
T3b	T3b: Mild invasion, 1-5 mm outside the muscle propria boundary
T3c	T3c: Moderate invasion, 5-15 mm outside the muscle propria boundary
T3d	T3d: Severe invasion, >15 mm outside the muscle propria boundary
T4a	Penetration through the surface of visceral peritoneum
T4b	Invasion to other adjacent organs and tissue

N: Lymph node involvement	
NX	Lymph node involvement can't be assessed
N0	No regional lymph node involvement
N1	Metastasis to 1-3 regional lymph node
N1a	Metastasis to 1 regional lymph node
N1b	Metastasis to 2-3 regional lymph node
N1c	Tumor deposits in subserosa, mesentery, or nonperitonealized pericolic or perirectal/mesorectal tissue
N2	Metastasis to 4 or more regional lymph node
	N2a: Metastasis to 4-6 or more regional lymph node
	N2b: Metastasis to 7 or more regional lymph node

M: Distant metastasis	
M0	No distant metastasis
M1	Metastasis identified
M1a	Metastasis in one organ or tissue identified without peritoneal involvement
M1b	Metastasis to more than one organ or tissue identified without peritoneal involvement
M1c	Metastasis to peritoneal surface identified alone or with other site or organ involvement

Nationellt vårdprogram tjock- och ändtarmscancer [68].8th edition of the UICC TNM Classification of Malignant Tumours.

Table 2: Tumor Stage

Stage	T	N	M
0	Tis	N0	M0
I	T1/T2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T2	N1/N1c	M0
	T1	N2a	M0
IIIB	T3-T4a	N1/N1c	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N2b	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b

Nationellt vårdprogram tjock- och ändtarmscancer[68]. 8th edition of the UICC TNM Classification of Malignant Tumours.

The optimal clinical assessment of patients often requires a tailored approach to imaging methods, based on clinical situation. Computed tomography (CT), magnetic resonance imaging (MRI) and less commonly positron emission tomography PET are techniques that can aid in understanding the spread pattern of the tumor [69]. Additionally, colonoscopic examinations are essential for identification and removal of pre-malignant polyps. This approach plays a crucial role in reducing the future risk of CRC onset in high-risk patients [70].

Pathological differentiation grade is another important aspect of tumor cells. This grading typically divides tumors into three categories based on the percentage of gland formation and their resemblance to normal cells [71]. Grade 1 (G1) tumors are well-differentiated and closely resemble normal tissue; they generally grow and spread at a slower pace. Conversely, grade 3 (G3) tumors are poorly differentiated, but proliferate and metastasize rapidly. Grade 2 (G2) tumors, being moderately differentiated, occupy the gap between grades 1 and 3 [72]. WHO also recommends grade 4 for undifferentiated group, however in many cases distinguishing between G3 and G4 is challenging. It is essential to approach these grades with caution, as a significant

limitation of this grading system is its inherent subjectivity of the pathologist [71].

1.2.5 Treatment

Currently, the only curative treatment for CRC patients is complete surgical removal of the tumor. This can be combined with preoperative (neoadjuvant) and/or postoperative (adjuvant) therapy. Adjuvant chemotherapy does not have a proven efficacy for rectal cancer, and for colon cancer, only 20% of patients have a survival benefit [73]. Chemotherapy protocols are either single- or multiple-agent regimens with the most commonly drugs used being 5-Fluorouracil, Oxaliplatin, Capecitabine and Irinotecan. Irradiation as a neoadjuvant treatment can be used either to reduce the risk for local recurrence in tumors that are resectable up front or to shrink unresectable tumors, but due to anatomical constraints it is *only* used in rectal cancer. Neoadjuvant treatment may even be so effective that the tumor is completely removed, referred to as complete response [74].

The treatment regimens differ depending on the aim of the treatment. To reduce the risk of local recurrence, plain radiotherapy five times a week (5 Gray; Gy) before surgery is often recommended in rectal tumors that are considered bad or ugly, that is, has an increased risk of recurrence [75]. For patients where the aim is to shrink the tumor, a longer period of radiotherapy (25 times 1,8-2 Gy) is often recommended in combination with Capecitabine (chemoradiotherapy), or the above described 5x5 Gy irradiation in combination with Capecitabine and Oxaliplatin [76]. However, neoadjuvant irradiation is also associated with adverse effects causing an increased risk for postoperative complications, urinary, bowel and sexual dysfunction. In addition, a significant variation in response to neoadjuvant treatment has been observed amongst patients. More specifically, studies show that complete and partial responses occur in around 10-30% and 60% of patients respectively, while the remaining don't respond at all [77-79]. Importantly, it is not yet well understood why some patients respond to (chemo)radiotherapy and why others show no beneficial effects from the neo-adjuvant treatment.

1.3 Tumor Immunology

1.3.1 Tumor immunology

In the early 20th century, Paul Ehrlich first proposed that the immune system, by eradicating abnormal cells, also plays a role in controlling the establishment of tumors [1,80]. This theory was based on clinical observations like those by William B. Coley, that linked tumor shrinkage following streptococcal infections and complete regression of tumors following intentional administration of bacterial extracts (vaccination) into solid tumors [1,81]. Fifty years later, using inbred mice, scientists were able to show that tumors were indeed distinct from normal cells in terms of immune recognition. These investigations led to the concept of “tumor specific antigens” [80] and served as a basic foundation for the cancer immunosurveillance hypothesis, as the findings confirmed that there must be identifiable features on tumor cells that the immune system could recognize. The idea of immunosurveillance - the detection and elimination of cancer cells by immune system - was formally reported by Burnett in 1970 [1,80]. However, there was a lack of agreement on the hypothesis for years, until it was unambiguously supported by higher cancer rates in mice with certain immune deficiencies [80].

Today, immunosurveillance is part of the broader "immunoediting" theory, which outlines three stages: elimination, equilibrium, and escape. The elimination is rooted in immunosurveillance, where newly transformed cells are identified and vigorously attacked by immune system. If the immune system cannot remove tumor cells, it will go to the equilibrium phase with a continuous battle at tumor site. During this phase, the tumor can eventually escape and become symptomatic due to exhaustion of the immune system and an increase in immune resistance mechanisms evolved by tumor [1,80] which will be described extensively in a separate chapter. All these processes affect the complexity of the immediate surrounding of the tumor due to the presence and absence of various cells, the amount of secreted cytokines, and levels of molecules expressed.

1.3.2 Tumor microenvironment

The immediate surrounding of tumor, known as the tumor microenvironment (TME), is the battleground of tumor and cells partaking in the immune response. Therefore, it is important not to consider a cancer only by the tumor cells that are present but also all other cellular compartments around the lesion. Furthermore, each of these components are able to secrete certain elements that may govern the TME as well as immune response. The complex landscape of the TME consists of cellular components, including tumor cells, unaffected nearby cells, endothelial cells, tumor associated fibroblasts, adipocytes, neuroendocrine cells and non-cellular components (soluble factors) composed of cytokines i.e., interleukins (IL), interferons (IFN), tumor necrosis factors (TNF) as well as chemokines, growth factors, extracellular matrix, exosomes and apoptotic bodies [82]. Research in the last 20 years has unveiled that the role of the TME in tumor formation and progression is in fact equally crucial to genetic mutation and epigenetic alteration within tumor cells [83]. However, the composition of the TME varies in tumors with different origins. Additionally, the distribution in malignancies of the same tissue may differ [84]. In the following section the focus is on the role of the most important immune cells in the microenvironment of CRCs.

1.3.3 Immune compartment of the TME

1.3.3.1 Tumor infiltrating lymphocytes

T cells, integral to the adaptive immune system, can have both inflammatory and anti-inflammatory effects depending on the context [85]. Together with tumor associated macrophages (TAMs), they are one of the most common immune cell types found in human tumors. Although certain T cells can have anti-inflammatory functions, the presence of infiltrating T cells often indicates a better prognosis in many cancer types. In the early tumor stages, in presence of adequate immunogenic antigens, effector T cells that have been activated in the regional lymph nodes, can enter the TME and drive the elimination of cancer cells [85,86].

Effector $CD8^+$ T cells, at their final differentiated state, are very effective in inducing tumor cell death and are called cytotoxic T cells (CTLs) [85]. Their priming and differentiation is highly dependent on IL-2 [86], especially during non-infectious conditions. The IL-2 is provided by $CD4^+$ T helper (Th) cells [85]. While the majority of the immune cells are located in tumor stroma, they have the best prognostic value when residing within the epithelial compartment in CRC, i.e. the tumor nest [87].

CTLs exert their anti-tumor function through two mechanisms. The main mechanism is secreting granzyme A and B, and perforin into the immunological synapse. Perforin forms transmembrane channels and creates a point of entry for granzyme into the targeted cells. In the cytosol, granzymes activate pathways which result in apoptosis through activation of caspase 3. [86,88] CTLs also kill the cells through cell-to-cell contacts. The binding of Fas-L on CTLs and Fas molecules on targeted cells activates pathways inside the target cells, which ultimately activate caspase 3 and 8. The process results in apoptosis and elimination of the targeted cell [86,89]. CTLs also amplify their killing efficacy by the autocrine effect of IL-2 and IFNγ production. These cytokines enhance the efficacy of natural killer (NK) cells [90].

After the initial immune response, a subset of antigen-experienced T cells survives for a long period of time and differentiates into memory T cells. Memory T cells respond more rapidly and effectively upon re-exposure to the antigen. Traditionally, depending on the signals the cells receive and their location, memory T cells are divided into two main subsets: central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). T_{CM} cells reside in lymphatic organs and are ready to proliferate and differentiate upon antigen re-challenging, generating effector cells. T_{EM}, also known as peripheral memory T cells (T_{PM}), patrol in peripheral tissue and are poised to quickly respond to antigen re-encounter and exert effector function [91]. Within the memory pool of $CD8^+$ T cells, a distinct population has been found. They are characterized by their capacity to reside and remain in a local tissue. This population is known as tissue-resident memory T cells (T_{RM}). They are identified by specific markers such as CD103, which binds to E-cadherin on epithelial cells. This binding facilitates the retention in the tumor area, especially when the tumor is of epithelial origin [86].

$CD4^+$ Th cells, a cornerstone of the immune cells, have long been recognized for their pivotal role in aiding in the priming of $CD8^+$ T cells, through the secretion of IL-2 fostering CTL maturation into effector and memory cells. This partnership is of significant importance, ensuring a robust response to infections but also against transformed cells [92]. Interestingly, studies by Nakanishi and colleagues in 2009 shed light on another crucial function of $CD4^+$ T cells in aiding the eradication of target cells. They can act as gatekeepers, regulating the entry of CTLs into tissues in mouse models [92,93]. This process is driven by various molecular interactions, including CXCR3 expression by $CD8^+$ T cells and IFNγ production by $CD4^+$ T cells, which prompts the secretion of CXCL9 and CXCL10 in the infected tissue and increased recruitment of T effector cells [92,93]. The coordination between $CD4^+$ and $CD8^+$ T cells isn't just limited to infections; it also extends to the priming of anti-tumor T cells in TME. For example, it has been observed that the presence of $CD4^+$ T cells significantly increases the accumulation of $CD8^+$ T cells in an experimental mouse model of pancreatic tumors [92].

The fate of activated Th cells, hinges on the extrinsic signals from the microenvironment they encounter during initial antigen contact [90,94]. This environment is rich in cytokines and other signaling molecules which can guide the Th cells down various differentiation pathways. The type of antigen encountered by dendritic cells (DCs), the co-stimulatory molecules expressed by these cells, and the cytokines that these cells release, can sway the path of the activated Th cells, which in the context of immunity is of paramount importance [94]. To understand the role of these Th lineages in anti-tumor responses, it is essential to recognize the cytokines they secrete and their subsequent impact. Historically, $CD4^+$ T cells were classified based on their functional subsets: Th1 cells, known for their high secretion levels of IFNγ and tumor necrosis factor-α (TNFα), chemotactic protein-1 (MCP-1 or CCL2), and macrophage inflammatory protein-1α (MIP1α or CCL3) [94,95] . Th1 cells play a vital role in the cell-mediated immunity against intracellular pathogens and tumor cells [94]. The IFNγ, IL-2, TNFα as well as chemokines that Th1 cells produce can either directly promote phagocytic activity of macrophages or augment priming and expansion of $CD8^+$ T cells which both may have anti-tumor effects [95]. In addition, Th1 cells have a crucial role in directing NK cells towards the tumor bed [85,95]. In the course of immunological activation of naïve $CD4^+$ T cells, IL-12

secreted by DCs and the autocrine effect of IFNγ drive the activation of STAT4 (signal transducer and activator of transcription 4) and T-bet transcription factors which induce the development of Th1 [94]. Although Th1 cells aid in the activation of $CD8^+$ T cells, due to secretion of IFNγ, Th1 cells can upregulate the expression of checkpoint inhibitor molecules on CTLs which dampen their cytotoxic effect [90]. However, higher infiltration of Th1 in CRC has been positively associated with a good prognosis for patients [90].

Th2 cells, characterized by the production of IL-4, IL-5, and IL-13, orchestrate humoral immunity and are key players in controlling helminthic infections through eosinophilic inflammation [94,95]. As the latter is an integral part of an allergic inflammatory response, Th2 cells are also a cardinal feature of this reaction [96]. The development of Th2 is driven by an environment enriched with IL-4 and the master transcription factor involved in this differentiation is GATA-3 [90]. Th2 cells have a more varied role in anti-tumor immunity. While potentiating anti-tumor effects by attracting macrophages to the tumor through the secretion of IL-4, in other scenarios, Th2 cells might instead promote tumor growth by recruiting eosinophils [90,95]. Furthermore, Th2 cells promote macrophages polarization toward the M2 subtype rather than an M1 subtype [90] leading to fibrosis instead of anti-tumor effect [97]. Intricate positive feedback governs the development of Th1 and Th2 cells. Th1 maturation is promoted by IFNγ, while IL-4 facilitates Th2 progression. Conversely, cross-regulation by IFNγ and IL-4 serves inhibitory roles, repressing the development of Th2 and Th1, respectively [94].

The initial understanding of CD4 T cell response was largely based on the binary Th polarization model (Th1–Th2). However, this model has been found insufficient in capturing the intricacies of the CD4 T cells. The discovery of other Th subsets, specifically T follicular helper (Tfh), Th17, and Th22 cells, has significantly refined our grasp of how CD4 Th cells contribute to various immune responses, including infection, autoimmunity, and even tumors [95]. The formation of the Th17 cells stimulated by the presence of TGFβ, IL-6, and IL-1β cytokines while IL-23 supports their stability and maintenance. These cytokines lead the differentiation of naïve $CD4^+$ T cells to Th17 effector cells by activating of transcription factors STAT3 and retinoic acid receptor γ T (RORγt)

[90,95]. Upon activation, the effector Th17 cells produce IL-17A and IL-17F, IL-21, and IL-22. IL-17A, secreted by Th17, plays a pivotal role in inflammation [90,95]. It triggers the expression of various chemokines and enzymes that boost inflammatory reactions. While these inflammatory responses are protective against some microbes, they can, paradoxically, also incite severe inflammation and even autoimmunity [95].

In the context of CRC, the role of Th17 cells remains somewhat ambiguous. It's still uncertain whether they promote tumor progression or rather aid tumor eradication [90]. IL-17A within tumors can contribute to tumor growth by promoting angiogenesis. In contrast IL17-F has been shown to have anti-tumor activity [90]. Thus, continuous exposure to Th17-associated cytokines might potentially expedite cancer progression, in some tumors [95,98,99] but this appears not be the case in CRCs [100]. Further evidence underscores the capacity of Th17 cells to enhance the movement of other essential immune cells, such as T and NK cells, to tumor sites [92,101]. Moreover, Th17 cells recruit macrophages and neutrophils [90]. It has been demonstrated that in the presence of IL-17, there is a notable increase in chemokines essential for leukocyte homing in mouse models of cancer [102]. In ovarian cancer, high levels of IL-17 were correlated with improved survival rates [92]. However, in CRC, these cells promote cancer stemness and resistance to chemotherapy through the secretion of IL-22 [90]. Hence, the role of Th17 in cancer resistance to therapy and immune cell-mediated eradication is still a topic of debate and demands more in-depth research.

Apart from the previously mentioned cells, the immune response landscape also features other subsets of $CD4^+$ T cells. Many of these subsets modulate or suppress immune responses. One such well-studied cell is the T regulatory cell (Treg). These cells are recognized for their importance in preventing autoimmunity and excessive reactions toward pathogens [95]. A minor proportion of $CD4^+$ T cells in peripheral lymphoid tissue, about 5% to 10%, express high levels of IL-2 receptor alpha chain (CD25), and the transcription factor forkhead box protein 3 (Foxp3) [94] and are thereby defined as Tregs. Tregs are also key players in cancer immunity. In the TME, the prevalence of Tregs increases to between 20% and 30%, varying with the tumor type [95]. A significant characteristic of intratumoral Tregs that might lead to their high

concentration in tumor tissue is their superior proliferation rate in comparison to other intratumoral effector T cells [95]. The migration of Tregs into tumors is driven by chemokines in the TME, for instance, CCL2 in ovarian cancer or CCL21 in melanoma [95]. Moreover, the presence of cytokines such as IL-2, IL-10, TGFβ assist the recruitment of Tregs [103].

Tregs employ a variety of mechanisms to dampen immune responses. They produce inhibitory cytokines like TGF-β, IL-10, and IL-35, and can change the phenotype of antigen presenting cells (APCs), toward more tolerant traits. This transition is facilitated by expressing checkpoint inhibitors and ligands, like CTLA-4, LAG-3, and PD-L1 molecules [90]. It has been described that Tregs, through a process called trans-endocytosis, mediated by CTLA-4, down-regulates co-stimulatory molecules on DCs. During this process, CTLA-4 binds to its ligands (CD80 and CD86), captures them away from the surface of the opposing cell and subsequently degrades them within the CTLA-4 expressing cell [104]. This process reduces the co-stimulatory signals required for effective antigen presentation and activation of effector T cells. Additionally, Tregs convert extracellular ATP into adenosine, by CD39 and CD73 ectoenzymes which in turn suppresses T cell activity. They also remove IL-2 from the immediate surroundings of tumor, limiting the activity of T and NK cells [105]. As tumors grow and the TME evolves, the immune system's ability to maintain tumor containment shifts. Tumor growth and the TME can suppress anti-tumor function, particularly by using immune checkpoints and recruiting Tregs which inhibit other immune cells [106]. Increased levels of circulating Tregs indicate a higher metastasis risk in several cancers [85]. The high frequency of Tregs can negatively impact cancer immunity. It has been suggested that the abundant presence of intra-tumoral Tregs can be attributed to the transformation of T effector cells into Tregs by increasing FoxP3 expression. Yet, there are inconsistencies among studies regarding the role of Tregs in both colon and rectal cancer prognosis. While in a meta-analysis from 2015, high infiltration of $FoxP3^{+}$cells was reported to be associated with longer survival in CRCs [107], other studies found no correlation or even negative association between the presence of Treg in TME and patients prognosis after treatment for CRC [87,90,108-110]. These inconsistencies might arise from the substantial heterogeneity of Tregs [111,112]. A recent study

indicated only one subpopulation of Tregs truly has suppressive role in TME [113]. Another potential reason for these discrepancies is the use of various markers to identify Tregs in different studies.

1.3.3.2 B cells

Tertiary lymphoid structures (TLS) are small lymphoid formations, containing both B and T cells that can be identified in solid tumors. Their presence is associated with a robust response and favorable prognosis, implying a close collaboration between B and T cells in the anti-tumor response [87]. While B cells are found in the TME of various carcinomas, their exact role in cancer progression remains less clear compared to T cells. There is evidence that B cells could support tumor growth by nurturing an anti-inflammatory TME [85]. These B cells can promote tumor growth through mechanisms like secreting immunosuppressive cytokines and directly stimulating tumor cell growth. Additionally, B cells stimulate angiogenesis and inflammation in the TME [85]. In contrast, several studies have noted a positive correlation between B cell infiltration and a favorable prognosis across different types of malignancies [114,115]. The prognostic significance of B cells in CRC is still a topic of debate. While some studies suggest higher infiltration of $CD20^+$ cells in tumor is associated with improved prognosis [87,116,117], other studies contradict these findings [118]. It remains uncertain about the various B cell phenotypes in the TME and whether they are inherently immunosuppressive or become so in the TME. Certain B cells have immunosuppressive qualities, called B regulatory cells, but there are no standardized makers for identification of these cells [85]. At present these cells are most often recognized by their capacity to produce immunosuppressive cytokines, or expressing markers such as PD-1, PD-L1 and FasL [119].

1.3.3.3 Natural Killer cells

Innate immune cytotoxic lymphocytes, known as NK cells, play an important role in anti-tumor responses [85]. IL-15 is known as a major recruiter of NK cells in the TME [85]. NK cells express activating and inhibitory receptors for detecting altered or infected cells [85]. Both virally infected and transformed cells may downregulate major histocompatibility complex I (MHC-I) to avoid killing by CTLs. This lack of MHC-I, or as originally coined “missing self”, is recognized by

NK cells [120]. Hence, a sufficient level on MHC-I is what protects healthy cells from activated NK cells. To target and destroy tumor cells, NK cells release cytotoxic factors such as granzyme and perforin as well as inducing apoptosis through TNFα or via direct interaction with cells involving Fas-L and TRAIL pathways [85]. The presence of NK cells in CRC tissue is most often a sign of a better outcome and improved survival rate [85,121,122] However, studies have shown that NK cells are scarce in CRC tissue[123]. Based on the expression of CD56 and CD16, two main populations of NK cells exist. $CD56^{dim}CD16^{+}$ NK cells which are highly cytotoxic [123], whereas $CD56^{bright}CD16^{dim/-}$ tumor-infiltrating NK cells are described as an important secretor of IFNγ upon activation. Under particular conditions, NK cells can also exhibit immunoregulatory properties [123,124]. NK cells in the blood of CRC patients display impaired function [123]. Notably, it has been observed that compared to the normal tissue, rectal cancer lesions have significantly lower amount of NK cells, both pre- and post-treatment [125]. Furthermore, in CRC tissues the expression of NK cells with activating-receptors, as well as $perforin^{+}$ NK cells, is lower compared to normal control tissue [123,126]. Yet, a higher percentage of NK cells in blood has been associated with prolonged survival [123].

1.3.3.4 Unconventional T cells

Unconventional T cells constitute a minor population of cells that serve as a link between innate and adaptive immunity. γδ T cells, natural killer T (NKT), and MAIT cells are the three main subtypes of this group of cells [127]. Unconventional T cells exert both pre- and anti-tumorigenic functions [128].

γδ T cells make up a small fraction of T cells in blood and account for 0.5 to 10% of the total T cells [129]. While γδ T cells identify a restricted amount of antigens compared to conventional T cells, γδ T cells in peripheral tissue are fully mature effector cells, and ready to quickly detect transformed cells [130]. In addition, unconventional T cells can also detect lipids, metabolites, and surface proteins; however, what the TCR receptor in γδ T cells precisely recognizes remains unclear [128]. The contribution of this minor cell population present in the TME of CRC is still controversial [131]. CRC progression can be perpetuated due to the secretion of IL17 from γδ T cells, which boosts angiogenesis and

suppressor mechanisms [131,132]. Additionally, a $CD39^+$ subpopulation of γδ T cells ($CD39^+$ γδTregs) has been identified in CRC, with immunosuppressive effect [133]. On the other hand, studies on CRC show that these cells also can have a strong cytotoxic potential through the production of IFNγ, which promotes DCs maturation and antigen presentation [131,134]. In rectal cancer, the misbalance between different subtypes of γδ T cells, with opposing roles, has been proposed as an important factor in cancer development [135]. In paper II of this thesis, we explored whether the presence of γδ T cells in TME of rectal cancer patients can aid in predicting their response to the neoadjuvant treatment.

1.3.3.5 Tumor associated macrophages

Blood monocytes can after migration into intestinal tissues differentiate into macrophages [85]. In other tissues macrophages can also self-renew from stem cells present in the tissue [136]. Macrophages in the TME can be polarized into proinflammatory (M1)-like or anti-inflammatory (M2)-like phenotypes depending on the cues presented [85,137]. Importantly, this polarization is reversible [85,137]. However, it is important to note that between these two extremes of polarization several intermediate phenotypic traits exist [137,138]. LPS and IFNγ are drivers for pro-inflammatory polarization, while M2-macrophages are induced by IL-4, IL-10 and IL-13 [85,138]. Due to the chronic inflammatory environment created by tumor in its early stages, M1 macrophages may have a tumor-suppressing effect by secreting IL-1β, IL-6, IL12, reactive oxygen species (ROS) and TNFα. In addition, M1-like macrophages possess high antigen presentation capacity at this early stage, but as the tumor progresses, M2-like polarization of tumor associated macrophages (TAMs) becomes dominant, promoting tumor growth and metastasis [85].

TAMs are one of the most dominant immune cells in the TME of CRC[138]. TAMs contribute to CRC progression through various mechanisms. They induce immunosuppression by secreting inhibitory cytokines such as IL-10 and TGFβ, recruit Tregs through CCL2 secretion, enhance cancer cell proliferation with the releasing of epidermal growth factor (EGF), and thereby promote angiogenesis. Additionally, TAMs regulate epithelial-mesenchymal transition (EMT)

via IL-6 and STAT3 signaling pathway, which diminishes treatment efficacy. In addition, these cells can remodel the extracellular matrix (ECM) and stimulate metastasis [85,138,139]. Moreover, TAMs can also inhibit T cells proliferation and cytotoxicity of $CD8^+$ T cells through IL-10 production. It has also been suggested that TAMs control T cell recruitment and limit their appropriate localization thereby indirectly suppressing their activity [83]. In rectal cancer, a correlation has been reported between the presence of M2 macrophages and reduced apoptosis in cancer cells [140].

The degree and polarization state of TAMs can influence the prognosis of both colon and rectal cancer patients. While some studies suggest that higher macrophage infiltration is associated with worse outcomes, others indicate improved survival. The balance between M1 and M2 macrophages in the TME appears to be critical [137,138,140,141].

Despite the complexity of the role of different subpopulations of TAMs in CRC, understanding what governs their polarization and subsequent function is essential for developing individualized treatment strategies and predicting patient response to neoadjuvant treatment.

1.3.3.6 Dendritic Cells

DCs are specialized professional antigen-presenting cells (pAPCs) that upon detection of microbes undergo maturation. This activation and induced maturation process restricts continued antigen uptake and instead activates pathways that upregulate co-stimulatory molecules on the surface of the DCs. While, processing the antigens, DCs simultaneously traffic to peripheral draining lymph nodes where they prime T cells to produce an antigen-specific response [84]. DCs not only detect microbial associated patterns but also cell damage through damage association molecular pattern (DAMP) receptors [142]. Recent findings suggest that DCs, depending on environmental factors such as the inflammatory context and expression of co-stimulatory markers, can help in activating naïve and memory T cells and their function can lead to either antigen tolerance or initiation of an effector T-cell response [85]. In various cancers, functional impairments of DCs have been reported, including CRC [143,144].

Two main types of DCs have been described: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs express the CD11c marker and are

instrumental in antigen uptake and T-cell activation. In contrast, pDCs express the CD123 marker and produce high levels of IFNα, highlighting their importance in creating pro-inflammatory milieu in anti-viral immunity and potentially also in anti-tumor activities [84]. Furthermore, newly identified regulatory DCs (DC regs) promote immune tolerance by primarily inducing proliferation and activation of Tregs [145]. Studies on DCs in CRC tissue show a direct correlation between the presence of intraepithelial mature tumor-infiltrating DC and better clinical outcomes [146,147]. Furthermore, such tumor infiltrating-DCs also show a notable association with increased infiltration of $CD4^+$ and $CD8^+$ immune cells in the same area [147].

1.3.3.7 Tumor associated neutrophils

Neutrophils are rapidly recruited to sites of local inflammation and are therefore among the first cells to be attracted to damaged tissue. Neutrophils exhibit various functions, where eliminating pathogens through phagocytosis and modulating inflammation by production of ROS and cytokines are the most well described [148,149]. The induced inflammation in the TME will has a large impact on both tumorigenesis and the progression of the tumor [150]. High levels of tumor associated neutrophils (TANs) have also been observed in the TME. These TANs have been suggested to be present as N1 and N2 subtypes and depending on their state of polarization, just like macrophages, they either inhibit or promote tumor growth. However, distinguishing between these populations remains challenging due to a lack of well-defined markers allowing separation of these subsets [85]. The general view of neutrophils in the TME is that they correlate with poor patient outcomes [151]. However, in CRC, longer survival has been reported in correlation with high levels of $CD66b^+$ TANs. This effect has been suggested to be promoted though amplifying the tumoricidal effects of CTLs [85,152]. On the other hand, neutrophil extracellular traps (NETs), networks released by neutrophils, have been associated with cancer progression. An increase in NET formation is associated with metachronous metastases in CRC patients, suggesting a pro-tumor role for neutrophils [85,153]. Moreover, in mouse models of colon cancer, TANs have been shown to promote angiogenesis [85]. The process of tumorigenesis has been suggested to initiate the recruitment of myeloid-derived suppressor cells (MDSCs) and to induce a change in the neutrophil polarization

towards a more tumor-promoting variant of neutrophils. This process is also paralleled with a rise in the number of neutrophils both within the tumor surroundings and in the blood circulation [150]. Overall, while neutrophils play a pivotal role at various stages of cancer progression, their exact function and how this links to the phenotypic variations within TANs need more in-depth characterization. Something which is challenging as these cells are fragile and therefore often lost during preparation of single cells suspensions from solid tumors or following cryopreservation.

1.3.3.8 MDSC

Myeloid-derived suppressor cells (MDSCs), consist of a heterogeneous group of granulocytes (PMN-MDSCs) and monocytes (M-MDSCs). The expansion of these cells is driven by infections, inflammation, and malignancies. MDSCs have been shown to play a pivotal role in cancer development, including CRCs. They exhibit immunosuppressive properties which protect the cancer cells from detection by the immune system, thereby propelling tumor progression [150]. Two essential enzymes, iNOS and ARG1, are linked with the suppressive activity of MDSCs [154]. These enzymes affect L-arginine metabolism which is vital for optimal T cell function [154-156]. Factors released within the TME, caused by local hypoxia, low PH, and chronic inflammatory conditions including the release of the chemokines, further stimulate MDSCs [154]. One of the important chemokines is CCL2, that has been linked to the growth, progression, and metastasis of various tumors, including CRC [154]. These cells also respond to other mediators like histamine, prostaglandins, as well as exosomes released by tumor cells. While histamine and prostaglandins activate MDSCs, exosomes can have varying effects on the activation or inhibition of these cells, depending on their cargo [154].

In CRC, MDSCs chiefly inhibit T cell proliferation and rather potentially boost Treg development. MDSCs activate Tregs by releasing cytokines, such as IL-10 and TGFβ, where the latter also has auto-amplificatory function on MDSCs [154]. Mouse studies have demonstrated that MDSCs stimulated Tregs via IL-10- and IFN-γ-dependent pathway both *in vitro* and *in vivo*[154]. However, the exact dynamics of the secretion of these factors by MDSCs in the TME and their effect on cancer progression in CRC remain to be elucidated.

MDSCs may even directly foster tumor growth and metastasis, by promoting processes such as angiogenesis and the EMT through TGFβ. The second of these processes is fundamental for late-stage metastasis [154]. Research indicates that in CRC, elevated levels of MDSCs in peripheral blood and tumor tissues correlate with higher histological grade as well as metastases to the lymph nodes [157]. Notably, high concentrations of PMN-MDSCs have been associated with poor prognosis for CRC patients [157].

1.3.4 Cancer immunity cycle and immune evasion

As described before, the uptake of antigens in the presence of danger detection of by the innate immune system (mainly DCs) triggers a series of events that leads to the activation and differentiation of immune cells belonging to the adaptive arm of the immune system. This ultimately enables the body to target and eliminate the detected threat while forming memory of the same antigen. While pAPCs transport the antigen to draining lymph nodes, these antigens are processed into smaller fragments that are then showcased on the surface of cells using MHC molecules. The T cell receptor interaction with the antigen-MHC complex on the DCs gives the initial activation signal (Signal 1) for T cells. This needs a second confirmation (Signal 2) through co-stimulatory molecules between the pAPC and T cell to prevent inadvertent activation of T cells that recognize peptides from self-antigens (self-peptides). In addition to these two signals, a third one determined by environmental cytokines, directs the T cell's specific function, either as a Th subset or a licensed CTL [84,158].

Nowadays, it is widely accepted that accumulation of genetic mutations and epigenetic transformations, leads to the development of CRC [159]. As a result of somatic mutations in cellular DNA, the regulation of signaling pathways may be disrupted. Therefore, tumors can arise through uncontrolled cell division. In addition, the mutations may lead to the expression of altered proteins; so-called ‘neoantigens’. These neoantigens contain new peptides, distinct from unaltered self-peptides found in normal healthy cells. When self-peptides are displayed on the surface of unmutated cells, they do not lead to an attack by the immune system, a mechanism in place to avoid autoimmune reactions and referred to as immune tolerance. Hence, neoantigens need to be

recognized by and result in activation of the immune system, in particular T cells, for effective eradication of the cancer cells to be initiated [160-162]. This can occur by necrosis in tumor cells resultsing in neoantigens being released in the presence of damage-associated molecular patterns (DAMPs), including tumor cell-derived DNA, that activate cGAS-STING (Stimulator of interferon genes) pathway in neighboring cells to produce type I IFN [163], and proinflammatory cytokines such as TNF-α and IL-1. This will lead to activation/maturation of DCs containing engulfed tumor antigens that upon arrival in the draining lymph nodes can prime neoantigen (peptide)-specific effector T cells.

Moreover, when the growing tumor disrupts the integrity of the gastrointestinal (GI) tract lining mucosa, gut microbiota products, e.g., Toll-like receptor (TLR) ligands, provide maturation signals for innate immune cells. This may further facilitate the processing and presentation of tumor derived neoantigens [160,161]. Once the tumor antigens are captured by pAPCs of the innate immune system - in particular DCs - in the presence of the abovementioned activation signals, DCs will become activated and migrate to the draining lymph node. Once reaching the lymph node, the DCs will meet naïve T cells and present the processed neoantigens on MHC-I and MHC-II molecules together with costimulatory molecules induced by the activation signals. This will prime and activate effector T cells clones specific for the tumor neoantigen [161]. In this process, the production of type I IFN facilitates cross-priming of tumor antigens on MHC-I by DCs, a process which is required for the activation of cytotoxic CD8 T cells with the capacity to induce cell death in tumor cells [164]. The activated effector T cells then travel to the site of the tumor and infiltrate the tissue to selectively detect and destroy the tumor cells that displaying neoantigens on MHC-I. This reduces the number of tumor cells but also importantly leads to further release of tumor associated antigens from the killed tumor cells. These can, in turn, after uptake and display by tumor DCs reaching the draining lymph node, initiate activation of additional T cell clones. Tumor-resident DC and macrophages that acquire the antigens but do not leave the tumor can also “re-present” the neoantigens to infiltrating T cells to reinvigorate their tumor-killing capacity. This will complete this cyclic process known as cancer

immunity cycle that thereby has the capacity to broaden and amplify the anti-tumor immune response [161,163] (Figure 4).

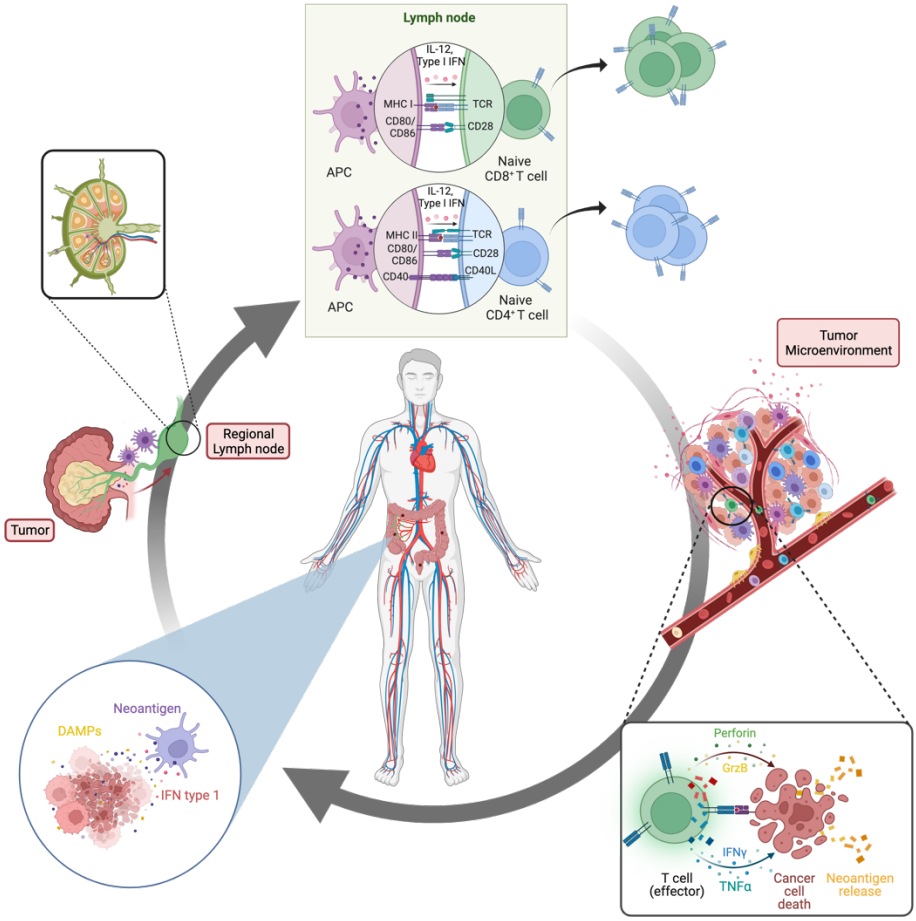

Figure 4: Cancer Immunity Cycle. This framework illustrates the sequential steps of the immune system's response to cancer cells. It begins with the release of cancer cell antigens, their presentation to T cells, T cell activation, trafficking to the tumor site, and infiltration into tumor tissue. Ultimately, cytotoxic T cells (CTLs) recognize and destroy cancer cells, leading to a continuous cycle of immune response. Created with BioRender.com

To reduce the risk of overactivation and immune-related damage in response to continuous, and amplified exposure to the tumor antigens, the immune system uses a variety of autoregulatory mechanisms. The process is mainly based on the expression of immune checkpoint molecules like PD-1 and CTLA-4 on immune cells, which serve as negative regulators of effector T cell function and keep the immune

response in a coordinated manner and also dampen the magnitude of the response [85].

In the TME - consisting of tumor cells, infiltrating immune cells, as well as stromal cells – every cell type has the capacity to abundantly express immune checkpoint receptors as well as their ligands. This can be in response to IFNγ produced and secreted by infiltrating immune cells, a direct impact of cancer-related intracellular signaling pathways [165], or arise from metabolic stress within the TME [166]. Cancer cells that manage to exploit this mechanism and inhibit the local cytotoxic response by T cells can escape from the host immune surveillance.

The process whereby $CD8^+$ T cells in response to persistent exposure to the antigen lose their ability to proliferate and exert effector functions, including production of cytokines upon stimulation, is called immune exhaustion [86,166]. Exhausted $CD8^+$ T cells are often characterized by the expression of PD-1 molecule that upon binding to its ligand PD-L1, suppresses T cell receptor (TCR) signaling [86,166]. The exhaustion is further maintained by other co-inhibitory receptors, including LAG-3, TIM- 3, and TIGIT [86,166]. In addition, increased antigen burden and reduction in specific T $CD4^+$ cells correlate with the severity of T cell exhaustion [86].

It has been demonstrated that tumors escape from immune system by recruiting tumor-growth promoting cells such as MDSCs and Tregs, while reducing the migration of anti-tumor cells to and from the TME [139]. Immune suppression mediated by Tregs involve dual mechanisms comprising a contact-dependent component involving immune checkpoint receptors and their ligands along with contact-independent component. The latter encompasses IL-2 sequestration and release of immunosuppressive mediators like TGF-β, IL-10, adenosine, prostaglandin E2, and galectin-1 [85,105]. Such cytokines along with macrophage colony-stimulating factor (MCSF), vascular endothelial growth factor (VEGF) and indoleamine 2,3-dioxygenase (IDO), inhibit the functions of DCs by negatively affecting the maturation, and dampen the ability of DCs to effectively present antigen [167]. Furthermore, some of the immunosuppressive factors induce apoptosis in tumor-infiltrating cytotoxic T cells by enhancing expression of Fas-L [167]. To control prolonged inflammation in the tumor environment, Tregs, compete with

CTLs in using tryptophan, which is a vital amino acid for the functional capacity of CTLs [82].

Another mechanism that tumors use to evade the immune system is mutations in genes that make cells incapable of presenting antigens on MHC-I. Therefore, cells become undetectable by immune system, which facilitates tumor expansion and metastasis [167]. Taken together, all these mechanisms can even in tumors with considerable infiltration of immune cells translate into local but severe attenuation of the anti-tumor activity.

In CRC, it has been shown that immune checkpoint markers in the TME are primarily expressed on innate immune cells such as DCs and macrophages rather than tumor cells [168]. This however is limited to a subgroup of patients with high levels of immune cell infiltration. Interestingly, a considerable number of these patients have shown an outstanding response to drugs that specifically inhibit the interactions between the immune check point receptors and their ligands, a blockade that unleashes the previously suppressed anti-tumor response [168-170].

1.3.5 Treatment induced immune responses

Radiotherapy – sometimes in combination with chemotherapy – is a part of the conventional treatment of rectal cancer in combination with surgery. However, for colon cancer chemotherapy has mainly been used after surgery. Cytotoxic agents, often part of chemotherapy, mimic shedding of tumor neoantigens as well as induce inflammation through inducing death in highly proliferative tumor cells [171]. However, these drugs are not yet as specific as cytotoxic T cells which will lead to off target effects in other cells that also frequently divide. Interestingly, irradiation, initially also used to target rapidly dividing cells, has now been shown to also result in release of activation signals (including type 1 IFN) and thereby also having the potential to initiate and reinvigorate anti-tumor responses [172]. Moreover, DNA damage, particularly double-strand breaks caused by cytotoxic drugs, induces death in target cells [172,173], and exposes tumor neoantigens in the presence of DAMPs to the immune system can promote an inflammatory anti-tumor response. Additionally, it has been observed in ex vivo irradiated rectal cancer tissue that short-course irradiation treatment is associated with

an M1-like TAM polarization and an enhanced phagocytic activity of these cells [174] which can then boost T cell activity. This effect appears to arise from the release of TLR agonists, like HMGB-1, which binds to TLR4, and other DAMP molecules including ATP, uric acid, heat-shock proteins, etc. all produced under the stress of chemo-irradiation treatment [172-176] (Figure 5). In extreme cases, the activated immune cells might migrate and suppress non-irradiated metastatic lesions, a phenomenon known as the abscopal effect [173,177]. In addition, the STING pathway can also be activated in malignant cells in response to DNA damage and trigger an inflammatory immune response in cells surrounding the damaged cells [176].

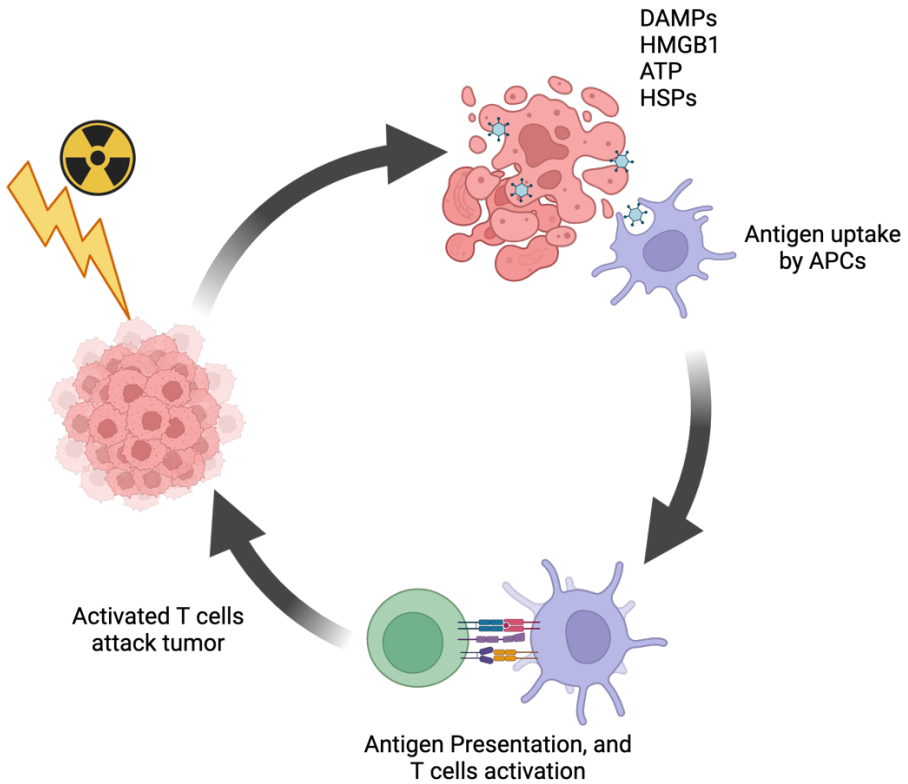

Figure 5: Chemo-irradiation Induced Anti-tumor Immunity. Cytotoxic drugs have the potential to trigger anti-tumor immune responses by releasing different danger-associated molecular pattern (DAMP) molecules. These molecules can activate dendritic cells that then drive the priming of tumor-antigen specific cytotoxic T cells. Created with BioRender.com [173,177]

While cytotoxic agents can induce inflammatory reactions, radiation can have the opposite effect by producing TGFβ, and augmenting Tregs in the TME [175]. The paradoxical effect of the irradiation on the TME spurs on research aiming to harness the immunogenic effects of the treatments and to possibly enhance their effect through combination with immunotherapy.

1.4 Carcinogenesis and Immune Contexture

1.4.1 Carcinogenesis

Most CRCs develop from adenomatous polyps, which are non-cancerous growths in the colon or rectum. Over time, some of these polyps may progress to become cancerous [26]. As somatic mutations, as well as other types of genetic alterations, accumulate within the stem cells residing in crypts of the colon and rectum, this progression leads to the development of adenomatous polyps, dysplasia, and ultimately malignant adenocarcinoma[49]. The transformation from a pre-malignant state to a fully malignant tumor occurs in all forms of CRC, regardless of whether they are sporadic or hereditary in nature [47,178]. The multistage predictable sequence of genomic alteration leading to this histology conversion was introduced by Faeron and Vogelstein [179] and is known as “adenoma-carcinoma sequence”(Figure 6) [159]. While the adenoma-carcinoma sequence has been a foundational model in understanding CRC progression, it is important to note that there are alternative theories and pathways that are not passing through the polyp stages, such as de novo colorectal cancer [180].

Genetic alterations have a profound impact on colorectal tumors that ultimately will influence the phenotype of the cancer. Proto-oncogenes as well as tumor suppressor genes play pivotal roles in this process, but in distinct ways. Both these sets of genes typically regulate intracellular signaling pathways that control cell proliferation and survival, thereby acting as guardians against uncontrolled proliferation. However, mutations can flip the switch, causing overexpression or activation of proto-oncogenes (KRAS and PIK3CA). On the other hand, mutations that inactivate tumor suppressor genes (APC, TP53, and SMAD4) will unleash uncontrolled cell proliferation. Therefore, whether through the “gas pedal” of [181] oncogenes or the failure of “brake” by tumor suppressor genes, genetic changes shape the course of CRC, albeit via distinct mechanisms[159].

Adenomatous Polyposis Coli (APC) gene is linked to the formation of polyps in both hereditary and sporadic cases [159,182]. Evidence of APC mutations has been observed in approximately 80% of CRC cell lines [183] and in 30-70% of sporadic adenomas and colorectal

adenocarcinomas [159,182]. Most mutations in the APC gene are either nonsense or frameshift mutations that impact the APC/Wnt/β-catenin signaling pathway [184]. Normally, this pathway ensures that stem cells at the base of colonic crypts remain undifferentiated [159]. Unmutated APC negatively regulates cell growth by degrading β-catenin in the Wnt pathway [159,185]. However, in the presence of an APC mutation, intracellular β-catenin levels remain elevated, leading to persistent Wnt pathway activation. This sustained activation allows the cells to maintain their progenitor characteristic, including the capacity for proliferation and renewal. Over time, this leads to accumulation of undifferentiated cells in colonic crypts, causing the formation of polyps. Subsequent mutations can then spur these polyps to develop into tumors (Figure 6) [159,181].

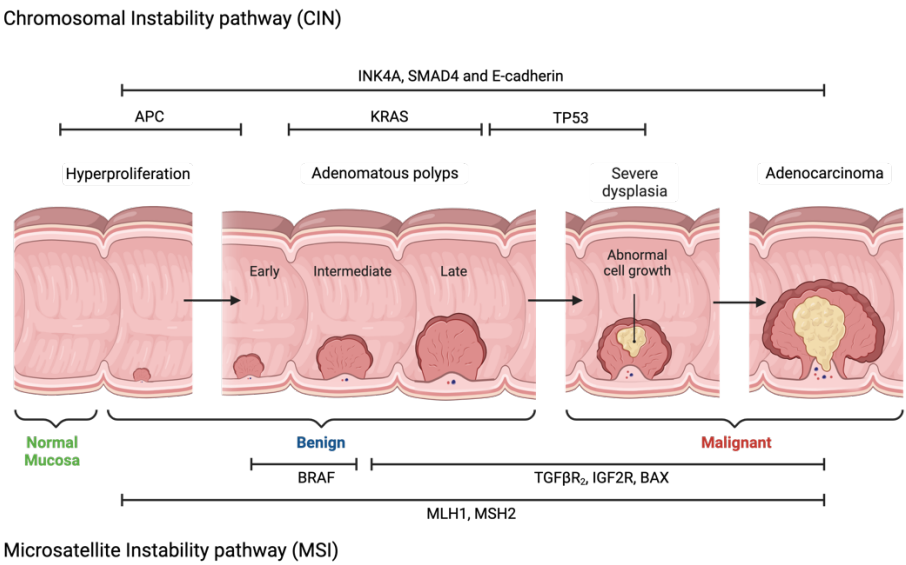

Figure 6: Adenoma-Carcinoma Sequence. Progression from non-cancerous adenoma to cancerous carcinoma. The CIN and MSI pathways, along with frequent mutations encountered by tumor cells throughout their evolution. Created with BioRender.com

CRC displays remarkable genetic diversity and is known for having one of the highest mutational burdens among all malignancies. This mutational load allows for a broad classification into more than 12 mutations and fewer than 8.4 mutation per 10^6 bases called hypermutated and non-hypermutated respectively [26,186,187]. However, a limited set of mutations (approximately 15) are considered to be true

"drivers" of tumor formation in CRC [188,189]. Further investigation into the genetic heterogeneity of CRC has provided insights for categorizing the tumors into distinct molecular subtypes.

1.4.2 Molecular subtypes

The main pathway behind the majority of sporadic CRCs is Chromosomal Instability pathway (CIN). This pathway is characterized by losing one or two alleles of a functional gene (loss of heterozygosity) and aneuploidy (imbalances in chromosome number) [188]. Genes that are involved in chromosomal segregation, DNA damage response, telomere stability, and centrosome formation can be primary contributors to this imbalance [9,188]. Cells with such significant genetic defects are more susceptible to further mutations in tumor suppressor genes, including APC, TP53, SMAD2/4 and DCC as well as proto-oncogenes KRAS, CTNNB1 and PIK3CA causing tumor formation [9,188]. The number of the CRC tumors that are affected by CIN pathway increases from the proximal to the distal part of the colon.

To maintain genomic stability, cells have a highly biologically conserved system called DNA mismatch repair (MMR) pathway. This system consists of heterodimers, including MLH1/PMS2 and MSH2/MSH6, with enzymatic activity which detects and rectifies errors during DNA replication and thereby prevents the arising mutations [190,191]. Through genetic investigations of hereditary non-polyposis colorectal cancer (HNPCC), the classification based on the mutations in the MMR system emerged as one of the earliest molecular classifications of CRCs [192-194]. Although various classification systems for CRC tumors have been introduced over the years [195], the MMR-based approach is still among the most prevalent methods for CRC classification today. An impaired mismatch repair system consequently leads to susceptibility to replication-associated errors and increase in mutation rate in DNA, particularly in short tandem repeat sequences such as microsatellites [9]. Mutations in the components of the MMR system have been found in 15% of colon cancers and 2-8% of rectal cancers. These are hence called mismatch repair deficient (dMMR) or microsatellite instable tumors (MSI). These tumors generally display high mutational rates (>20 mutations/Mb) and a median number of neoantigens that is 20 times greater than microsatellite stable tumors (MSS). MSI tumors can be further classified

based on their mutational burden. Tumors with a high mutational load are termed MSI-H, while those with a lower burden are known as MSI-L [195]. It has been reported that MSI-H tumors are associated with a favorable clinical outcome before metastasis [191]. Interestingly, these tumors often display a lower differentiation grade and a deeper invasion into adjacent tissue, while also presenting with a generally lower stage compared to MSS tumors [191,196]. In rectum, it is uncommon to observe sporadic tumors harboring MSI mutations [48]. Another genetic instability pathway is characterized by a unique epigenetic feature known as the CpG island methylator phenotype (CIMP). In this pathway, hypermethylation occurs within CpG-rich promotor sites of DNA, leading to the silencing of impacted tumor suppressor genes [9,197,198]. Notably, only 3-12% of CRCs with CIMP phenotype are found in the distal colon [195]. It is worth noting that an overlap can be observed across all these subtypes e.g., hypermethylation of MLH1 promotors, which leads to silencing of the MMR system has been found in up to 80% of MSI tumors [9,197].

The enhanced tumor mutational burden can lead to increased display of peptides derived from neoantigens, which in turn improves the chances of tumor recognition by T cells (as described previously in the cancer-immunity cycle). Hence, increased immune cell infiltration is generally found in MSI compared to MSS tumors that have a proficiently functional MMR system (mismatch repair proficient; pMMR) [9]. A tumor with a high infiltration of immune cells is called a hot tumor, while a tumor with a low immune cell infiltrate is described as a cold tumor. Patients carrying MSI tumors constitute the subgroup that shows a very beneficial response to immune checkpoint inhibition treatment. Regulatory authorities have approved the use of MSI status as one of the few indicators for clinical applications in CRC. Generally, most patients are advised to undergo MSI testing following a CRC diagnosis[191,199]. However, studies of CRC tissues have revealed that some MSI tumors are cold tumors, and correspondingly, some MSS tumors are hot [9,72,200].

Considering genetic and epigenetic alterations, as well as clinical and phenotypical criteria, six independent research groups have proposed different molecular subtyping systems for classification of CRC [9,201-206]. Although there was interconnectivity between these classifications, achieving full consistency was challenging. [9,72]. Thus, in 2015, a

classification of CRCs based on genetic mutations, intra-tumoral immune phenotype, metabolic features, and mesenchymal alterations were presented by the CRC subtyping consortium [72]. This defines four consensus molecular subtypes (CMS) (Figure 7). CMS1, or "Immune activated" with high intra-tumoral immune cell infiltration including CTLs, Th1, Tfh, memory T cells, γδ T cells NK cells, M1 macrophages and activated DCs [9,207,208]. Moreover, genes associated with chemotaxis of T cells have robust expression in this subtype [9,208]. In contrast to CMS1, CMS2, known as "Immune desert", exhibit limited infiltration of immune cells. The immune contexture within this tumor subtype mainly comprised of resting NK cells, naïve CD4 T cells and B cells. In addition, the expression of PD-1/PD-L1 is notably low[207]. CMS3, or "Immune excluded" subtype is characterized by low infiltration of immune cells. Th17, naïve B and T cells, and resting T cells constitute the majority of the immune cells found in this subtype and display an enrichment in expressing PD-1 [9,207,208]. CMS4, or "Immune inflamed" [9,72] is characterized by high lymphocyte and macrophage infiltration. However, these cells predominantly display phenotypes of Tregs and M2 macrophages. In addition, the tumor microenvironment of the mesenchymal subtype contains monocytes, eosinophils, myeloid cells, and resting DCs [9,207,208]. Approximately 76% of the CMS1 are MSI tumors with an immune activate profile, but this profile can also be detected in 2%, 16%, and 6% of CMS2, CMS3, and CMS4 subtypes respectively [9,72].

Currently, the CMS classification stands as the most reliable system for CRC categorization, based on present biological understandings [72]. It offers promise in tailoring cancer treatments. While these systems use genetic analyses to provide information about the mutational rate in the tumor as well as a quantitative indication of which immune cells that are present in the biopsies, it conveys no information about the spatial distribution of the immune cells and the tumor cells. Furthermore, the use of this system in clinical settings and the interpretation of the results is a complex task. 13% of the patients remain unclassified, potentially indicating heterogeneity within a tumor or a phenotype transition [72]. Up to now, various models, both *in vitro* and *in vivo* related to these CMSs have been created to better understand tumor variability and to bridge the gap between experimental researchers and clinical practice [209-211].

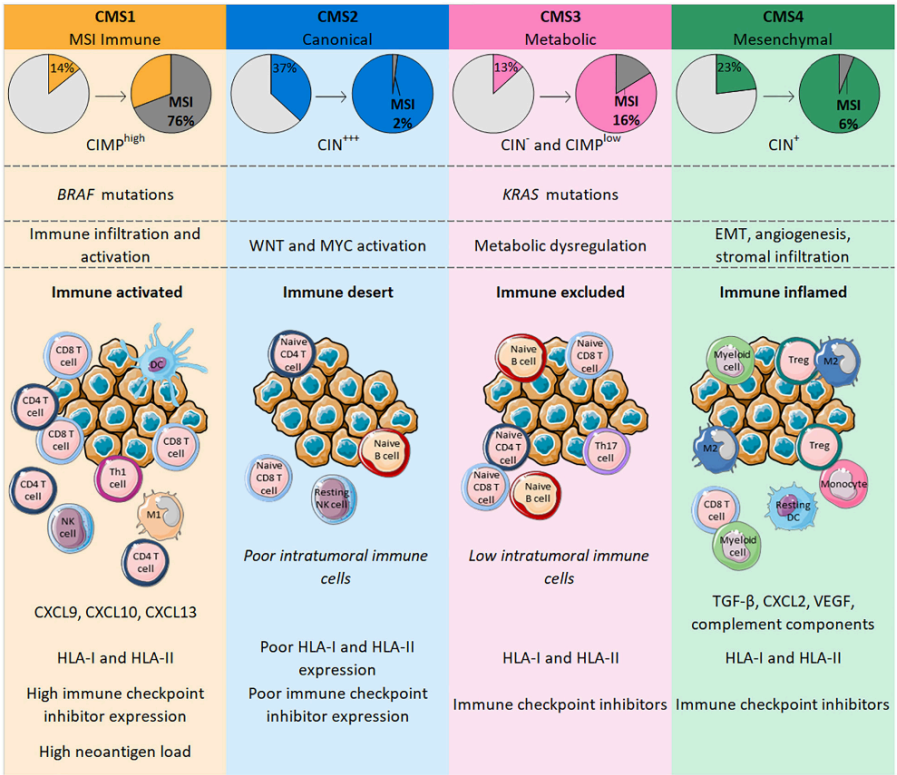

Figure 7: CMS Classification. CMS1 predominantly features MSI tumors with high antigen load, and an increased influx of the immune cells. CMS2 and CMS3 are characterized by limited intra-tumoral infiltration of the Immune cells. In contrast to CMS2, CMS3 has high expression of MHC I and II. Both are associated with CIN tumors. CMS4 has a suppressive environment with high infiltration of inhibitory immune cells and comprises of few MSI case. ©2020 Picard, Verschoor, Ma and Pawelec (Licensed under CC BY 4.0)[9]

1.4.3 Immunoscore

From a prognostic perspective, the predictive accuracy of the TNM classification has not been improved by any genes or genomic signatures. [1] Assessing tumor markers is often more challenging than measuring immune cells because only a subset of tumor cells express specific tumor antigens. Measuring staining intensity accurately and thereby determining protein expression using immunohistochemistry presents inherent challenges. A significant factor contributing to this difficulty is the vast range and diversity of genomic changes; for

instance, individual colorectal tumor cells can have as many as 11,000 genomic modifications [1]. On the other hand, determining the numbers of immune cells, such as T cells identified by $CD3^+$, is simpler due to the presence of clear markers that enable cell-by-cell counting [5]. Immunoscore (IS) is an immunohistochemistry-based system that stages tumors based on the degree of immune cell infiltration within the tumor. In 2006, Galon et al. [212] highlighted the importance and the prognostic accuracy of this in situ enumeration of infiltrating T cells in CRC tissues. IS was derived by studying patients with prolonged disease-free survival (DFS) who showed high gene expression linked to CD8 T cells cytotoxicity and Th1 orientation [212,213]. Immunohistochemical evaluation using CD3, CD8, Granzyme B, and a marker for memory T cells (CD45RO) in the tumor core (CT) and invasive margin (IM) confirmed that for CRC patients without metastasis, a high level of this immune infiltration in each tumor compartment correlated with reduced recurrence risk, irrespective of the tumor stage [212]. In contrast, low cell density in the CT and IM was associated with unfavorable outcomes, even in minimally invasive lesions. Due to technical difficulties in immunohistochemistry (IHC) staining (DAB chromogen staining) such as background noise of CD45RO and granular staining of granzyme B, the assessment was simplified to the quantification of T cells using markers CD3 (all T cells) and CD8 (potentially cytotoxic T cells) in the core and at the IM of primary tumors [66]. These parameters yielded a scoring system ranging from Immunoscore 0, which indicates a limited influx of both cell types in both regions, to Immunoscore 4, with abundant CD3 and CD8 immune cells in both regions. The cutoff value in this system was determined using the minimum *P*-value approach [214,215]. In pursuit of a more clinically-friendly approach to immune contexture, a "consensus Immunoscore" was developed based on the mean of four percentiles – considering both markers and regions [1]. A score below 25% was deemed low, above 70% high, and all other patients between 25% to 70% were considered as intermediate.

Studies using the Immunoscore have reported that it has a prognostic and predictive value superior to the TNM classification [216,217]. Most international guidelines for treatment of rectal cancer recommend neoadjuvant treatment prior to surgical excision for selected patients [218]. Given the potential of neoadjuvant treatment to alter the immune landscape in the tumor, it could be essential to evaluate local immune

cell infiltration in biopsies at the time of diagnosis. Studies have shown that pre-existing immune cell infiltration in the whole pre-operative biopsies of rectal cancer is associated with tumor regression following neoadjuvant treatment [215,219]. The inherent limitations of biopsies make accessing specific regions of a tumor e.g., tumor core and invasive margin, challenging. Identification of the invasive margin in small pre-operative biopsies is considerably more difficult than in resected tumor after surgery and has been abandoned in the biopsy-adapted Immunoscore (IS_B) recently described by El Sissy et al [220]. In this simplified scoring system, which is reduced to mean percentile of two markers (CD3 and CD8) in the tumor biopsies there is no need to distinguish between CT and IM [220]. (Paper II, Supplementary Figure 5a).

While the TNM classification helps guide treatment choices, the IS may offer insights into the response to the treatment and clinical outcomes [5]. This could influence both treatment choices and post-treatment follow-up intervals, could reduce unnecessary adverse effects from ineffective treatments, enhance patients' quality of life, decrease healthcare costs, and facilitate more informed discussions between clinicians and patients regarding expectations and future management plans. Building on its potential advantages, IS meets most requisites of a practical clinical biomarker: it is feasible, requiring only two sequential FFPE tissue sections, and is cost-effective, rapid, robust, pathology-based, and quantitative [5,66]. However, it remains relative to the cohort which makes it challenging to implement in the clinic [216,217].

1.4.4 Future stratification for precision treatment in colorectal cancer

It has been mentioned before (chapter 1.2.5) that response to neoadjuvant treatment varies, with 10-30% achieving complete response among rectal cancer patients. Most patients, however, tend to show a resistance to treatment, leading to a potential relapse or metastasis even after surgical intervention. The recent emergent immunotherapies that potentiate T cell responses are now in clinical use [170,221]. Consequently, the discovery of these immune checkpoint inhibitors (ICIs) has revolutionized cancer therapy. In CRC, several

immune cells within the TME express checkpoint molecules. Neutralizing immune checkpoint molecules, with monoclonal antibodies unleash the immune system. Ipilimumab and Nivolumab, which are anti CTLA-4 and PD-1 inhibitors respectively, have been approved by FDA in treatment of unresectable, metastatic (stage IV) CRC patients with MSI-H tumors [139,222-224]. It is worth noting that higher density of immune cells is overrepresented in MSI-H tumors therefore more immune checkpoint molecules exist in the TME. However, the extent of cellular exhaustion within the tumor also plays a pivotal role in determining the efficacy of immunotherapy. Cells that reach the terminal levels of exhaustion may become anergic, which severely attenuates their effector functions [225]. Thereby, patients may still respond dramatically differently to ICIs.

Adaptive cell therapy is another approach that has made encouraging progress in the treatment of CRC patients. However, this method still needs to pass clinical trials before being approved. The method is performed by re-infusion of *in-vitro* expanded and activated T cells (non-engineered), obtained from the patient's blood post-surgery [226,227]. This is typically in combination with *in-vitro* treated monocyte derived DCs. The results are promising particularly for MSS patients and 5 years increased in patient survival was observed [226]. Genetic modification of antigen receptors on immune cells, mainly T cells and NK cells, that express chimeric antigen receptors (CAR) is another approach in adaptive cell therapy [227]. These therapies have not yet been approved for clinical use in CRC. Many other immunotherapy strategies have been introduced by researchers over the past decade, including cancer vaccines, bispecific T-cell engagers [86], targeting MDSCs [154], etc. though challenges like on-target off-tumor effects remain.

To maximize the benefits of any treatment, either conventional chemo-irradiation or innovative immunotherapy strategies, predicting patients' responsiveness to the treatment is crucial. Several challenges persist in the realm of precision treatment for CRC. Although a high neoantigen load may stimulate immune response, additional factors present in the TME in CRC can have inhibitory effects. Therefore, the addition of mutational analysis of the MMR status to the existing TNM scoring might not be sufficient guidance for improving personalized treatment of CRC. Immunoscore, would be one additional important predictive

tool to use, especially regarding the surveillance regime after surgery. Indeed, a strong correlation between a high frequency of tumor-infiltrating T cells and improved clinical outcome in CRC patients have been reported [212,216]. However, to improve the choice of neoadjuvant treatments or potentially immunotherapy for the individual patient, increased knowledge of the interactions between immune cells as well as with the tumor cells and how this may influence the response to the chosen neoadjuvant treatment is likely crucial. For example, the clinical response to radiochemotherapy varies between patients with rectal cancer, but the nature of the inter-individual diversity in response to the treatment, is still unknown. Consequently, there is yet no assessment of the genetic characteristics of the tumor or the extent of immune cell influx made in clinical practice to guide the choice of neoadjuvant therapy in rectal cancer. Moreover, the need for comprehensive biomarker identification and validation is essential to truly personalize treatment. This approach, with artificial intelligence and machine learning, might provide predictive tools for patient stratification, guiding clinicians in choosing the best therapeutic combinations for individual patients.

2 AIM

In this thesis, we delve deep into the immunological aspects of colorectal cancer by focusing on the role and function of professional antigen-presenting cells and T cell subsets in the tumor microenvironment and comparing them to the adjacent colorectal tissue from the same individual. Our overall objective is to understand the interactions between these immune cells and their surrounding environment and how this may determine treatment outcomes to thereby pave the way for personalized pre-operative treatments. Specifically, our objectives are as follows:

1. *Characterize the functional capacity and the activation status of professional antigen presenting cells in the tumor and adjacent tissue in colorectal cancer patients and determine if the professional antigen presenting cells are differentially influenced by factors secreted by tissue residing T cells following activation.*

2. *Determine if the spatial distribution of T cell subsets in combination with a "footprint" of type I IFN intra-tumoral activity in preoperative rectal cancer biopsies can aid prediction of neoadjuvant treatment response.*

3. *Determine if phenotypic and functional assessments of T cells present in preoperative rectal cancer and paired adjacent rectal biopsies can identify patients that will not respond to neoadjuvant treatment.*

3 PATIENTS AND METHODS

The tumor microenvironment and tumor-infiltrating immune cells were investigated using various techniques in this thesis, including flow cytometry, immunohistochemistry, cell culture, cell stimulation, and ELISA-based immune assays for functional analysis of the cells. The study involved samples from both healthy donors and patients (Figure 8).

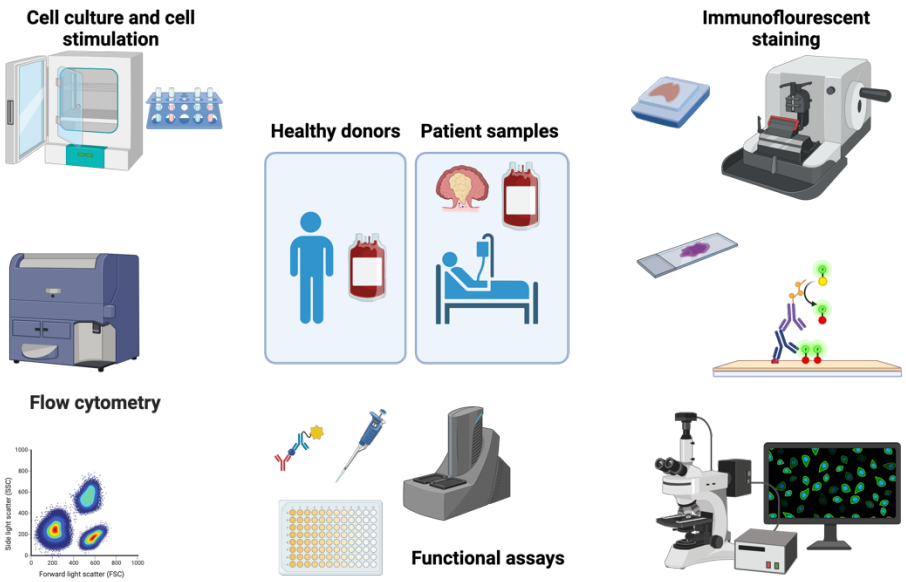

Figure 8. Methods Overview. In this thesis we used different methods to analyze tumor infiltrating immune cells both functionally and phenotypically. Methods such as flow cytometry, immunohistochemistry, and various functional assays were utilized to explore tumor microenvironment. Created with BioRender.com

Human samples:

Biopsies from malignant lesions as well as normal adjacent tissue (5 - 10 cm away from the tumor) from patients with CRC were collected at initial endoscopic examination at diagnosis or during surgery. Adipose and connective tissue were carefully removed from the biopsy before further dissection and digestion. Blood samples were collected from patients during the surgery and from healthy volunteer donors during a

visit in “Ge blod” (a non-profit organization, responsible for collecting and distributing blood products to hospitals throughout Sweden). Informed consent was obtained from all individual participants included in the study. Fresh tissue was utilized in study I, while in study III we used cryopreserved tissue. Formalin-fixed paraffin-embedded tissues (FFPE) were used in both study II and III.

Fresh tissue:

Tumor and adjacent colon/rectal (control) tissues were transported at +4°C in complete media. The complete media contains 88% RPMI 1640, 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin, 1% HEPES, and 0.1% Gentamycin. Subsequently, the material was processed into single cell suspensions in study I.

Cryopreservation of tissue:

To minimize interexperimental differences and to facilitate the comparison of material collected before and after surgery, the samples were cryopreserved for study III. Cryopreservation provides the opportunity for further or re-analysis of selected samples. However, it comes at the cost of losing some populations of cells including PMNs and APCs. The effect of cryopreservation on immune cells and their function was investigated in a separate study in our group. (not included in this thesis [228]). For cryopreservation, tumor tissue and adjacent colon/rectal control tissues were cut into 1x1 mm pieces. The small pieces were submerged in cryoprotectant solution containing 90% FBS and 10% Dimethyl Sulfoxide (DMSO) added to cryovials and placed in Mr. Frosty control rate cell freezing container at -80°C to regulate the freezing rate and minimize cell damage caused by ice crystal formation. This also guarantees uniform cooling rate for all the samples. After 24 hours the vials with the cryopreserved material were transferred to liquid nitrogen tanks, until use (Figure 9).

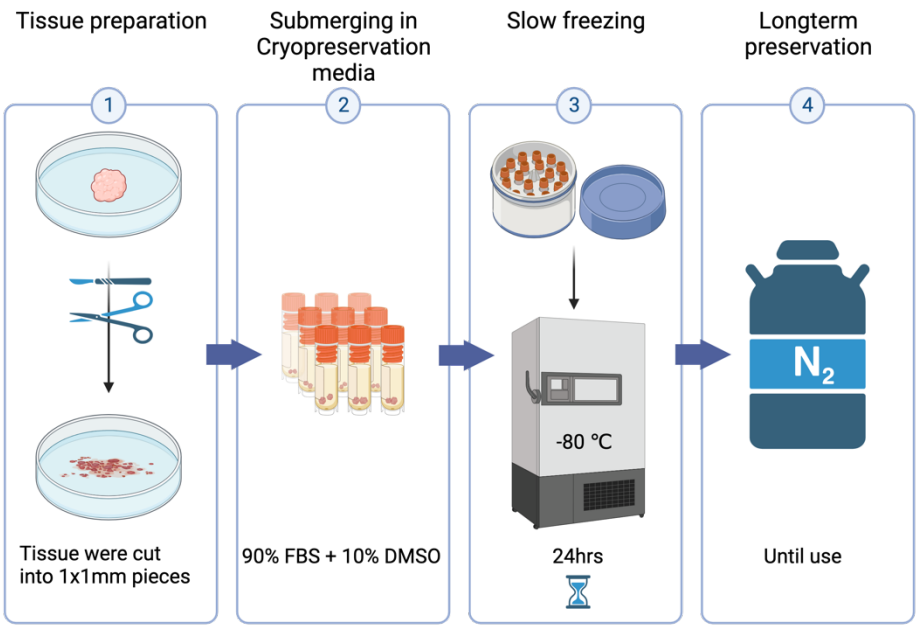

Figure 9. Overview of the Cryopreservation Process. Tissue samples were manually cut into smaller pieces (1x1mm), resuspended in cryoprotectant media in cryovials and placed in Mr. Frosty container for gradual freezing and preservation of the cells' viability. After 24 hours, cryovials were transferred to liquid N_2. Created with BioRender.com

Peripheral Blood Mononuclear Cell (PBMC) isolation:

Peripheral blood mononuclear cells (PBMCs) were utilized in study I, in parallel to tumor and unaffected tissue. Density gradient centrifugation is used widely in isolation of immune cells from blood samples. This technique yields a substantial quantity of cells while causing minimum or no changes to the cells. PBMCs were separated from red blood cells (RBCs) and platelets using a Ficoll gradient. Venous blood was drawn into EDTA vacutainer tubes and stored at room temperature prior to isolation. To initiate the separation whole blood was diluted with an equal amount of PBS (1:2), or in case of using buffy coat from healthy donors, a ratio of 1:5 was used. The diluted blood was gently laid over the Ficoll gradient. PBMCs were isolated from the interface between serum and Ficoll layers following centrifugation for 20 minutes at 2000 rpm, with minimum acceleration speed and without brake. Subsequently, the isolated PBMCs were purified through two rounds of washing with PBS in centrifuge. The

PBMCs were then used fresh or cryopreserved in cryoprotectant media for later use, as explained above.

Single cell suspension:

Single cell suspensions were generated from fresh or cryopreserved tissues through enzymatic digestion (Figure 10) in study I and III. In this commonly used technique in biological research, we can obtain small clusters of cells from tissue. By using enzymes, this process breaks down extra cellular matrix and cell-to-cell connections that hold cells together. Thereafter, cells released from tissue and can be collected for various downstream applications. Optimization and proper control are two crucial factors in enzymatic digestion since the deterioration of some of the cell surface markers stands as a notable drawback of the enzymatic digestion. To address this concern, we included a parallel peripheral blood sample in our experiments. This approach enables us to verify any observed loss of markers is not a result of damage occurring during the digestion process.

In this process, we first treated the tissue samples with EDTA buffer for four rounds of 15-minutes at 37 °C. EDTA buffer dissects and removes the epithelial fraction by facilitating cell-cell disruption and weakening the calcium bonds. The buffer contains HBSS without Ca^{2+} and Mg^{2+}, 2% FBS, Hepes buffer, and 2mM EDTA. Following treatment with EDTA buffer, the buffer was completely removed to prevent inhibition of enzymatic activity in the subsequent step. Then, the remaining tissue (lamina propria) was enzymatically digested in media supplemented with Liberase TM and DNase I. The digestions were performed at 37°C for 60 minutes. The digested tissue was then passed through a cell strainer, and washed once with PBS before resuspension in media and use in experiments (Figure 10).

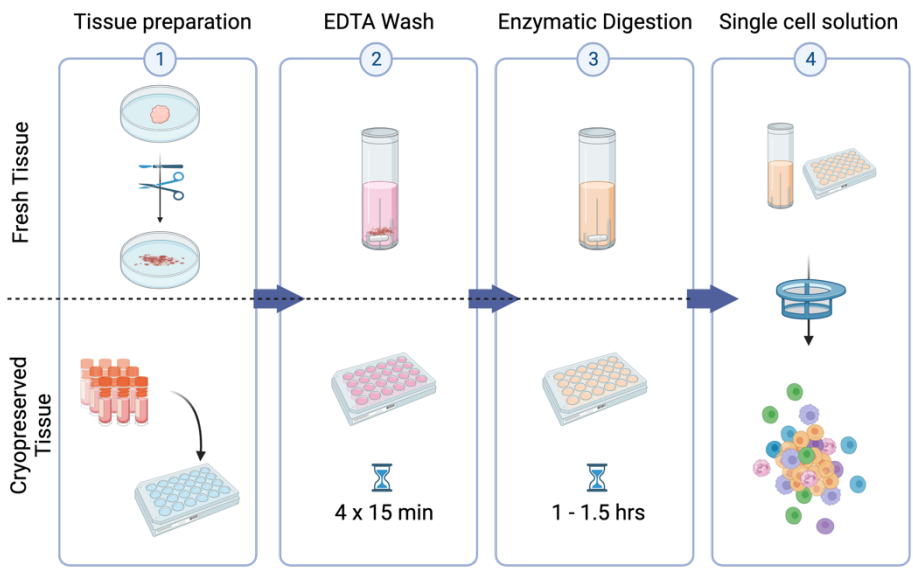

Figure 10. Cell Isolation Process. Fresh or cryopreserved manually processed tissue were washed with EDTA buffer for four rounds and then digested using Liberase TM and DNAse I. The digested tissue was passed through a 40µm cell strainer and was then ready to use for various analyses. Created with BioRender.com

Thawing of cryopreserved tissue:

Cryopreserved tissue biopsies were thawed at 37°C in a water bath, washed and centrifuged twice with PBS at room temperature to remove DMSO. The tissues were then ready for further processing and preparation of single sell suspensions, as described above.

Flow cytometry:

Flow cytometry is an effective method for single-cell analysis that enables scientists to collect comprehensive data regarding quantity and properties of cells within a heterogenous sample suspension. In the field of immunology, this is one of the most significant and widely used techniques. It can offer a wide range of benefits including the ability to analyze multiple parameters simultaneously, perform high-throughput analysis, detect rare populations of cells, sort cells, conduct functional assays, etc.

In this method, cells are labelled with fluorescent-conjugated commercially available antibodies, which are specific for cell surface, intracellular or intranuclear protein markers. The cells are illuminated by an arrangement of 3-5 consecutive laser beams as they pass through the flow cytometer in a stream of single cells. Detectors gather the light signals that the fluorochromes linked to the cells emit. To identify and characterize particular cell components, several detectors evaluate the intensity of fluorescence emission at various wavelengths. Typically, the excitation wavelengths are shorter than the emission wavelengths. The detectors generate data on each cell's fluorescence intensity as well as other characteristics like cell size and granularity, called forward scatter, and side scatter respectively. These real-time data are converted to digital signals and are usually displayed as scatter plots or histograms using a software. Compensating for overlaps between fluorochrome spectra and fluorescence spillover is extremely essential, specifically when evaluating multiple parameters on one cell. However, a proper panel design can also help limit these effects.

In this thesis in Study I and study III flow cytometry used as one of the main methods. Single cell suspensions were stained with fluorescently conjugated monoclonal antibody mixtures for 20 minutes to detect molecules expressed on the cell surface. The stained cells were washed once with PBS, fixed in 2% paraformaldehyde (PFA), then washed again with PBS before storage at +4°C. Staining of intracellular antigens was done after resuspending the cells in fixation/permeabilization buffer for 20 minutes, before addition of fluorescently conjugated monoclonal antibodies mixed in permeabilization buffer. For simultaneous assessment of total number of cells, AccuCount beads were added to the samples before acquisition in the flow cytometer. The stained cell samples were acquired on BD LSR Fortessa 20X and analyzed using FlowJo software.

In vitro stimulation:

In vitro stimulation is described the process of artificially inducing biological response outside of a living organism in a controlled environment. With this method we can investigate particular biological processes in a laboratory setting. In vitro stimulation might involve exposing cells or tissues to various substances such as growth hormones, medication or other stimuli, to examine their reactions and behavior in

the context of cellular or tissue research. In our case, cells were transferred to 5 ml polystyrene tubes and resuspended in complete media containing 88% RPMI 1640, 10% FBS, 1% Penicillin/Streptomycin, 1% HEPES, and 0.1% Gentamycin with or without stimulation using beads coated with anti-CD2, -CD3 and -CD28, or recombinant IFNγ. Then, samples were incubated at 37°C and 5% CO_2. The duration of the culture stimulations varied between 6 hours (Paper I) to overnight (Manuscript III) regarding each experiment. After the incubation, the conditioned media were collected and stored in -20°C for further analyses.

During radiotherapy and/or chemoradiotherapy, T cells are targeted directly or indirectly irrespective of their specificity. To mimic this broad cell activation, we used micro beads coated with anti-CD2 -CD3 and -CD28 antibodies. Anti-CD2 -CD3 and -CD28 is a polyclonal pan T cells stimulus. The antibodies on the beads engage with corresponding receptors on the T cell surface, mimicking the interaction between T cells and pAPCs independent of specific antigens. This stimulation triggers a series of intracellular signaling events that result in T cells activation and ultimately, changes in surface markers and cytokine secretion.

This method is mainly suited for exploring the functional capabilities of the cells rather than the response triggered by a particular antigen. Since certain antigens might produce various intensities of responses or even distinct cytokines, to note this difference is very crucial. Furthermore, stimulating the CD3 receptor along with different cytokines can activate unique transcriptional patterns in Th cells. While polyclonal stimulation offers many insights, it might miss some responses specific to certain antigens or surface receptors [11,229]. Yet, it is often impractical to examine every single antigen or receptor present in the TME or on an individual cell.

IFNγ is a cytokine secreted from activated T cells and plays an essential role in immune response to various antigens. This molecule helps in the regulation of immune reactions and inflammation. Recombinant IFNγ (rIFNγ) is produced through genetic engineering. It can play the same role as human IFNγ in a laboratory setting. rIFNγ can stimulate the APCs by increasing the MHC molecules on the surface of these cells, enhancing co-stimulatory molecules, and increasing the phagocytic

activity of macrophages. In the study of the interplay between pAPCs and T cells in the TME, this substance can help us differentiate between unresponsive T cells and pAPCs.

Functional assays:

The concentration of cytokines in supernatants from tissue cultures in paper I was determined using U-Plex immunoassay (Meso Scale Diagnostic - MSD) and Genieplex. Both of these assays are ELISA-based techniques. In the U-Plex immunoassay, a multi-spot panel with self-assembled unique linkers is located at the bottom of each well, which allows us to detect up to 10 cytokines in the samples simultaneously. The sandwich complex composed of a biotinylated capture reagent, a targeted molecule, and a detection antibody that conjugated to SULFO-TAG binds to the linkers (Figure 11). Thus, the MSD instrument can read the electrochemiluminescence - that comes from SULFO-TAG labels - and determines the concentration of cytokines in the sample. The measurements were done according to the manufacturer's protocol.

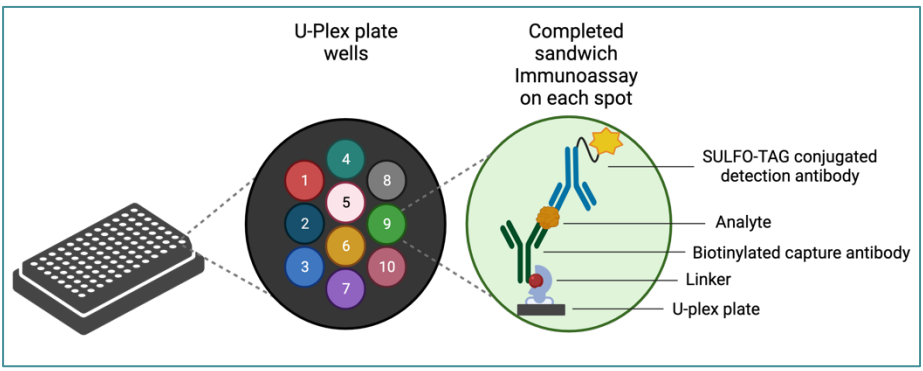

Figure 11. Assay Protocol Overview; U-Plex Multiplex Assay: Each well in the plate features 10 distinct spots. A specific linker, associated with each spot, binds to biotinylated antibodies tailored for individual analytes. The sandwich formation is completed upon addition of the SULFO-TAG conjugated detection antibody, which is specific to the analyte. Created with BioRender.com

Briefly, a coating solution was made by adding equal amounts of each linker-biotinylated antibody solution to a single tube and mixed properly. Then, the coating solution was added to the plate wells and the plate was incubated at room temperature for 1 hour. Following three

washes with PBS-0.05% Tween20, samples were diluted with an equal volume of MSD diluent buffer and added to the wells. After one hour incubation at room temperature, wells were washed with PBS-0.05% Tween20. Detection antibody was added to the wells, followed by one hour incubation at room temperature. After the final wash with PBS-0.05% Tween20, MSD Gold read buffer was added to the wells and the plate was analyzed with the MSD instrument.

Another method employed to analyze cytokines in the conditioned media in paper III was the Genieplex Multiplex ELISA assay from Assay Genie. This bead-based immunoassay is similar to the principle of a sandwich assay and enables simultaneous detection of up to 24 analytes using a minimal sample volume with a sensitivity of less than 2 pg/ml. The Genieplex approach combines beads with conjugated antibodies to target and quantify the desired analytes using a flow cytometer. In this assay, the wells were first coated with pre-mixed antibody-bead populations. Samples containing analytes were then added to the wells and incubated for one hour at room temperature. The residual solution in the wells was removed using a flow-through filter plate washer and the wells were subsequently washed with a wash buffer. Next, biotinylated detection antibodies, which recognize the bound analytes, were added to the wells and incubated for 30 minutes at room temperature. After this incubation, the remaining solution was removed, and the wells were washed. Streptavidin-PE was then added, binding to the biotinylated detection antibody, and the mixture was incubated for an additional 20 minutes at room temperature. Samples were then ready for analysis on a flow cytometer after the final wash and the addition of the reading buffer. Data were processed using FCAP Array Infinite (Soft Flow). The bead-analyte populations are differentiated by the different intensity level of the fluorescence and the size of the beads. To determine the protein concentration in the samples, fluorescent signals can be compared to those from the reference standard curve, derived from serial dilution of an analyte with a known concentration (Figure 12).

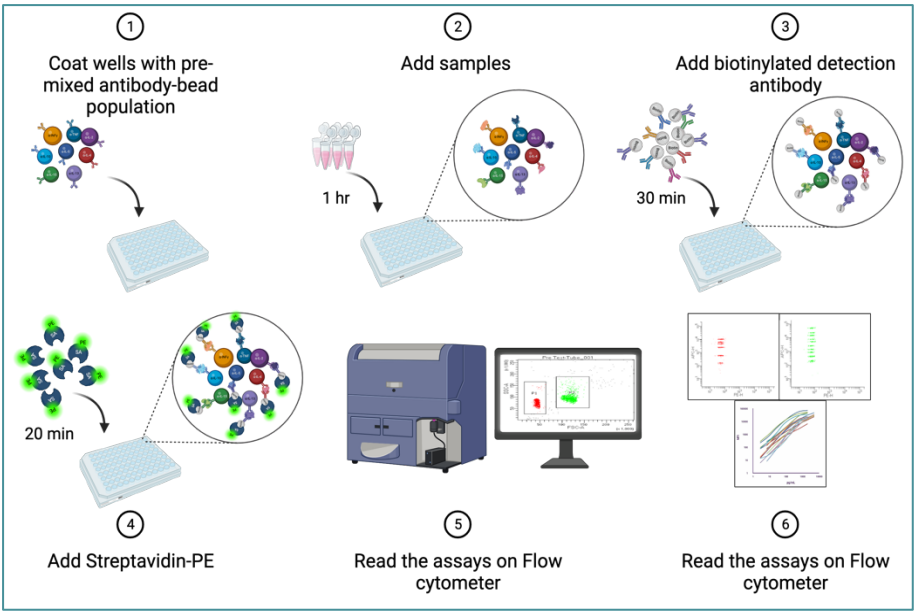

Figure 12. Assay Protocol Overview; GeniePlex. Wells are coated with an antibody-bead mixture, followed by sample introduction. Biotinylated detection antibodies are then applied. Afterwards, Streptavidin-PE is added. Samples are prepared using reading buffer for reading with flow cytometer. Finally, data are interpreted with FCAP Array to determine protein concentration using a reference curve. Created with BioRender.com

Immunohistochemistry (IHC):

Immunohistochemistry is a microscopy-based technique, using fluorescent labeled antibodies specific for markers of interest in the tissue. Exploiting this method, allows us to visualize and localize different molecules and cellular components in our sample under a fluorescent microscope. Unlike flow cytometry, this technique maintains tissue integrity, enabling us to investigate the spatial distribution of cells within the tissue. Still, it operates on a principle similar to flow cytometry, where protein targets are labeled using fluorescently-conjugated antibodies. The detection of signals in tissue sections depends on the excitation of the fluorochromes attached to the antibodies by laser beams. The resulting images are captured through various filters and cameras. In this thesis, this method has been carried out on formalin-fixed paraffin embedded (FFPE) tissue (Paper II and III) and cryopreserved tissue (Paper I).

Prior to the staining, we need to deparaffinize the slides and retrieve the antigens. Removing the paraffin wax, enables the antibodies to bind to the tissue antigens. Incomplete deparaffinization can lead to poor or uneven staining. For this aim, FFPE tissue sections were immersed in xylene followed by stepwise incubations in 99.5%, 95%, 70% ethanol before the slides were left in distilled H_2O. To enhance the detection of the antigen, the slides were subjected to two rounds of incubations with the antigen retrieval solution (10mM Tris, 1mM EDTA, pH 9.0) in a pressure cooker. Once the slides had cooled down to room temperature, sequential staining procedures were started. Antigen retrieval unmasks the target epitopes by reversing some effects of the fixation and breaking the cross-links between proteins. As a result, the antigenic sites of the protein are made accessible, allowing antibodies to stain the tissue (Figure 13).

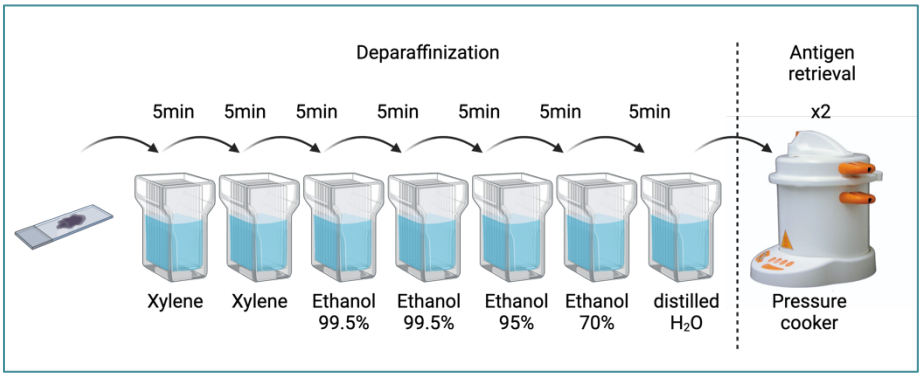

Figure 13. Deparaffinization and Antigen Retrieval. Slides were immersed sequentially in xylene (twice), ethanol 99.5% (twice), ethanol 95%, Ethanol 70%, and distilled water before being treated in a pressure cooker for two rounds, in retrieval buffer. Created with BioRender.com

Immunofluorescence staining: Unconjugated primary antibodies were added to the tissue sections for 1 hour at room temperature. The slides were then washed with PBS once before secondary antibodies (anti-mouse and anti-rabbit) conjugated to horseradish peroxidase (HRP) were added to sections and incubated for 10 minutes at room temperature. After one more wash, fluorescently labeled-tyramide was added to the sections for 10 minutes. The HRP present on the secondary antibody enzymatically converts the inactive tyramide to an active form that covalently binds to tyrosine residues on proteins in the tissue section in proximity to the secondary antibody. The Tyramide Signal

Amplification (TSA) protocol thereby results in an efficient deposition of fluorescent dye, giving an enhanced fluorescent signal compared to staining using directly conjugated antibodies.

For multiple color staining, the sections were then placed in a rack with IHC antigen retrieval buffer and placed in microwave oven for 1 minute at 700W and then 10 minutes at 70W, followed by 30 minutes at room temperature to cool down. The slides were then washed with PBS, before staining with the next primary antibody. This cycle was repeated 6 times and after the last round sections were counterstained for 10 minutes with DAPI. The stained sections were mounted with Prolonged Gold Antifade Reagent and were scanned with the Axio Imager Z2 microscope equipped with a Hamamatsu C13440-20CU digital camera and TissueFAXS software (TissueGnostics) (Figure 14).

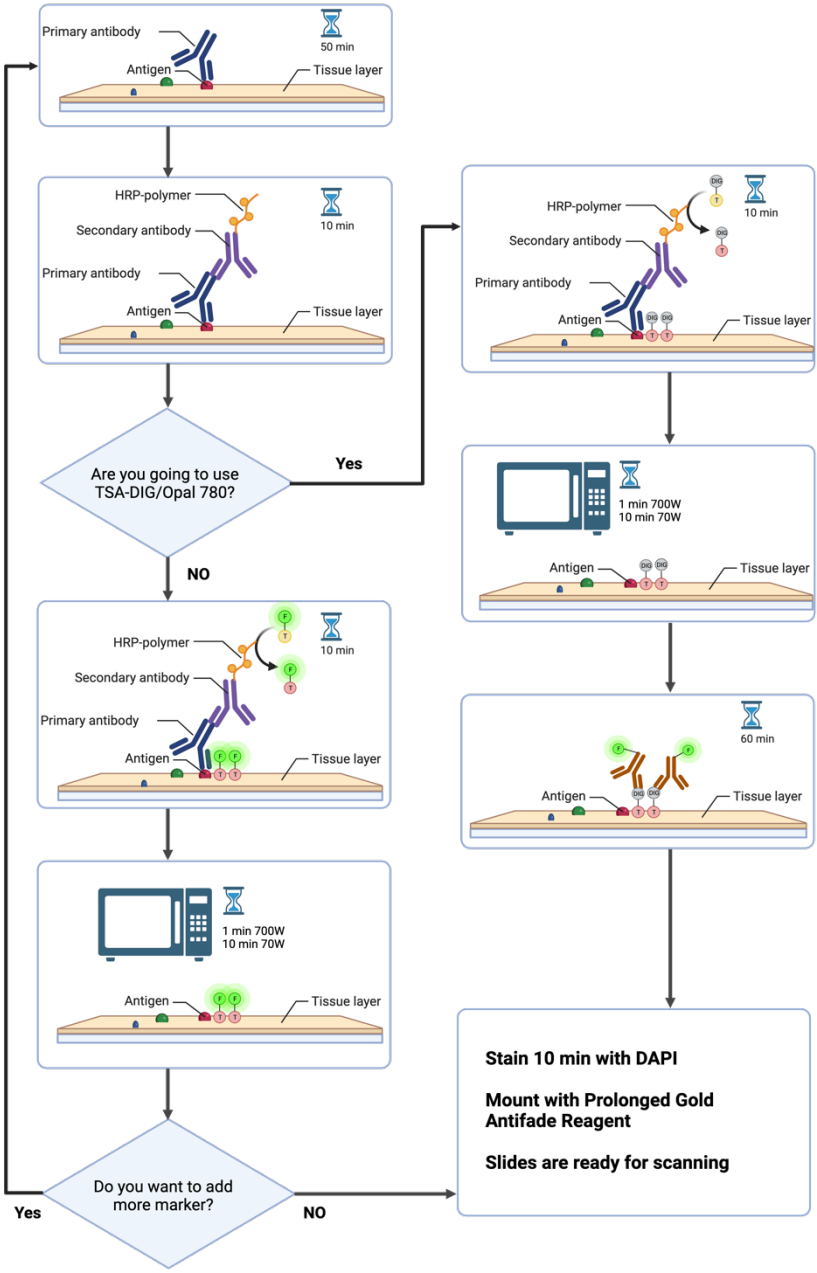

Figure 14 Multiplex Immunofluorescent Flowchart: The targeted surface marker is stained with a primary antibody. The secondary antibody, which is conjugated with horse radish peroxidase (HRP), specific for the constant fraction of the primary antibody, binds to the primary antibody. The peroxidase activates the tyramide-fluorophore when it is added to the sample. To make the other surface markers accessible for the next primary antibody, the slides should be stripped

with a microwave. The same logic applies if TSA-DIG is utilized. Since TSA-DIG does not have fluorescence activity, a fluorescent conjugate antibody should be added separately to the sample. Multiple staining of the samples cannot continue after utilizing the TSA-DIG. Created with BioRender.com

The images were then analyzed and intratumoral cell populations were quantified by StrataQuest (TissueGnostics) after the border between tumor and non-transformed tissues had been determined by a pathologist (Figure 15).

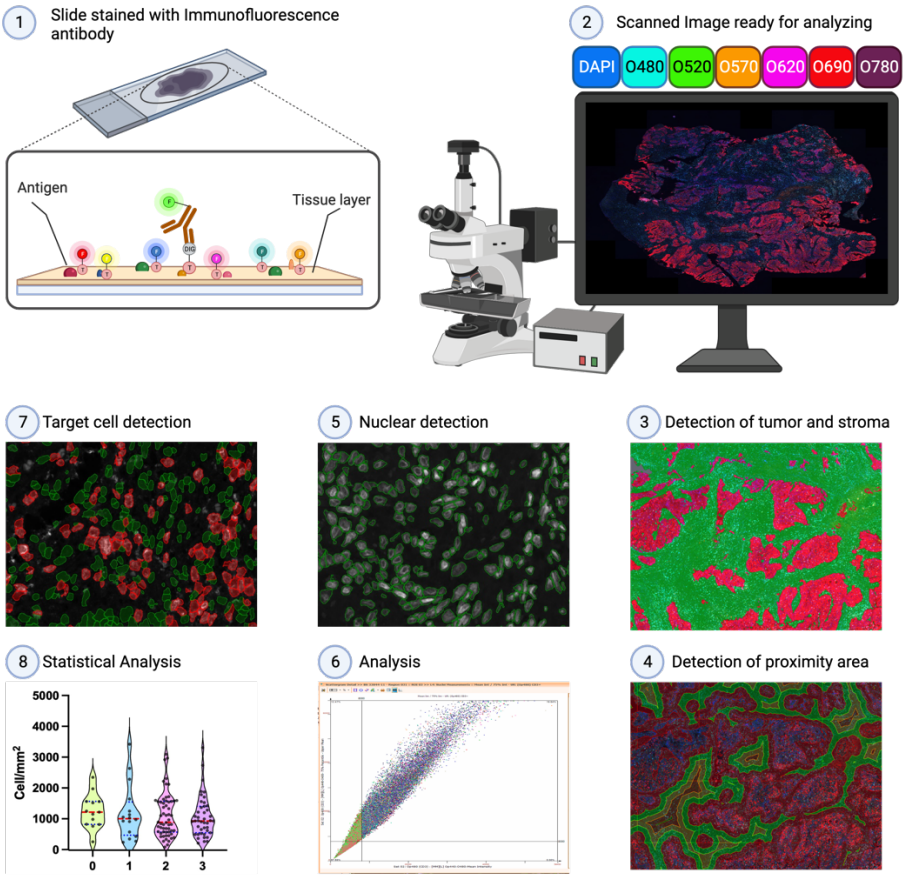

Figure 15.Workflow From Tissue Staining to Statistical Analysis. 1)Slides were stained by using tyramide amplification methods. 2) The entire tissue was scanned with a scanning microscope equipped with a camera. 3) The tissue was classified into tumor (epithelial fraction) and stroma region. 4)The proximity area around the tumor was defined. 5) Cell nuclei were differentiated based on DNA staining with DAPI. 6) A detection threshold was set for each marker. 7) Target cells were detected and evaluated based on cellular indicators including surface and

intracellular expressions, as well as proximity to tumor cells. 8) Raw data were subjected to statistical tests for interpretation. Created with BioRender.com

Hematoxylin and Eosin staining (H&E): To allow examination of sections by a pathologist to determine diagnosis, differentiation between normal and tumor tissue, as well as staging, sections were stained with Hematoxylin and Eosin that label cell nuclei and cytoplasm, respectively. This is the most widely used technique used in medical diagnosis and provides a comprehensive picture of the microanatomy of cells and tissue [230]. In this thesis (paper II and III), H&E staining was performed on separate sections. The slides were stained with Hematoxylin for five minutes, followed by a three minutes wash with water and next stained with Eosin for 30 seconds. Finally, the slides were placed, in the following order, in 70%, 95% and 99% ethanol for 10 quick dips followed by two x 5-minute incubations in xylene prior to mounting with Pertex.

Statistical analyses:

Several types of statistical analyses have been performed in the studies presented in this thesis, and all tests were considered significant at $p < 0.05$. In study I, paired comparisons were performed using Wilcoxon signed-rank test and unpaired comparison were made using Mann-Whitney test. Parametric Pearson correlation analyses and linear regression have been used for generating bubble plots and correlation matrixes. In study II, Wilcoxon signed-rank test was utilized for paired comparison. For testing the association between markers, the Pearson correlation coefficient was used and ordinal data were analyzed by linear-by-linear association and the Glm function in R was used for logistic regression analysis. In study III, for parametrical and non-parametrical paired analysis, t-test and Wilcoxon signed-rank test were used. Non-parametrical unpaired comparison were analyzed by Mann-Whitney test. Kruskal – Willis statistical test was used for analysis between TRG groups. GraphPad Prism software version 10.0.2. (San Diego, CA, USA) was used for data visualization and statistical analysis.

4 RESULTS AND DISCUSSION

Paper I:

Professional APCs (pAPCs) comprise of macrophages, DCs and B cells. These cells play an important role in activating anti-tumor T cells in secondary lymphoid tissues. In addition, their role in reinvigorating TILs, where an immunosuppressive environment prevails, has also become recognized in experimental animal models [231]. CRC tissues can contain considerable numbers of T cells and their infiltration and location have been shown to correlate with overall survival after surgery [212,232-234]. However, if the functional capacities of pAPCs in CRC tissues and their crosstalk with activated T cells are distorted compared to adjacent colonic tissue, thereby potentially revealing an underlying incapacity to induce proper anti-tumor activity of TILs in patients, is not yet known. Hence, the objective of this investigation was to assess whether the performance and activation of pAPCs in reaction to cytokines released by T cells present in the tumor differed from those in the nearby unaffected tissue of CRC patients.

To address these aims, pre-operative untreated biopsies from a total of 55 CRC patients, divided into three separate cohorts (n=28, 10, 17 respectively) were used. The biopsies obtained were separated into three parts; one part was embedded in OCT and stored at -80°C for further analyses of pAPC and T cells subsets with mIF, another part was snap-frozen in liquid nitrogen for MSS/MSI profiling, and the remaining part transported to the lab for generation of single cell suspension and analysis with flow cytometry. In parallel, venous blood was collected for PBMC analysis. PBMCs as well as single-cells suspensions of tumor and unaffected adjacent tissue were stained with a panel of antibodies recognizing activation as well as subset markers. Staining was performed immediately *ex vivo* or after *in vitro* T cell activation with anti-CD2, CD3 and CD28 coated beads after which the tissue culture supernatants were collected for multiplex analyses of the released cytokines.

First, we investigated the presence of pAPCs in tumor and unaffected tissue of CRC patients using immunofluorescent staining of tissue biopsies from one group of patients in our cohort. To identify pAPCs,

we utilized anti-CD64, CD163 as well as CD11c fluorescent antibodies. While DCs express only CD11c, macrophages could be identified by the co-expression of CD64 and CD163 within the population of $CD11c^+$ cells (Paper I Figure 1B). Delving further into our research, we determined the frequency and functional profiles of pAPCs in tumor, unaffected adjacent tissues, as well as peripheral blood samples from the CRC patients in the second cohort of our study. Analysis of the single-cell suspension by flow cytometry, using similar markers demonstrated a consistent finding of macrophages and DCs in the CRC tissue.

pAPCs are heterogenous and comprised of different subsets with opposing functions, like macrophages with pro-inflammatory and anti-inflammatory characteristics. Thus, for simplicity, we focused on the entire population of macrophages that are $CD11c^+$, $CD64^+$, and DCs that are $CD11c^+$ but lack CD64 expression. Compared to the adjacent colonic tissue, the tumor displayed a significant dominance of macrophages. This finding aligns well with previous studies [235-237]. In contrast to macrophages, a concomitant decrease in the frequency of DCs was observed (Paper I Figure 1E, F). Dividing the pAPC into subpopulations, revealed that a substantial proportion of the macrophages expressed CD14 and remained prevalent in the tumor. While the DCs were categorized into $CD141^+$ cDC1 and $CD1c^+$ cDC2 subsets, a significant decline was observed in cDC2 cells in tumor, but a slight elevation was noted in the cDC1 subset (Paper I Figure G).

In the fluorescently stained tissue sections, $CD8^+$ T cells were studied and we found that the majority of $CD8^+$ TILs and pAPCs were co-located in the stroma of the tissue (Paper I Figure 1A). This distribution, suggested interactions, potentially frequent, between pAPCs and T cells. Activation of the naïve T cells in the lymph node cannot occur unless CD80 or CD86 molecules on the pAPC bind to CD28 on T cells. PD-L1 is another molecule on pAPC that is upregulated in response to stimulation and through interactions with activated T cells that express PD-1 to control the immune response. As we were interested in what happens in the TME, we examined the expression of CD80 and PD-L1 on pAPCs, co-stimulatory and co-inhibitory molecules respectively, in tumor as well as adjacent macroscopically unaffected colonic tissue as control. Within the tumor, macrophages exhibited higher CD80 and lower PD-L1 levels compared to those in the adjacent tissue (paper I Figure 2B). This contrasting expression pattern was also evident in the

$CD14^+$ subset of macrophages (paper I Figure 2C). A similar trend, among DCs, most apparent regarding the expression of CD80 and in cDC1 was also observed (paper I Figure 2).

Our characterization of pAPCs revealed a pronounced infiltration of macrophages in tumor tissue. In addition, the TAMs expressed higher levels of CD80 and reduced PD-L1 compared to macrophages in the colonic tissue counterparts. These findings led us to postulate several underlying mechanisms that could be driving this phenotypic distinction. The colon is not just a passive tissue; it's a bustling hub of microbial activity. The presence of commensal microbes, which coexist in a finely tuned balance with our immune system, might hold clues to the pAPC phenotype we observed. Given their constant interaction with these microbes, it is conceivable that colonic pAPCs have adapted to ensure that the immune system does not overreact to the microbiota, i.e a restricted CD80 and elevated PD-L1. The increase in macrophage infiltration seen in the tumor may be a direct result of chemoattractant signals produced by cells in TME. Previous studies have underscored the role of chemokines in the recruitment of immune cells, such as macrophages, thus potentially fostering an immune tolerant or even immunosuppressive niche [238]. Moreover, the enhanced expression of CD80, a co-stimulatory molecule, and downregulation of PD-L1 might imply that TAMs are ready for facilitating T cell activation, suggesting an intensified anti-tumor immune response within the TME. This would be consistent with a body of evidence proposing that signals from the TME can potentiate local immune responses against malignancies (the cancer immune cycle).

The observed TAM phenotype in the CRC tissue might also be influenced by feedback from T cells present in the TME. Given that T cells may release an array of cytokines, including IFNγ, their presence might induce phenotypic alteration in macrophages. Therefore, we examined the early activation marker CD69 and inhibitory marker PD-1 on T_{RM}s cells in both tumor, adjacent colonic tissue as control, as well as PBMC. Lower expression of both CD69, and PD-1 on T cells in blood circulation of the patients compared to both tumor and colonic tissue was observed (paper I. Figure 3B). This suggests that T cells within the tissue – both in tumor and colon – are either in a more activated state or have a history of activation. While the proportion of $CD8^+$ T cells was higher in both unaffected and tumor tissue, the latter exhibited a

noticeable increase in proportions of total T cells and $CD4^+$ T cells (paper I Figure 3C). The analysis of subpopulations of T cells revealed that the frequency of T_{RM}s, characterized as $CD103^+CD39^-$ T cells, was comparable in tumor and colon regardless of being CD4 or CD8 expressing. A high presence of $CD4^+CD39^+$ T cells in tumor has been reported regardless of whether the $CD39^+$ T cells expressed CD103, a marker of tissue residency [239]. The $CD4^+CD39^+$ subset are potentially Treg as CD39 has been introduced as a marker for Treg [239]. No difference was observed in the frequency of $CD8^+CD39^+CD103^+$ T cells in tumor compared to adjacent colonic tissue, even though they are potentially known as tumor-specific T cells. Importantly, this subpopulation of T cells displayed greater PD-1 and reduced CD69 levels (paper I Figure 3D-E), suggesting that these cells might be functionally suppressed due to the chronic inflammatory state in the TME which inhibits the entrance of new cells into the tumor and suppresses the existing cells. Furthermore, the expression of PD-1 or CD69 might be under the influence of interaction with other cells in TME like APCs.

Interestingly, activation markers on T cells and pAPCs in tumor and unaffected tissue followed the same pattern and they were negatively associated, which was significant between certain subpopulations of cells. However, the correlation between inhibitory markers PD-1 and PD-L1 on T cells and pAPCs, respectively, in tumor and colonic tissue was very different. In the tumor higher PD-L1 on pAPCs was correlated with higher PD-1 on T cells, suggesting a suppressive environment, while in the colon, higher PD-L1 was negatively correlated with PD-1 expression (paper 1, Figure 4). This might be a signature established as a consequence of the different microenvironments in colon and tumor. Furthermore, analyzing the functional capacity of pAPCs in antigen uptake and degradation using the model protein ovalbumin demonstrated that intratumoral pAPCs, in comparison to colonic pAPCs are less proficient in protein uptake and degradation. We also observed that the co-stimulatory capacity appears compromised in the TAMs that degraded the antigen, as these cells were unable to upregulate co-stimulatory markers to the extent that the counterparts from the colonic adjacent tissue did (paper I Figure 5). We also measured the functional capacities of T cells in tumor and unaffected tissue through stimulation single-cell suspensions with microbeads coated with anti-CD2, CD3,

CD28 for 6 hours. These beads mimic antigen presentation to all T cells, allowing us to monitor the behavior of pAPCs in both tumor and unaffected colonic tissue after exposure to the factors secreted by the T cells present at the two locations. In contrast to TAMs, T cells residing in the CRC tissue showed maintained capacity to activate pAPCs following polyclonal activation. In addition, the increase in pAPC-specific cytokines, e.g., IL-1β, TNF, IL-6, suggests that the pAPC in the tumor are able to respond to activated T cells. The only measured cytokine that was secreted at different levels by TILs and colonic T cells was IFNγ. This demonstrated a reduced capacity of TILs from CRC, compared to colonic T cells, to produce IFNγ upon activation (paper I, Figure 6C). Furthermore, stimulation of the T cells resulted in the upregulation of PD-L1 by pAPCs (both macrophages and DCs) from colonic tissue, but in tumor, it was only DCs that upregulate PD-L1 expression in response to pan T cell stimulation (paper I, Figure 6A). Conversely, CD80 was significantly downregulated exclusively by tumor macrophages and this appeared to follow the same trend among DCs.

As IFNγ is a cytokine capable of influencing macrophages, and also known from murine studies to result in upregulation of PD-L1, it was readily detected in the Pan T cell stimulated cell cultures [240]. We directly stimulated the suspensions of cells with rIFNγ, to assess how it would affect pAPCs. We noted a significant upregulation of PD-L1 on both tumor and unaffected tissue pAPCs, but CD80 was only upregulated in colonic macrophages (paper I, Figure 6B). This suggests that when the cells were stimulated with anti CD2-3-28, something beyond IFNγ but not among the 8 cytokines measured, was produced. Consequently, we decided to investigate if colonic pAPCs would respond differently to conditioned media collected from the tumor and vice versa. To ascertain the proper volume of conditioned media, we titrated different colon and tumor conditioned media at 1%, 10%, 30% on PBMCs obtained from a healthy individual. We observed response at concentrations over 10% (not shown). Based on this, we transferred 10% of the tumor conditioned media to colon cells and tumor cells, and 10% of colon-conditioned media to tumor and colon cells. These two conditioned media were also added separately to PBMCs as a control.

The cells were incubated overnight and the expression markers on the pAPC present in the cultures were assessed (Figure 16 – Left). We found no significant difference in the response by colonic or TAM to either of the conditioned media. Similarly, blood monocytes of DCs exhibited comparable shifts in marker expression following coculture with either tumor or colon conditioned media. This shows that any potential difference in conditioned media generated from either tumor or colon could not be detectable by monitoring CD80 and PD-L1 on pAPCs (Figure 16 – Right). Additionally, these differences in the conditioned media derived from tumor and colon cells could not be detected in in our cytokine measurements.

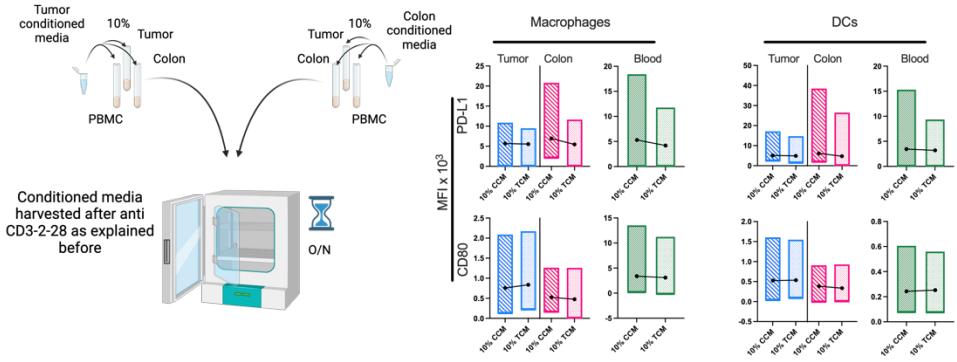

Figure 16: The Response of Antigen-presenting Cells to The Conditioned Media of Tumor And Colon. Experiment workflow (left), and changes in expression markers, CD80 and PD-L1 on tumor, colon, and blood APCs in response to 10% of tumor conditioned media and colon conditioned media. Created with BioRender.com (left).

Moreover, a positive relationship was observed between the expression of CD80 and PD-L1 on pAPCs in both tumor and colonic tissues. This correlation indicates a potential negative feedback mechanism in which pAPCs expressing CD80, after interacting with activated T cells, might increase PD-L1 levels to dampen excessive T cell stimulation. This pattern of CD80 and PD-L1 expression on pAPCs was also replicated when a portion of the culture media was replaced with conditioned media, as observed in PBMC cultures (paper I, Figure 6 D and E). In summary, we conclude that intratumoral and colonic pAPCs are functionally and in activation status distinct yet similarly responsive to activated T cells.

Paper II and III:

Inter-patient heterogeneity in response to the current standard treatment for rectal cancer as well as complications and adverse effects of radio/chemotherapy, necessitate the need for stratification and development of novel means to predict the response to neoadjuvant therapy. Immunoscore and its biopsy adapted approach (IS_B) - discussed in chapter 1.4.3 - are tools that have drawn a lot of attention to the role of the immune system in (colo)rectal cancer treatment [220]. Although it may be a promising prognostic tool to stratify patients according to their possible response to neoadjuvant therapy and perhaps even more importantly to predict tumor recurrence in non-surgically treated watch and wait patients [220], the IS_B is based on relative frequencies of cell populations and quartiles within a given a cohort which makes clinical applicability harder.

Our aim in these two studies was to identify accurate and reliable predictive immune markers capable of predicting outcomes, to guide patient treatment decisions with a focus on complete responders in paper II, and non-responders in study III.

We explored the distribution of T cell subsets in combination with the functional activity of these cells in preoperative rectal cancer biopsies. In paper II, we determined the distribution of T cell subsets ($CD8^+$, $CD8^+GrzB^+$ and $\gamma\delta TCR^+$) in combination with a "footprint" of type I IFN, and in paper III we replaced γδTCR and GrzB markers with CD4 and TCF1 in a separate cohort. In both studies, Pan Cytokeratin was utilized as a marker to differentiate the epithelial compartment (the tumor center or "nest") from the stromal area of the tumor.

It has been reported that the best prognostic capacity is achieved if the TILs reside in the tumor nest [87]. Our initial results obtained from the identification of cells through multiplex immunofluorescence staining of the formalin-fixed paraffin-embedded tissue (FFPE) showed a higher density of TILs in the tumor stroma than in the tumor nest in both studies (paper III Supp Figure 1D). Our observation was aligned with other studies of rectal cancer tissues [241-244]. This could reflect an accumulation of T cells in the stroma prior to infiltration between tumor cells. Alternatively, it suggests a suppressive environment in the tumor that prohibits further recruitment of the immune cells into the core of the

tumor. However, studies in other GI malignancies suggested that the assessment of TILs in stroma could be beneficial in differentiating the biological behavior in patients rather than intra-tumoral TILs [245,246]. To explore potential differences in immune cell phenotypes based on treatment response, a pathologist classified patients by their tumor regression grade (TRG) post-treatment. This can be done using several different scoring systems and we used the American Joint Commission for Cancer (AJCC). TRG 0 indicates a complete pathological response while TRG 3 denotes no detectable response. The density of T cell subsets was then plotted for each patient across these TRG categories (paper II Figure 1a, paper III, Supp Figure 1E).

The number of $CD3^+CD8^+$ cells was not significantly different in the preoperative tumor biopsies across TRG groups. This was consistent for *complete responders* compared to other patients with TRG 1,2, and 3 (paper II, Figure 1). Determining the density of $CD3^+CD8^+$ cells could not assist in identifying *non-responders* among other patients either (paper III, Figure 1B). This was regardless of whether the cells were located in the tumor nest or within the surrounding stroma. It has been observed that a high pre-treatment presence of $CD8^+$ T cells in rectal cancer is linked to pathological complete response [219]. This finding has later been supported by multiple studies of rectal cancer [241,242,244,247,248]. However, it should be noted that some studies using rectal cancer biopsies have not been able to find such a correlation [109,249]. These discrepancies could be attributed to variations in evaluation methods. Upregulation in GrzB gene expression in complete as well as partial responders to neoadjuvant treatment has been reported [250]. To pinpoint our analysis to cells with lytic capacity, we included in addition to antibodies against CD3 and CD8, also anti-GrzB in the mIF staining. This did however not significantly improve the capacity to correlate TILs and response to neoadjuvant treatment (paper II, Figure 1).

γδ T cells are among the most abundant unconventional T cells. Despite being a relatively small population of TILs, the presence of these cells has been shown to be correlated to anti-tumor responses in several different malignancies. However, detection and enumeration of these cells in our study, did not show any association with tumor regression, as there was no difference in the number of γδ T cells across TRG groups or when comparing complete responders versus TRG1, 2 and 3 pooled

together (paper II Figure 1). This could be because γδ T cells have been suggested to have dual roles in TME [251] (discussed in chapter 1.3.3.4). In paper III, in addition to $CD8^+$ T cells, when comparing non-responders (TRG 3) to a dichotomized group of patients with better or complete responses (TRG 0-2), we also included Th cells ($CD4^+$) and $TCF1^+$ (T cell transcription factor 1) T cells. This factor is expressed at different stages but mainly during memory formation and exhaustion [252,253]. We found that neither quantification of Th nor TCF-expressing T cells could assist in identifying patients that would not benefit from neoadjuvant treatment i.e TRG3.

A type I IFN rich TME is beneficial for inducing of T cells with lytic potential. One reason is that the cytokine can promote cross presentation on MHC-I through activation of cGAS-STING pathways [164]. A trait that could be of importance for anti-tumor responses. Thus, we enumerated cells responsive to type I IFN by assessing the presence of the response element Myxovirus resistance protein A (MxA), which is upregulated following exposure to type I or III IFN. We focused on tumor stroma as cells in the stroma are responsive to type I IFN, while cells in epithelium can respond to both type I and type III IFN [254]. Quantification of MxA-expressing cells in the tumor stroma revealed a significant difference between TRG 0 and TRG 3 (paper II, Figure 2). This suggested a higher exposure to type I IFN in this area in complete responders to neoadjuvant treatment. While there was a significant difference when comparing TRG 0 (complete responders) to grouped TRG 1-3 (paper II, Figure 2), no significant difference was observed when comparing TRG3 (non-responders) to TRG 0-2 (paper III, Supp Figure 1F). This leads us to hypothesize that quantification of MxA^+ cells in the tumor stroma might assist in detecting patients with a complete pathological response to neoadjuvant treatment. To build such a model and leverage a tool like the IS_B, introduced by El Sissy et al [220], our cohort was divided into three ranked quartiles (paper II, supp Figure 5a). However, in our model rather than using CD3 and CD8 separate quantification in the biopsy, we solely focused on the number of MxA^+ cells per mm^2 in the stroma. This approach yielded a significant association between the density of MxA^+ cells and more efficient tumor regression following neoadjuvant treatment. It also showcased a sensitivity comparable to the IS_B (paper II, Figure 3). Exchanging CD3 for MxA in the IS_B approach and using CD8 and MxA

to create the relative quartiles resulted in blunted sensitivity to predict the clinical response to neoadjuvant treatment (paper II, Figure 3). This finding implicated an independent role of MxA as a surrogate marker for type I IFN in predicting patients' response to neoadjuvant treatment. We therefore reasoned that using an approach where relative quartiles of the densities of stromal MxA and CD8 cells were assessed separately and then weighed equally could improve the quality of the prediction. For this purpose, after determining the ranked percentiles of CD8 and MxA and categorization according to IS_B, we created a heat-map. We used color codes where dark red represented high value for both CD8 and MxA, dark blue for both low values and additional shades in between these extremes for other values, to represent the categorization (paper II, Figure 3B). The heat-map based IS_B, which was derived from high $CD8^+$ and/or MxA^+, was significantly associated with complete tumor regression following neoadjuvant treatment. These results suggest that patients with high numbers of TILs prior to neoadjuvant treatment may exhibit a stronger response due to initial tumor-specific CTL priming, although this alone is not adequate to counteract the suppressive TME. Post- neoadjuvant treatment dying cells might release tumor antigens that could be captured by professional pAPC and potentially reactivate CTL in patients with abundant TILs. Type I IFN-driven inflammation in the tumor has also been suggested to foster a TME conducive to tumor elimination by TILs [255]. A high density of MxA^+ cells might be a characteristic of a tumor with such a propensity and neoadjuvant treatment might amplify this trait by inducing cell death and propelling the cancer immunity cycle.

MxA-expressing cells, either alone or in combination with CD8, can assist in identifying complete responders to neoadjuvant treatment, potentially guiding inclusions in the "watch and wait" strategy. If identification of the patients with a high risk of regrowth within this group, who have opted out of surgery, is achieved, these individuals could have more frequent follow ups with endoscopy or be counselled to receive surgery. The other group of individuals that would highly benefit from identification are the non-responders. However, despite modifying our multiplex immunofluorescent (mIF) panel by adding marker for Th and exhaustion markers in T cells, neither MxA-expressing cells nor spatial analysis of T cells could aid in identifying the non-responders in our cohorts. To delve deeper, we expanded our

analysis by generating single-cell suspensions from cryopreserved rectal tumors and corresponding rectal tissues of the same patients by choosing five patients of each TRG score from our biobank. After confirming that the mean proportions of $CD8^+$ and $CD4^+$ T cells from single-cell suspensions aligned with mIF results (data not shown), we proceeded with our investigation. Consistently with mIF findings, T cells proportions remained unchanged across patient groups with different treatment responses. Similarly, the proportion of T cells, including regulatory T cells (Treg), showed no significant differences between normal and tumor tissues (paper III, Figure 2 B-D).

The diagnostic potential of CD103 in the evaluation of the response to neoadjuvant treatment in rectal cancers has been highlighted by studies linking increased numbers of $CD8^+CD103^+$ TILs with better survival outcomes in both CRC [256,257] and other cancers [258,259]. Our analysis revealed no significant difference in the proportion of $CD103^+CD39^+$ T cells across different TRG scores in either tumor or rectal tissues (paper III, Figure3). While the proportion of $CD8^+CD103^+39^-$ T cells remained consistent in unaffected rectum across all TRG scores, their proportion within tumors varied notably depending on TRG scores. Specifically, a pronounced decline in the frequency of $CD8^+CD103^+39^-$ T cells was evident in patients with TRG 1, followed by TRG 0 (paper III, Figure3). In contrast, TRG 3 patients did not exhibit a significant drop in these cells compared to their unaffected tissue (paper III, Figure3). The unchanging trait in TRG 3 makes this group stand out compared to TRG1 and grouped TRG 0-2. Furthermore, it potentially introduces a signature to identify patients that will not benefit from neoadjuvant treatment. $CD8^+CD103^+$ cells have been described as T_{RM} and further categorized into two subsets based on CD39 expression. While CD39 is used as a marker on regulatory CD4 T cells, its expression on CD8 T cells indicate a chronic inflammatory state possibly driven by recurrent exposure to presented tumor antigens [260]. CD39-expressing cells exhibit a strong exhausted phenotype [260,261] and commonly co-express PD-1 [261]. In some malignancies, including CRC, the presence of $CD8^+CD103^+39^+$ cells in the TME is linked to a favorable prognosis [260,262], possibly indicating a robust tumor immune response. In CRC, higher frequencies of these cells have been reported in MSI tumors [260]. On the other hand, the absence of CD39 on T_{RM} cells suggests a lack of continuous antigen stimulation at the tumor site. This population was

described by Simoni et al [263] as "tumor-unrelated bystander CD8 T cells" [263] and suggested to be detecting viral rather than tumor epitopes presented on MHC-I [263]. Hence, non-responsiveness to the neoadjuvant treatment in patients with a predominant $CD8^+CD103^+39^-$ phenotype of T_{RM}, might at least partially be explained by an inability to effectively recognize tumor antigens. Despite the fact that a large proportion of this subset of T cells resided in the tumor, they would be unable to detect tumor antigens and initiate a potent immune response. Interestingly, it has been reported that these bystander CD8 TILs express fewer inhibitor molecules like PD-1, which is consistent with our observations that non-responders to neoadjuvant treatment have lower PD-1 than responders. However, we could not reliably directly assess the expression of PD-1 on the $CD8^+CD103^+39^-$ subset due to the limited number of cells in the biopsies. Other potential reasons for the higher frequency of $CD8^+CD103^+39^-$ in non-responders could be a lack of proper co-stimulation, presence of inhibitory signals, high sensitivity to immunosuppressive factors released in the TME, or inefficiency of pAPCs in antigen processing and presentation.

In Paper I, we observed elevated PD-1 and diminished CD69 expression on TILs, a pattern which was further corroborated in Paper III for both CD4 and CD8 T cells compared to their counterparts found in non-tumorous rectal tissue (paper III figure 4A). Notably, this increased PD-1 expression was evident in TRG 0 and TRG 1 groups for both T cell types, whereas this was not discernable in the cells from TRG 2 or TRG 3 biopsies (paper III, Figure 4B). The greatest decline was observed when TRG3 was compared to grouped TRG 0-2 for both Th and cytotoxic TILs (paper III, Figure 4C). Earlier studies have also reported a link between higher expressions and density of $CD8^+$ and PD-1^+ TILs and a better prognosis in CRCs [264,265]. We determined the PD-1 expression ratio between tumor and unaffected tissue to enhance the reproducibility and applicability of these markers in clinical practice. This confirmed that, only CD8 T cells from preoperative biopsies of non-responders, had a significantly lower ratio of PD-1 compared to those of other TRG groups in these tissues (paper III, Figure 4E).

Given PD-1's crucial function in modifying immune checkpoint pathways, increased PD-1 expression on T cells can suggest activation as well as possibly exhaustion and/or an immunosuppressive environment. To determine if these disparities extended to gross

functional attributes, single-cell suspensions received overnight pan T cell stimulation. In line with study I, T cells in both tumor and rectal tissue, were similarly responsive to this stimulation and able to release cytokines into the conditioned media (paper III, Figure 6 and Supp Figure 4). In addition, in this study, we observed certain surface activation markers increased post-stimulation (paper III Figure 5 A). However, uniform upregulation was observed across the groups with different TRG scores (paper III Figure 5C-E). These results indicate that identification of immunological and potentially functional reasons to the non-responsiveness to neoadjuvant treatment in the tumors of these patients cannot be found using pan activation of T cells followed by global measurements and would rather need focusing on the response in tumor-peptide-specific T cell clones.

5 CONCLUSION AND FUTURE PERSPECTIVES

In study I, with a focus on tumor immunity, we assessed the interaction between T cells and pAPCs. More specifically, we addressed how pAPCs respond in co-culture with pan activated T cells when single-cell suspensions were derived from tumor or adjacent colon/rectal tissue of the same individual. We found that both pAPCs as well as T cells in these two tissues demonstrated different phenotypical characteristics. TAMs expressed a lower level of co-inhibitory molecule PD-L1 and the co-stimulatory molecule CD80 compared to their colonic paired cells. On the other hand, on the potentially tumor-specific TILs ($CD8^+$ $CD39^+$ $CD103^+$), activation and exhaustion markers were elevated and reduced, respectively. Additionally, the results revealed that although the secretion of IFNγ from T cells was significantly lower in tumor tissue compared to the colon, pAPCs in the TME were capable of efficiently responding to T cell stimulation by upregulating CD80 and PD-L1 molecules on their surface.

In study II, our exploration of the tumor landscape in pre-treatment biopsies of rectal cancer patients with different responses to neoadjuvant treatment, showed that a heat-map could be created to stratify the patients based on the density of $CD8^+$ TILs in the whole tumor tissue and MxA^+ cells in the stromal compartment. This heat-map generated a score that correlated with tumor regression. However, this approach is cohort-dependent and requires validation. This validation is underway in study III, where we are keeping the core markers but also refining it by exchanging other markers in our mIF panel. This approach could assist in identifying patients who don't require surgery following neoadjuvant treatment. It may also guide clinicians in adjusting treatment and follow-up protocols for complete responders.

Identification of non-responders to neoadjuvant treatment is the primary objective of study III. These patients would most likely benefit from alternative treatments. Opting for earlier surgery could be more beneficial than investing time in a treatment that offers no obvious therapeutic value but rather creates potential adverse effects. Enrichment of the TME with TILs with characteristics of T_{RM} cells ($CD8^+CD103^+CD39^-$) was pronounced in non-responders. Moreover,

TILs in non-responders exhibited a reduced expression of PD-1. We intend to validate these finding using mIF staining of FFPE tissue from the same individuals. Expanding our study to include more patients is also part of our agenda, especially considering the limited size of our current cohort. In addition, the $CD8^{+}CD103^{+}CD39^{-}$ population has previously been introduced as being viral-specific rather than reactive to tumor antigens. Thus, shifting the focus from studying pan T cell responses to assessing antigen-specific responses could be beneficial, as our present approach has an eminent risk of overshadowing the potential nuanced differences among tumor antigen-specific T cell clones in the samples. Unfortunately, this is not easily done as there are very few, if any, common tumor antigens in CRC described in the literature. This could possibly be circumvented to a certain extent by combining Pan T cell activation with single-cell and TCR sequencing technologies.

Presently, IS_B is the only available immunological assessment for predicting the response to neoadjuvant treatment. It exhibits the potential to identify complete responders to neoadjuvant treatment. However, it is worth noting that this tool is intra-cohort dependent. One potential solution to this issue is to compare different parameters in the tumor tissue with paired adjacent normal tissue in pretreatment biopsies obtained during endoscopic examinations from each individual. This would allow for defining an index for each patient that could then be compared with other patients. Another way to classify patients according to their responsiveness to neoadjuvant treatment is by comparing the TME of the patients pre- and post-treatment. However, collecting post-treatment samples from complete responders is challenging due to clinical guidelines. To address this, emerging methods like organoid models might be instrumental in bridging the gap. Furthermore, the TME is composed of compartments beyond just immune cells. These cells, previously considered to be merely building blocks, can in fact directly or indirectly influence the behavior of the immune cells as well as the growth of the tumor. Thus, methods like single-cell RNA sequencing, for detailed cellular data, can offer invaluable insights. Moreover, multi-dimensional analysis that integrates various parameters from diverse methods might pave the way for developing a more practical assessment tool. This integrated approach could be pivotal in driving personalized therapies for CRC.

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