Regulatory T cells and mucosal-associated invariant T cells in colon adenocarcinomas; Phenotype and function

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Gothenburg 2019

Cover illustration:
CFSE-stained original $CD39^-$ Treg (magenta) and autologous responder T cells (green), both originating from peripheral blood, were analyzed by flow cytometry after 5 days of co-culture.

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ISBN 978-91-7833-346-2 (PRINT)
ISBN 978-91-7833-347-9 (PDF)

Printed in Gothenburg, Sweden 2019
Printed by BrandFactory

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ABSTRACT

In many solid cancers, and also in colon adenocarcinomas, an increased accumulation of lymphocytes is beneficial for the patient. However, tumor-infiltrating immune cells may be either pro- or anti-tumorigenic and the balance between these two counteracting forces partly determines patient outcome. Boosting of the anti-tumor immune response by immunotherapy, e.g. by immune checkpoint blockade, has been highly successful in several types of cancer but less so for colon cancer. In the interest of developing new cancer immunotherapies also for the treatment of colon cancer, additional studies of tumor-infiltrating lymphocytes in colon cancer are warranted. In this study, we used flow cytometry and flow cytometric cell sorting as well as *in vitro* cell culture assays to examine the phenotype and effector functions of two distinct immune cell populations which we have shown to accumulate in tumors of colon cancer patients, $CD39^+$ regulatory T cells ($CD39^+$ Treg) and mucosal-associated invariant T (MAIT) cells. Treg reduce the activity of other immune cells and can express the surface molecule CD39, an ectoenzyme involved in converting extracellular ATP to immunosuppressive adenosine. MAIT cells recognize bacterial metabolites and are innate-like T cells which are believed to provide a first line defense at epithelial surfaces. This thesis comprises an extensive phenotypic and functional characterization of these two subsets in colon tumors, and also preliminary survival data on their respective impact on patient prognosis.

We show that $CD39^+$ Treg constitute a highly activated and immunosuppressive Treg subset. In particular, surface expression of immunomodulatory mediators were increased in the $CD39^+$ Treg subset, while cytokine production was similar in $CD39^+$ and $CD39^-$ Treg. We also present preliminary survival data which suggests a correlation between high levels of CD39 expression on intratumoral Treg and a worse patient prognosis, thus highlighting $CD39^+$ Treg as a potential candidate for targeted immunotherapy. With regard to MAIT cells, we could demonstrate accumulation of MAIT cells in colon adenocarcinomas. However, there were reduced frequencies of IFN-γ-producing cells among tumor-associated MAIT cells compared to MAIT cells from unaffected colon tissue. MAIT-cell infiltration into colon tumors has been correlated with poor patient prognosis and in an independent appendix of the thesis, we present preliminary data actually showing a positive impact of MAIT cell infiltration into colon tumors on patient survival.

Keywords: colon cancer, regulatory T cells, CD39, adenosine, immune checkpoint molecules, immunosuppression, MAIT cells, cytokines, cancer-specific survival

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SAMMANFATTNING PÅ SVENSKA

Koloncancer orsakar årligen en omfattande cancerrelaterad död och det finns ett stort behov av utveckling av nya alternativa behandlingsstrategier inom området. Medan behandlingen av flertalet cancerformer, såsom avancerat malignt melanom, lungcancer och prostatacancer, har revolutionerats genom införandet av tilläggsbehandling i form av immunterapi med T-cells aktiverande antikroppar (immune checkpoint inhibitors), har koloncancer svarat sämre på den här behandlingsformen. Endast hypermuterade tumörer, som utgör en mindre andel av all koloncancer och karakteriseras av en förhållandevis hög grad av immuncellsinfiltration, har i detta sammanhanget uppvisat ett lovande behandlingssvar. T-cells aktiverande antikroppar verkar genom att blockera hämningen av T celler i tumören och närvaro av ett rikt immuncellsinfiltrat i tumören anses vara en förutsättning för ett bra behandlingssvar vid den här behandlingsformen. Ett rikt immuncellsinfiltrat i kolontumörer är även i sig självt förknippat med en fördelaktig patientprognos, tydligt visat när varje enskild kolontumör klassificeras enligt ”Immunoscore”, ursprungligen definierat av Jerome Galón. I takt med en ökad kunskap om samspelet mellan tumörinfiltrerande immunceller i koloncancer och deras bidrag till tumörtillväxt, samt en ökad förståelse av T-cells aktiverande antikroppars verkningsmekanism och även andra alternativa immunterapier, finns en förhoppning om att en större andel av koloncancer ska svara på immunterapi inom en snar framtid.

I den här avhandlingen har vi karaktäriserat och studerat funktionen hos två olika immuncellspopulationer som båda utgör en del av immuncellsinfiltratet i kolontumörer, i.e. regulatoriska T celler (Treg) som uttrycker enzymet CD39 på sin yta ($CD39^+$ Treg) och mucosal-associated invariant T (MAIT) celler. Båda de här celltyperna förekommer i såväl kolonslemhinnan hos friska individer som i kolontumörer. CD39 är delaktig i omvandlingen av extracellulärt ATP till adenosin och balanserar de extracellulära nivåerna av dessa molekyler, en viktig funktion då extracellulärt ATP frisätts i samband med vävnadsskada och aktiverar ett påföljande pro-inflammatoriskt värdsvar. Adenosin är även en av effektormolekylerna Treg använder för att hämma andra immunceller. MAIT celler, å andra sidan, är en okonventionell typ av T-celler, och tros ha en slemhinneskyddande effekt mot invaderande mikrober. I syfte att klargöra funktionen hos de här två celltyperna i kolontumörer, och deras inflytande på tumörtillväxten, har vi med hjälp av flödescytometrisk teknik, kartlagt och jämfört egenskaper och funktion hos tumörinfiltrerande $CD39^+$ Treg och MAIT celler, med motsvarande celler isolerade från normal kolonvävnad och blod hos koloncancer patienter. Vi visar att både $CD39^+$ Treg och MAIT celler ansamlas i kolontumörer jämfört med övriga studerade lokaler. I funktionella experiment har $CD39^+$ Treg en uttalat hämmande effekt på tillväxten av konventionella T celler och ett högre uttryck av immunhämmande proteiner ses hos $CD39^+$ Treg jämfört med Treg som saknar CD39-uttryck. Detta talar för att $CD39^+$ Treg i kolontumörer är en särskilt immunhämmande Treg population med stor potential att hämma ett för patienten annars skyddande immunsvar mot cancern. En hög frekvens av CD39-uttryckande Treg bland totala antalet tumörinfiltrerande Treg, förefaller även vid en preliminär överlevnadsanalys att vara kopplat till en sämre överlevnad hos koloncancer patienter. Specifik eradikering av CD39-uttryckande Treg i kolontumörer, i syfte att släppa på T-cells ”bromsen”, kan vara ett framtida alternativ till T-cells aktiverande antikroppar som än så länge fungerat sämre vid koloncancer. MAIT cellers funktion i kolontumörer är mindre studerat jämfört med funktionen hos tumörinfiltrerande Treg. Våra resultat visar nedsatt produktion av IFN-γ hos tumörinfiltrerande MAIT celler men hur detta förhåller sig till ett eventuellt prognostiskt inflytande av MAIT celler på patienters prognos vid koloncancer är för tidigt att uttala sig om. Till skillnad från andra studier som kopplar samman MAIT celler med en försämrad patientprognos, visar våra preliminära överlevnadsdata att en hög grad av MAIT-cells infiltration i kolontumörer är kopplat till en bättre patientprognos.

PAPERS

This thesis is based on four studies, referred to in the text as Paper I, II, III and Appendix:

I. Ahlmanner F, Sundström P, Akeus P, Eklöf J, Börjesson L, Gustavsson B, Lindskog EB, Raghavan S, Quiding-Järbrink M.

$CD39^+$ regulatory T cells accumulate in colon adenocarcinomas and display markers of increased suppressive function.

Oncotarget. 2018; 9(97): 36993-37007.

II. Ahlmanner F, Sundström P, Gustavsson B, Lindskog EB, Wettergren Y, Quiding-Järbrink M.

Intratumoral $CD39^+$ regulatory T cell accumulation may predict disease recurrence in colon cancer patients.

Manuscript.

III. Sundström P, Ahlmanner F, Akéus P, Sundquist M, Alsén S, Yrlid U, Börjesson L, Sjöling Å, Gustavsson B, Wong SB, Quiding-Järbink M.

Human mucosa-associated invariant T cells accumulate in colon adenocarcinomas but produce reduced amounts of IFN-γ.

Journal of Immunology. 2015; 195(7): 3472-3481.

APPENDIX

Ahlmanner F, Sundström P, Rodin W, Gustavsson B, Lindskog EB, Wettergren Y, Quiding-Järbrink M.

Intratumoral mucosal-associated invariant T cells and disease outcome in colon cancer patients.

CONTENTS

ABBREVIATIONS

5-FU	5-flurouracil
APC	Adenomatous polyposis coli
APC	Antigen-presenting cell
BM	Bone marrow
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus molecular subtype
CRC	Colorectal cancer
CTL	Cytotoxic T lymphocyte
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
eATP	extracellular ATP
FAP	Familial adenomatous polyposis
Foxp3	forkhead box P3
GALT	Gut-associated lymphoid tissue
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocyte
ILC	Innate lymphoid cell
LP	Lamina propria
MAIT	Mucosal-associated invariant T
MALT	Mucosa-associated lymphoid tissue
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility Complex
MLN	Mesenteric lymph node
MSI-H	Microsatellite instability high
MSS	Microsatellite stable
PBMC	Peripheral blood mononuclear cell
PP	Peyer's Patch
PRR	Pattern-recognition receptors
pTreg	peripheral Treg
STAT1	Signal transducer and activator of transcription 1
T-bet	T-box binding transcription factor
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper
TLR	Toll-like receptor
Treg	Regulatory T cell
tTreg	thymic-derived Treg

1 INTRODUCTION

1.1 Brief introduction to cancer

Cancer is the malignant transformation of healthy human cells leading to disruption of tissue homeostasis and uncontrolled and invasive cancer growth [1]. In this process, control mechanisms counteracting cancer transformation and cancer growth are impaired or circumvented, such as for example regulation of cell proliferation and cell death, and cancer immunosurveillance [2,3]. Once a cancer has been established it is generally firmly rooted and if not treated by therapeutic intervention patients most often succumb due to the cancer. The risk factors to developing cancer are multiple, e.g. genetic predisposition, age, diet, and smoking [4], but importantly, also chronic inflammation may promote cancer development of certain cancer types [4,5].

As originally established by Hanahan et al., the different traits of an established cancer are referred to as the hallmarks of cancer [6]. These hallmarks have since been revised [2], but the fundamentals of the original hallmarks largely remain the same. Six original hallmarks comprise, sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis, and the two newly defined hallmarks, are reprogramming energy metabolism and evading immune destruction [2]. In addition, the importance of the tumor microenvironment and the interplay between cancer cells and other neighboring cells, e.g. stromal cells and immune cells, has recently been established in many types of cancer [2,7].

While great progress has been made in cancer research leading to improved treatment strategies for many types of cancer, interventions to these hallmarks are, to a large extent, still missing.

1.2 Colon cancer

The disease of interest in this thesis is colon adenocarcinoma, a subset of colon cancer that constitutes the major part of all colon cancer. However, due to its close resemblance with rectal cancer, these diseases are often looked upon as one entity in studies of molecular biology, which is colorectal cancer (CRC). Of CRC, 90% are adenocarcinomas originating from epithelial cells in crypt foci of the colorectal mucosa [8]. In turn, mucinous adenocarcinoma and signet-ring cell carcinoma, constitute the majority of the remaining CRCs [9]. In several instances, studies in the field have been conducted in a mixed cohort of colon and rectal tumors, but important distinctions between colon and rectal cancer are present [9-11].

1.2.1 Incidence and mortality

The incidence of CRC is ranking as the third highest in the world with over 1.8 million estimated cases in 2018, and out of these cases approximately 61% are colon cancers [12]. Incidence is higher in Western or transitioned countries, about 3-fold higher compared to transitioning countries, but as transitioning countries take on a more westernized lifestyle the global incidence rates of CRC are expected to increase [12].

CRC is inarguably a major malefactor to human health, ranking as the second highest contributor to all cancer mortality worldwide [12]. Currently, the combined mortality rate of colon and rectal cancer is the most accurate, since, at least in the United States, the mortality cases of rectal cancer are still often classified as colon cancer [13].

1.2.2 Etiology and risk factors

The largest part of colorectal tumors, arise spontaneously, but at least 5% of all CRC cases have identifiable genetic predisposition associated with hereditary cancer syndromes, including for example Lynch syndrome and familial adenomatous polyposis (FAP) [14,15]. To have a first degree relative with CRC highly increases the risk of developing cancer and the risk is further enhanced with a combined heredity of first, second and third degree relatives with CRC [16]. Lynch syndrome, previously classified into the group of “nonpolyposis hereditary cancer” (HNPCC) [17], is the most common of the hereditary cancer syndromes [18]. It is associated with mutations of DNA repair mechanisms, a common trait also

for sporadic CRC and thus of high interest to current research in various clinical implications of CRC [17,19]. Also FAP, the second most common group of syndromes associated to hereditary cancer [18], display similarities with sporadic CRC, and constitutive activation of the Wnt signaling pathway due to truncation of the adenomatous polyposis coli (*APC*) gene is present in both FAP and the majority of colorectal tumors [20]. In more than 70% of human colon cancer cases, a mutation in the tumor suppressor *APC* gene is considered as the initiating event of the transformation into aberrant intestinal crypt foci and adenomas [21]. The specifics of the genetic and molecular landscape driving carcinogenesis in CRC is further elaborated on in the next section.

The risk factors of CRC are several and can for simplification be divided into unmodifiable and modifiable risk factors. Unmodifiable risk factors, i.e. risk factors which cannot be influenced by the patient, include age, male sex, genetic predisposition and inflammatory bowel disease, while modifiable risk factors, that can be influenced by the patient, include smoking, excessive alcohol consumption, high consumption of red and processed meat, obesity and diabetes [4]. Genetic predisposition and inflammatory bowel disease are less frequent risk factors on a population basis but associated to a higher relative risk of developing CRC [16,22].

1.2.3 Carcinogenesis and subtyping

Carcinogenesis

During the malignant transformation of healthy tissue cells, the cells gradually acquire somatic mutations as well as other types of genetic alterations, and after acquiring a critical amount of these genomic alterations the tumor will convert from a pre-malignant state into a fully malignant state with uncontrolled cancer growth [1] (*Figure 1*). In the colon, this process is best exemplified by the adenoma-carcinoma sequence; the gradual dysplastic transformation of a pre-malignant adenoma into a malignant adenocarcinoma [23]. Both hereditary cancer syndromes associated with CRC and sporadic CRC are believed to develop through these steps of transformation [18,24]. The phenotype of genetically altered colorectal tumors is dependent on the translational effects of mutations in oncogenes and tumor suppressor genes, i.e. genes that harbors the potential to cause cancer and becomes either activated (oncogenes) or inactivated (tumor suppressor genes) upon genetic alteration [2]. Oncogenes (e.g. *KRAS*, *PIK3CA*) and tumor suppressor genes (e.g. *APC, TP53, SMAD4*) in CRC, in turn affects intracellular signaling

pathways regulating for example cellular proliferation and survival [25,26]. In a recent large exome-sequence analysis of 224 colorectal cancer samples by The Cancer Genome Atlas Network, twenty-four genes were significantly mutated in colorectal tumors [10], but a single colorectal tumor may harbor up to 70 genomic alterations per tumor affecting protein-coding genes [1]. Of these altered genes, the majority are however passenger mutations and only a small fraction are actual "driver" mutations, i.e. mutations associated with a selective growth advantage to the tumor cell [1].

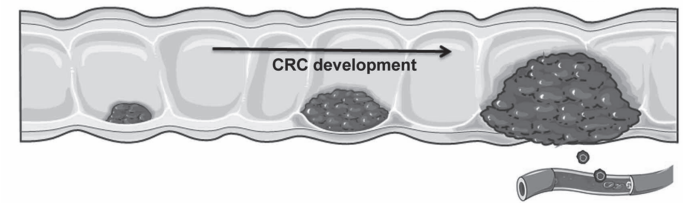

Figure 1. CRC development. Wang et al. Sci Rep 2017;7:4281. Adapted.

Genetic instability pathways

At least three major pathways of genetic instability have been identified in CRC: the chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) pathways.

The CIN pathway is characterized by large chromosomal alterations leading to imbalances in chromosomal number and loss of heterozygosity (LOH), i.e. loss of one of the two alleles of a functional gene, e.g. a tumor suppressor gene, rendering the cell more sensitive to subsequent mutation of the remaining intact allele [27]. Colorectal tumors arising through the CIN pathway constitute the majority of colorectal tumors, accounting for approximately 65-70% of all sporadic CRC cases and also the inherited syndrome FAP [27,28].

The MSI pathway is characterized by tumors with hypermutation and ubiquitous somatic mutations at repetitive sequences (microsatellites) of specific DNA markers, and accounts for about 15% of all CRC cases, of which 3% are associated with Lynch syndrome [29]. These MSI tumors have defective mismatch repair (MMR) systems and mutations in genes responsible for enzymatic DNA repair, e.g. the mammal gene homologues of the prokaryotic *mutS* and *mutL*; the *Mut S homologue* (*MSH*) and *Mut L homologue* (*MLH*), respectively [29,30].

The CIMP pathway, the last of the three major pathways of genetic instability, stands for DNA hypermethylation in CpG-rich promotors which results in silencing of affected genes [31]. It is highly associated with mutation in the proto-oncogene *BRAF* [32]. However, overlap exists between these genetic pathways, e.g. 70-80% of MSI tumors also display gene promoter hypermethylation and silencing of the *MSL1* gene associated with the CIMP pathway [31].

Consensus molecular subtypes

Based on a high degree of heterogeneity among colorectal tumors on the gene expression level, an alternative classification system, the consensus molecular subtypes (CMSs) of CRC has been formed, including four CMSs with distinguishing features: CMS1 (microsatellite instability immune, 14%): hypermutated, microsatellite unstable, strong immune activation and enriched for *BRAF* mutations; CMS2 (canonical, 37%): epithelial, microsatellite stable with high CIN, marked WNT/MYC signaling activation, EGFR amplification or overexpression and mutant *TP53*; CMS3 (metabolic, 13%): epithelial, evident metabolic dysregulation, low CIN, moderate WNT/MYC pathway activation, mutant *KRAS* and phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha gene (*PIK3CA*), and insulin-like growth factor binding protein 2 (IGFBP2) overexpression; and CMS4 (mesenchymal, 23%): CIN/MSI heterogeneous, prominent transforming growth factor beta (TGF-β) activation, neurogenic locus notch homolog protein 3 (NOTCH3)/vascular endothelial growth factor receptor 2 (VEGFR2) overexpression, stromal invasion and angiogenesis" [9,33,34]. Even though the CMSs classification system of CRC cannot stratify all colorectal tumors into CMSs (13% of samples display mixed features), it is nowadays considered as the most robust stratification system of colorectal tumors with regard to biological interpretability [33]. To date, several *in vitro* and *in vivo* models of these CMSs have been developed to account for this tumor heterogeneity and allow for improved translation between experimental and clinical studies concerning for example drug development [35-37]. Interestingly in this context, further differentiation between colon and rectal cancer may also unravel from the CMSs classification system. Colon and rectal cancers have recently been shown to have highly similar patterns in terms of genomic alteration, excluding the hypermutated tumors which are rarely present in the rectum [9,10], while tumor clustering into CMSs has proven more difficult across anatomic boundaries [11]. In addition to the mechanisms of carcinogenesis accounted for in this section, colon cancer may also undergo additional transformation at later time-points during disease, due to for example

immune escape mechanisms or treatment associated alterations [3,38]. This will be discussed in the following chapters of the thesis.

1.2.4 Diagnosis and clinical parameters

The prevailing clinical diagnostical or histopathological classification of CRC is undoubtedly the TNM-classification, recently updated in the 8th edition of the TNM Classification guidelines of malignant tumors by the Union Internationale Contre le Cancer (UICC) [39]. It contains tumor classification with regard to local invasion depth (T stage), lymph node spread (N stage) and distant metastases status (M stage); and these three stages are combined into an overall TNM-stage [4]. The T stage of the tumor (Tis-T4b) refers to depth of tumor invasion into the surrounding tissue, and spans from in situ localization (Tis) to the serosa (T4a) or neighboring tissues/organs (T4b) [39]. Importantly, tumor size is currently not incorporated into the TNM-stage [40]. The N stage of the tumor (N0-N2b) spans from no lymph node involvement (N0) to cancer in 7 or more regional lymph nodes (N2b) [39]. The M stage (M0-M1c) spans from no distant metastases to peritoneal metastases with or without organ involvement [39,41]. Common sites of metastasis in CRC are to organs such as the liver, lungs, bone, brain, intra-abdominal organs and the peritoneum [9,41]. Finally, the combined TNM-stage (0-IVB) spans from in situ carcinoma with no affected lymph nodes and no distant metastases to any T or N stages but with peritoneal metastases with or without organ involvement [39].

In addition to the TNM-stage of colorectal tumors, tumor differentiation grade and tumor location are also important diagnostic factors in CRC as described below [23,42]. The grading system in CRC (grade 1-3) entails well-differentiated (grade 1), moderately differentiated (grade 2), and poorly differentiated (grade 3) tumors [33] (*Figure 2 and 3*). In addition to the routine based grading of colorectal tumors during histopathological examination, also pre-malignant lesions are graded, but according to histological type, size and grade of dysplasia rather than grade of differentiation and stage as in CRC [43]. Despite a more favorable prognosis for patients with MSI-H tumors compared to MSS tumors in CRC [44], as discussed in the following section, MSI-H tumors are generally more poorly differentiated and present with a greater depth of invasion compared to MSS tumors [30,45]. Interestingly also, the likelihood of presenting with an MSI-H tumor is higher in colorectal tumors of low TNM-stage (stage I-II) [30]. In addition to the TNM-stage and the tumor differentiation grade, tumor location is also an important diagnostic

factor in CRC. Conventionally, tumors proximal to the splenic flexure are defined as right sided and tumors distal to the flexure as left sided [42]. This division has important clinical bearings and the potential advantages with a more precise classification according to tumor location is currently under evaluation [42,46].

The majority of colorectal tumors are single primary tumors, but in rare cases and more frequently in patients with inflammatory bowel disease (IBD) and CRC associated to hereditary cancer syndromes, a patient initially presents with more than one primary tumor, referred to as synchronous CRC [47]. Metachronous CRC, another important diagnostic subgroup of CRC, refers to a consecutive colorectal tumor occurring more than 6 months after the index tumor [47]. For optimal classification according to the TNM-stage, pre-operative evaluation usually entails a custom selection of imaging techniques dependent on the clinical scenario, e.g. computed tomography (CT) colonography, magnetic resonance imaging (MRI), and positron emission tomography (PET)/CT colonography [48]. Furthermore, colonoscopic examination to detect and remove pre-malignant lesions, such as serrated polyps and adenomas, is an important measure to reduce the risk of later CRC development, prone to occur in a minority of these patients [42,43].

Colorectal tumors develop slowly, granting an opportunity for yet another diagnostic tool which is molecular biomarkers, involved in secondary prevention of CRC and early cancer detection [4]. However, despite intense research, the currently available biomarkers, e.g. fecal hemoglobin, carcinoembryonic antigen (CEA), and CA19.9, in many instances cannot provide prognostic details for individual CRC patients [49]. Analysis of the MSS/MSI status of colorectal tumors is recommended, but has not yet been fully introduced in clinical practice [30]. For both CRC and pre-malignant lesions associated with CRC, several studies are currently evaluating a wide spectrum of potential biomarkers, ranging from microRNAs (miRNAs) [26,50] to biomarkers associated with the consensus molecular subtypes of CRC [34,43,51].

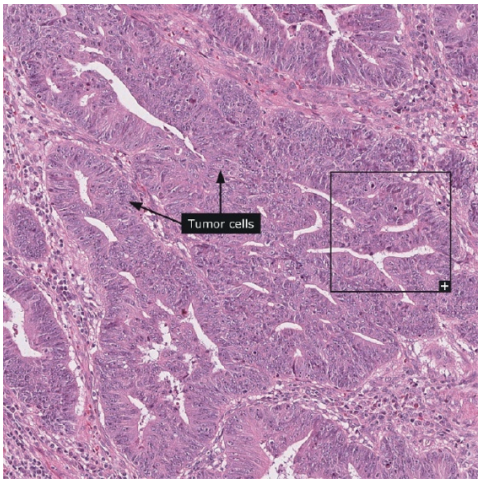

Figure 2. Colorectal adenocarcinoma, moderately differentiated, Stage I.
Human Protein Atlas. www.proteinatlas.org

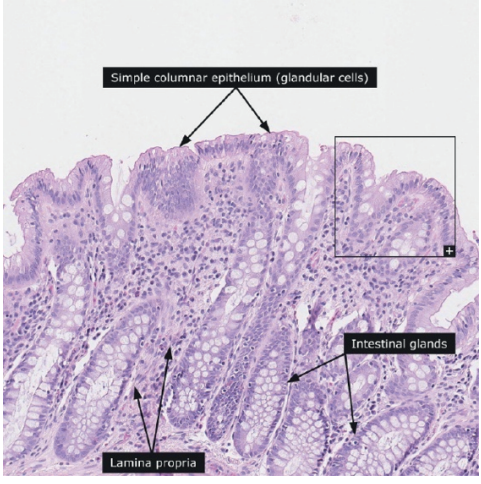

Figure 3. Normal colon. Human Protein Atlas. www.proteinatlas.org

1.2.5 Treatment and prognosis

Treatment

The predominant therapeutic method of choice in CRC patients is surgical removal of the tumor [4]. Surgery is performed in virtually all patients apart from those with severe un-operable metastatic disease. In addition to surgery, the other forms of therapeutics in CRC are radiotherapy, chemotherapy and targeted molecular therapy [52,53]. For both colon and rectal cancer, the specific treatment protocol for each cancer is largely dependent on the TNM-stage classification. The common chemotherapeutic agent of choice in colon cancer is 5-flurouracil (5-FU), which is routinely used as adjuvant chemotherapy in stage III colon cancer and some stage II colon cancers of high risk of cancer relapse (T4 tumors) [4,9]. The standard treatment protocol of colon and rectal cancer have some distinctive differences. The surgical procedure to treat colon cancer lack international standardization with regard to the tumor resection margin during a standard partial colectomy [54], while the surgical procedure to treat rectal cancer is highly established world-wide, i.e. with proctectomy or proctocolectomy and a total mesorectal excision (TME) [9]. Also, neoadjuvant chemotherapy, i.e. chemotherapy given before the main treatment, is the standard treatment for stage II and III rectal cancers, but commonly not used in colon cancer [55]. Targeted therapy in CRC, e.g. molecular targeting to inhibit the activities of vascular endothelial growth factor (VEGF) and epidermal growth factors (EGFRs), is mostly used in advance metastatic disease [56]. In addition, specific immunotherapeutic targeting is highly related to the scope of this thesis and cancer immunotherapy will be discussed in a later chapter.

Prognostic markers

Several combined factors determine patient prognosis in CRC, and apart from determining tumor stage, it is also important to account for tumor heterogeneity by determining the specific genetic and molecular subtype of each individual tumor. In this context, it has been shown in several studies of stage II and III colon cancer, that MSI-H tumors, also classified as CMS1, have a more favorable prognosis compared to MSS tumors, i.e. tumors typically classified as CMS2 and CMS3 [34,57]. However, despite improved survival of CRC patients with MSI-H tumors compared to MSS tumors, and a reduced overall risk of metastasizing [58], MSI-H tumors may also progress to metastatic adenocarcinoma [59]. In addition, MSI-H colorectal cancers are differently enriched between tumor stages (20% of all colorectal tumors in stage II) but more rarely diagnosed at later stages of disease

(3-5% of all colorectal tumors in stage IV) [58,60]. It is thus essential that patient groups are carefully stratified in survival studies. Of note, one study of 2720 stage III colon cancer samples, found a similar prognosis of MSI-H cancers (CMS1) and MSS cancers without *KRAS* or *BRAF* mutation (CMS2), while those MSS cancers harboring mutant *KRAS* or *BRAF* (CMS3) had a comparatively shorter 5-year survival [61]. Prognostic impact of mutant *BRAF* in MSI-H cancers, present in approximately 50% of these patients, has so far been contradictory [62,63]. Furthermore, colorectal tumors characterized by activation of signaling pathways related to epithelial-mesenchymal transition (EMT), constitute the subtype CMS4 cancers and have a less favorable prognosis compared to CMS2 cancers [33,64]. In a recent study, the prognostic biomarker PBX3, expressed in tumor cells, was required for EMT transition and may be useful to identify potentially aggressive stage II colon cancers and late progression in CRC [65]. In addition, other prognostic biomarkers that serve to detect miRNAs and posttranslational modifications in colorectal tumors, such as glycosylation and ubiquitylation, are currently also under evaluation [66-68].

Apart from the tumor stage and the specific genetic and molecular subtype of each colorectal tumor, as accounted for above, also other tumor characteristics play an important role in determining patient prognosis, in addition to the impact of the immune system and the purinergic signaling system on patient prognosis which will be covered in later parts of the introduction. Even though 75-90% of the MSI-H cancers are located in the proximal or right sided colon, depending on how the tumors were classified [9,69], left sided colon cancer has a favorable prognosis compared to right sided in advanced stages of disease [70]. Importantly, right and left sided tumors have a different embryonical origin which may explain these prognostic differences [42]. As a consequence, a designated study to compare right and left sided tumors, carefully needs to consider group stratification, as reflected in a recent adjuvant chemotherapy trial where patient with stage II colon cancers, enriched for MSI-H tumors, but not stage III colon cancers, relapsed less frequently in patients with proximal cancers [71]. Interestingly, among MSI-H cancers only, patients with proximal colon tumors had a more favorable prognosis compared to patients with distal colon tumors [69]. Additional traits affecting patient prognosis in CRC are tumor size and type of distant metastasis. Tumor size correlates negatively with patient survival and advanced disease with peritoneal metastases correlates with a shorter overall survival compared to CRC patients with other sites of metastases [40,72].

Predictive markers

Predictive biomarkers in CRC, commonly referred to as biomarkers of treatment response, is outside the scope of this thesis, apart from predictive biomarkers of cancer immunotherapy which will be discussed in a later chapter of the thesis. However, a brief summary of the topic is motivated before closing this introductory section on colorectal cancer. An important feature of MSI-H colon cancers is poor response to 5-FU-based adjuvant chemotherapy, as shown when comparing patients with MSI-H tumors receiving adjuvant chemotherapy compared to surgery alone [73]. Furthermore, the heterogeneity of colorectal tumors with regard to tumor location and activated pathways, also advocates a correlation between different molecular subtypes and treatment response of targeted therapies. In this context, the first acknowledged treatment biomarker of metastatic CRC is poor response to EGFR-targeted therapy in tumors with *KRAS* exon 2 mutation (CMS3) [74]. Also, colon cancers with mutated *BRAF*, commonly present in MSI-H and right-sided tumors (CMS1), have a poor treatment response to anti-EGFR therapy [70,75]. In contrast, colorectal tumors wild-type for *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* (quadruple-negative tumors) have been shown to respond better to anti-EGFR therapy [38,76]. Predictive biomarkers are currently also under evaluation with regard to inhibitors of the VEGF:VEGFR2 pathway associated to angiogenesis in advanced CRC disease [77]. In addition, also in CRC patients with advanced disease, a polymorphism in the Vitamin D Transporter gene has been shown to affect treatment response of both anti-EGFR and anti-VEGF therapy [78].

1.3 Overview of the immune system

After this introductory part on colon and colorectal cancer, we now switch focus to the immune system. The immune system composes a major organ system and serves to detect and fight off a broad variety of foreign insults to the human body. As such, it is highly engaged in combating infections and wound-healing, but also in internal organ stress of various origin and in cancer growth. In addition, a dysregulated immune system may result in chronic inflammation, autoimmune diseases, or allergy. The major organs of the immune system include primary lymphoid organs, i.e. the bone marrow and the thymus, and secondary lymphoid organs, i.e. lymph nodes, spleen, tonsils, Peyer's patches and mucosa associated lymphoid tissue (MALT). Highly simplified, immune cells are formed in primary lymphoid organs and activated in secondary lymphoid organs. Prior to introducing cancer immunology, the core research field of the thesis, this section provides a basic overview of the composition and function of the healthy immune system.

1.3.1 The innate versus adaptive immune system

The response of the immune system to an invading pathogen, or an encounter perceived as foreign to the body, consists of principally two distinct responses. A direct response, referred to as the innate immune response, and an acquired or late response, referred to as the adaptive immune response [79]. Distinct immune cell subsets are involved in the innate versus the adaptive immune response, but they are highly interconnected via dendritic cells (DCs), a major subpopulation of antigen presenting cells (APCs) [80].

When a foreign invader, for example a pathogen, manages to breach the epithelial protective barrier of a random organ, it will be directly exposed to innate immune cells [81]. The innate immune cells are phagocytic cells such as tissue resident macrophages, neutrophils and DCs, but also other types of immune cells such as natural killer (NK) cells, mast cells, eosinophils and basophils. In order to sense foreign encounter, phagocytic cells express surface-bound and intracellular pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) [82]. Also epithelial cells express some PRRs and play a significant role in innate immunity by producing proinflammatory cytokines [81]. PRRs are basically categorized into three major subtypes, which are surface-bound and intracellular Toll-like receptors (TLRs), intracellular NOD-like receptors (NLRs), and retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs) which

are cytosolic helicases [82]. Upon stimulation of these receptors, a signaling cascade is initiated, often activating mitogen-activated protein kinases (MAPK) and the master transcription factor NF-κB, which results in the secretion of proinflammatory cytokines by innate immune cells, such as TNF-α, IFN-γ and IL-1, but also activation of various other effector cell functions [82-84]. Furthermore, most immune cells express TNF-α receptors, and TNF-α itself can activate NF-κB [85]. Importantly, PRRs also respond to danger-associated molecular patterns (DAMPs) which are endogenous products of stressed or necrotic cells, and DAMPs thus serve as alarm signals to the body in case of foreign encounter or different types of diseases [86]. An excess of DAMPs leads to a pro-inflammatory state in the tissue, due to activation of MAPK and NF-κB, and also activation of the inflammasome, i.e. a cytosolic multimeric signaling complex involved in immune responses towards foreign or host derived danger signals [86,87]. In addition, changes in tissue homeostastis upon pathogen exposure or other types of diseases, also affect complement signaling and the release of acute-phase proteins such as for example C-reactive protein (CRP), both highly involved in the first phase of the immune response and a link to adaptive immunity [88,89]. Yet another important signaling system in the first response is the interleukin-1 (IL-1) family of cytokines and receptors, and these receptors share similar functions with TLRs, both using the intracellular adaptor MyD88 for signaling via IL-1R-associated kinase (IRAK) family kinases to activate MAPK and NF-κB [90,91].

While innate immune cells respond upon first recognition of a foreign invader and detect targets that are commonly shared between different types of invaders, adaptive immune cells react to more specific targets and require days to develop. The adaptive immune cells consist of T and B lymphocytes, and these are largely dependent on DCs to become activated [79]. From the large pool of preformed T and B cells, always present in the blood and lymphoid tissues of a healthy individual, only the cells with specific receptors recognizing towards the invader will clonally expand upon activation in the lymph node and migrate towards the site of invasion [92,93]. In this manner, the adaptive immune response can generate a strong and well-directed response towards the invader, and the precise mechanisms of this response will be presented in the following sections. Notably, the adaptive immune response serves as an additional level of protection in those instances when the innate immune response does not suffice. In addition, adaptive immunity may also generate memory that is a long-term protection through the formation of long-

lasting memory cells and antibodies during the first response which can easily be reactivated upon re-exposure to the same pathogen [94,95].

1.3.2 Intestinal immunology and the microbiome

Due to the topic of the thesis, this brief summary of the mucosal immune system will focus solely on the intestinal immune system, and not specifically address the immune system at other mucosal sites, such as the urogenital tract or the respiratory system. The large intestine, i.e. colon and rectum, forms the distal part of the intestine and is protected from the outside by two mucus layers, one inner and one outer mucus layer, and a mucosal surface consisting of a single layer of epithelial cells supported by intercellular tight junctions and an underlying lamina propria (LP) [96,97]. In contrast to the small intestine, the colonic wall is flat and lacks protruding villi, but the main tissue layers of the colon are otherwise similar to the small intestine, with three distinct layers underlying the mucosal surface, i.e. the muscularis mucosae, the submucosa and a muscular layer [98]. The colonic epithelium is a glandular epithelium made up of several types of intestinal epithelial cells (IECs), including absorptive enterocytes but also stem cells located in the colonic crypt, numerous mucin-producing goblet cells, neuroendocrine cells and intraepithelial lymphocytes (IELs) [96,98]. Also the LP consists of several different cell types, and in addition to its supporting role to supply the epithelial cells with blood vessels and lymph drainage, it contains numerous immune cells of different types.

Homeostatic immune control at the intestinal mucosal site entails a variety of functions, ranging from protective functions, such as fighting of intestinal pathogens, to tolerogenic functions, such as preserving unresponsiveness to food antigens and commensal bacteria [99]. As previously described, innate immune cells contribute significantly in this process, and for example macrophages are highly important to intestinal immune homeostasis [100]. Also cytokines play a critical role, and in particular interleukin-10 (IL-10) have important immunoregulatory properties for gut homeostasis [101]. Furthermore, the innate and the adaptive arms of intestinal immunity, are linked together in a complex structure of organized lymphoid structures (*Figure 4*). These include sites of antigen-presentation and lymphocyte activation, which are mesenteric lymph nodes (MLNs) and gut-associated lymphoid tissue (GALT) such as for example Peyer's Patches (PPs) and isolated lymphoid follicles (ILFs), but also lymphocyte effector sites such as the lamina propria (LP) [98]. M cells, a subset of intestinal epithelial cells (IECs)

commonly present in the follicle-associated epithelium in the small intestine [102], are highly specialized at taking up and transporting antigen to the underlying DCs present in GALT or PPs, and similar mechanisms are believed to occur in ILFs of the colon [98,103]. After antigen-uptake, DCs migrate to the MLNs of the small intestine or colon were they prime T cells [104,105]. A more detailed description of the specific mechanisms behind these processes will be provided in the following chapters of the introduction.

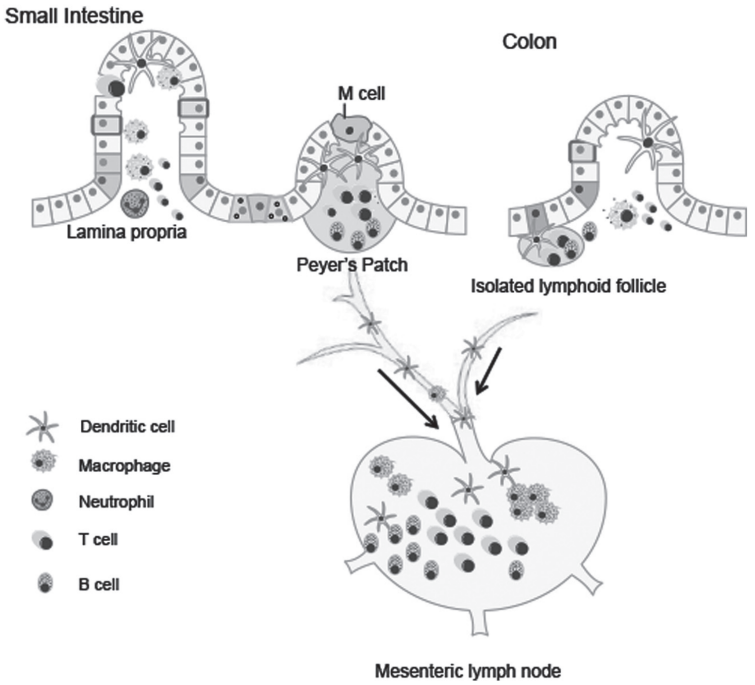

Figure 4. Immune cells in the gastrointestinal tract. Meng J, Sindberg GM and Roy S. Front Microbiol 2015;6:643

Also the gut microbiota is highly interlinked with mucosal immunity. The microbiota consists of numerous commensal or non-pathogenic bacteria which live in synergy with the host and the host is dependent on commensal bacteria for a wide range of different functions, e.g. food digestion, vitamin synthesis, and lymphocyte development and differentiation [98]. A state of tolerance between the mucosal immune system and the gut microbiota is therefore essential, and commensal bacteria-specific $CD4^+$ T cells are highly important to this process [106]. Also important in this context are a subset of innate immune cells, innate lymphoid cells (ILCs), which provides important cross-talk with other immune cell subsets such as $CD4^+$ T cells to confer protection against both pathogenic and non-pathogenic bacteria residing in the GALT upon epithelial barrier breach [107]. IgA secretion by mucosal B cells also has an important role for establishing a healthy microbiota [108]. Epithelial barrier breach in the intestine may overturn tolerance to commensal bacteria and lead to microbiota dysbiosis, i.e. an altered composition of the microbiota, eventually leading to various degree of immune hyperactivation [109]. This in turn may have implications for disease development and an altered microbial composition has been observed in IBD and CRC patients [109]. Indeed, epidemiological studies have revealed an increased risk of CRC development in patients with IBD, which may partly be caused by the dysbiosis [110]. Furthermore, a recent study by Grivennikov *et al.*, observed increased epithelial permeability of bacteria in colorectal tumors, together with increased expression of IL-23 and IL-17 [111]. These findings were confirmed in a mouse model of colorectal tumorigenesis, indicating a potential involvement of microbially driven tumor growth mediated by IL-23 and a tumoral IL-17 response.

1.3.3 Lymphocyte activation and antigen-specificity

This subchapter is a brief summary on the processes behind lymphocyte activation and specificity, and includes the development of both B and T lymphocytes (B and T cells) and their subsequent activation in draining lymph nodes by DCs upon antigen-presentation. Both B and T cells originate from pluripotent haematopoietic stem cell progenitors in the bone marrow (BM) and after their initial development in the BM, T cell progenitors migrate to the thymus, while B cells remain in the BM for their continued development [112]. During their continued development in the BM and thymus, and prior to their release into the circulation as mature naïve B and T cells, they undergo a sequential maturation and selection process, resulting in the generation of naïve B and T cell subsets with highly specific B and T cell antigen receptors, BCRs and TCRs, respectively [93,113]. Importantly, lymphocytes

with a strong responsiveness to self-antigens, i.e. antigens originating from proteins normally present in the host itself, will undergo negative selection to prevent the development of autoreactive B and T cells [93,113].

The subsequent activation of naïve B and T cells occur in the secondary lymphoid tissues. In contrast to B cells, T cells cannot bind to native antigens by themselves and are dependent on APCs for their activation. Lamina propria DCs sample antigens and present them to T cells in the draining lymph nodes as small peptides on Major Histocompatibility Complex (MHC) class I or II molecules. Depending on the type of antigen, antigens are processed and loaded onto MHC class I and II molecules via different loading routes, i.e. in the endoplasmic reticulum for MHC class I peptides and in endosomes for MHC class II peptides [80]. Apart from these normal routes of peptide loading, an additional MHC class I route exists for cross-presentation of peptide antigens originating from other phagocytosed cells, such as tumor cells and virus-infected cells [114-116].

In addition to its role in antigen-presentation, MHC class I expressed on the cell surface also inhibits targeting by cytotoxic NK cells [117]. While MHC class I molecules are present on all human cells, MHC class II molecules are only present on professional APCs, such as DCs, macrophages, and B cells. The classical DCs (cDCs) in the intestine express the integrine CD103 and cDCs are required for tolerogenic as well as protective immune responses [116,118]. Importantly, only those naïve T cells with a peptide-MHC specific receptor are activated by the DCs and form large clones of T cells towards the antigen with TCRs of identical specificity. In addition to TCR-signaling upon cognate antigen encounter (signal 1), naïve T cells also require co-stimulation by APCs (signal 2), and cytokines from APCs and other neighboring cells (signal 3) to become activated [119,120]. More specifically, APCs provide co-stimulation by CD80 and CD86 which bind to CD28 on naïve T cells. Cytokine signaling (signal 3) during activation will be addressed in a later section in the introduction.

In contrast to T cells, naïve B cells are able to take up antigen themselves in the draining lymph nodes via their BCRs, process it, and later present it to antigen-specific T helper (Th) cells to gain specific help from T cells [93,121]. This pathway of B cell activation thus represents the T cell dependent pathway of B cell activation, but in some instances B cells can also be directly activated by some types of antigen independently of T cells, commonly in response to large polysaccharide structures [93]. B cells that receive T cell help usually migrate into

germinal centers (GC) of PLNs or MALT, where they further differentiate and become highly specific antibody-secreting B cells [108,121].

1.3.4 Immune cell migration

Immune cell migration is similar between all subsets and involves three major steps, i.e. rolling, adhesion and transmigration. Whilst innate immune cells such as neutrophils are able to respond and migrate directly towards inflammatory signals in the tissue, initiated by epithelial cells and tissue-resident macrophages and mediated by endothelial cells, lymphocytes first need to undergo antigen-induced activation and priming in peripheral lymphoid organs [122]. Hence, naïve B and T cells circulate between blood and PLNs and mucosal associated lymphoid tissue (MALT) in search for cognate antigen. Already upon their original release into the circulation, both naïve B and T cells destined for mucosal tissues, express a key integrine $\alpha4\beta7$ which allows them to bind to endothelial cells of high entothelial venules (HEV), a specific endothelium present in peripheral lymphoid organs [123]. More specifically, $\alpha4\beta7$ on naïve lymphocytes binds to mucosal addressin cell adhesion molecule 1 (MADCAM-1) and peripheral lymph node addressin (PNAd), expressed on HEVs [124,125]. Whilst the interaction between $\alpha4\beta7$ and MADCAM-1 is essential for both lymphocyte rolling and adhesion to the endothelium, also additional interactions between selectins and oligosaccharide ligands promote this process [123]. Upon firm adhesion to the HEVs, the lymphocytes are able to transmigrate into PLNs and MALT where they encounter migratory DCs, e.g. $CD103^+$ DCs, as well as free antigen, both recently arrived via the draining lymphatic vessels [99,123].

Importantly, during B and T cell activation also specific homing properties will be acquired by lymphocytes, and for example vitamin A is metabolized into retinoic acid by DCs in the intestinal LP and confers gut homing-properties to both B and T cells [124,126]. These homing properties consists of enhanced surface expression of the gut-homing adhesion molecule $\alpha4\beta7$ but also upregulation of various chemokine receptors on the lymphocyte cell surface, e.g. CCR9 and CCR10 [124,126], and license activated lymphocytes to migrate from the site of activation to their final destination in the intestinal tissue. Typically, lymphocytes express a combination of several different chemokine receptors and migrate towards a gradient of chemoattractants in the affected intestinal tissue, such as specific chemokines [127]. Depending on differences in the concentration of their chemokine receptor targets along the length of the intestine, chemokine receptors confer

specific homing properties, e.g. $CCR9^+$ and $CCR10^+$ lymphocytes home preferentially to the small intestine and colon, respectively [125]. This allows for specific recruitment of antigen-specific B and T cells to their final destinations.

1.3.5 Effector lymphocytes

Intestinal lymphocytes

As previously described, antigen-specific B and T cells that encounter their cognate antigen in secondary lymphoid organs of the intestine, i.e. GALT and MLNs, will first undergo activation and clonal expansion and then migrate to their mucosal effector sites in the LP or the intestinal epithelium [93,119]. As a consequence, the majority of lymphocytes present at these mucosal effector sites will be antigen-experienced effector cells [95,128]. Also effector memory T cells are present here, and can be directly activated in the intestinal tissue upon re-encounter with their cognate antigens [129]. However, the mucosal surface of the intestine is a shared site between adaptive and innate immune cells, and as such it composes a highly heterogenous environment with a broad range of different immune cells with both protective and tolerogenic functions. During immune homeostasis, specialized tissue-resident lymphocyte subsets include tissue-resident memory T (TRM) cells, ILCs, unconventional or "innate-like T cells" (e.g. natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, γδ T cells, and $CD8\alpha\alpha^+$ intraepithelial lymphocytes (IELs) [128,130,131]. Importantly, some of these immune cell subsets are able to self-renew in the intestine and are not dependent on circulatory precursors for this process [130]. MAIT cells will be further discussed at a later stage in the introduction.

In the context of this thesis, also conventional T cells are of great importance and different T cell subsets are highly involved in a wide range of mucosal immune responses in the intestine, e.g. pathogen clearance, inflammation, autoimmunity, allergy and tumor immunity [132-135]. T cells can be divided into three main types, i.e. $CD4^+$ T helper (Th) cells, $CD4^+$ regulatory T (Treg) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs). In turn, $CD4^+$ Th cells can be further divided into subtypes, of which Th1, Th2 and Th17 cells are the most studied [128,133]. During T cell activation in the MLN, naïve $CD4^+$ T cells will be exposed to a distinct cytokine environment (signal 3) provided by DCs and neighboring cells, which will decide what subset the naïve $CD4^+$ T cell will differentiate into [119]. During this process, each Th cell subset will acquire specific effector functions governed by lineage-

specific transcription factors and epigenetic gene modifications [133]. Furthermore, due to changes in the cytokine milieu in the intestine, T cell subsets may also, in some instances, display plasticity and overdrive its previous lineage commitment to convert into the phenotype of another T cell subset [133,136]. In contrast to $CD4^+$ Th cells and CTLs, which provide protective immunity towards foreign insult, the major function of $CD4^+$ Treg is to modulate immune responses and Treg are particularly important in immune homeostasis due to their ability to suppress the function of other T cell subsets [137]. In this section, features and function of each of these T cell subsets will be presented.

Th1 cells

In order for naïve $CD4^+$ T cells to differentiate into Th1 cells in secondary lymphoid organs they are dependent on a specific cytokine milieu (signal 3) consisting of primarily IL-12 and IFN-γ, but also TNF-α [92,138]. IL-12 and IFN-γ, in large produced by neighboring DCs and NK cells, activate the transcription factors STAT1 (signal transducer and activator of transcription 1) and STAT4, respectively, which in turn activates T-box binding transcription factor (T-bet), the master transcription factor of Th1 cells [92,139]. Whilst T-bet leads to the upregulation of a specific set of genes in the naïve $CD4^+$ T cells and is required for Th1 cell differentation, it is however not a specific transcription factor for Th1 cells and is shared between several different immune cell subsets [139,140]. TNF-α is also important for Th1 differentiation, but more with regard to co-stimulation (signal 2) [92]. Upon T-bet activation, Th1 cells acquire the ability to produce IFN-γ and IL-2, their main effector cytokines, and autocrine IFN-γ signaling by Th1 cells thus also reinforces commitment to the Th1 lineage. In addition, T-bet itself also suppress the differentiation into other Th cell lineages by indirectly inhibiting other key transcription factors [92].

Once fully developed, antigen-specific Th1 cells promote a type of cellular immunity, often referred to as type I immunity [141]. Th1 effector cells are highly involved in protective immunity towards both infectious diseases and cancer [135,138]. Importantly, in the context of infectious disease, IFN-γ secreted by Th1 cells enhances the phagocytic function of macrophages and promote CTL development [142]. However, in other settings, IFN-γ may also promote autoimmunity and inflammation, due to its proinflammatory function [133].

Th2 cells

Th2 cells stimulate type II immunity, often characterized by high IgE antibody titers and activation of various innate immune cells such as mast cells, basophils and eosinophils [141]. Th2 effector cells are thus implicated in the immune response towards extracellular infectious agents and parasites, but also in allergy [134,138]. The cytokines IL-4 and IL-2, drive Th2 differentiation by activating the transcription factors STAT6 and STAT5, respectively. STAT6 in turn upregulates GATA3 (GATA-binding protein), the master transcription factor of Th2 cells [92]. Upon GATA3 activation Th2 cells acquire the ability to produce the effector cytokines IL-4, IL-5, and IL-13 [138]. Furthermore, similar to T-bet which suppresses other key transcription factors, also GATA3 has been shown to downregulate STAT4 and suppress Th1 differentiation [92].

Th17 cells

Th17 cells, yet another important Th cell lineage which stimulate type III immunity, are characterized by activation of mononuclear phagocytes, neutrophil recruitment and epithelial antimicrobial responses [141]. Th17 effector cells are involved in immune responses towards infectious diseases but are also implicated in autoimmune diseases [143]. While type III immunity constitutes a highly pro-inflammatory immune response, in large accounted for by secretion of IL-17A by the majority of Th17 cells [141], it has also been shown that Th17 cells in the intestine are a highly heterogeneous population and not always associated with type III immunity [144]. Indeed, on rare occasions, IL-17A$^+$ Th17 cells in the intestine have been shown to co-express either IFN-γ or forkhead box P3 (Foxp3), the master transcription factor of Treg, and Th17 cells may still have unrecognized functions in intestinal immune homeostasis [144]. Nevertheless, a clear pro-inflammatory Th17 phenotype is evident in the majority of Th17 cells and increased numbers of IL-17A$^+$ Th17 cells have been observed in the intestinal mucosa of patients with IBD [144]. In addition to IL-17A, Th17 cells also frequently secrete IL-21 and IL-22, and may also secrete IL-17F depending on environmental context [144]. IL-22 provides antimicrobial defense and promotes epithelial cell homeostasis [141].

The specific cytokine milieu that drives differentiation of naïve CD4$^+$ T cells into conventional Th17 effector cells, as defined by IL-17A expression, consists of IL-6, IL-21, IL-23 and a low concentration of TGF-β [92]. These cytokines activate downstream STAT3 signaling which further activates the retinoic acid receptor-related orphan receptor gamma-T (RORγt), the master transcription factor of Th17

cells [92]. Interestingly, TGF-β also promotes the differentiation of naïve $CD4^+$ T cells into peripheral Treg (pTreg) but only in a setting of high TGF-β concentration, and in the absence of IL-6 which otherwise counteracts Treg development. In this context, an overproduction of IL-6 will lead to a dysregulated Th17 response and autoimmunity [145]. Also Treg, which frequently secrete TGF-β, may contribute to Th17 differentiation in the presence of IL-6 [146].

Treg

As suggested by the name, regulatory T cells (Treg) are regulatory lymphocytes with a main function of maintaining immune homeostasis by regulating the activity of both self-reactive lymphocytes and other effector immune cells [147]. As such, they are important in preventing autoimmunity and allergy, but also in executing anti-inflammatory responses to control and limit inflammation [133,147,148]. In contrast to Th cells, Treg come in two flavors which are either thymus-derived Treg (tTreg) or peripheral Treg (pTreg) [92]. Of these, thymus-derived Treg are already lineage committed Foxp3-expressing T cells upon their release from the thymus [92]. They are also more easily activated compared to peripheral Treg, i.e. via TCR engagement and IL-2 signaling alone, while peripheral Treg are activated in a similar fashion to the other Th cell subsets [149]. In addition, thymus-derived Treg are preferentially self-reactive with TCRs of higher affinity towards self-antigen compared to other naïve $CD4^+$ T cells [147].

pTreg are highly present at mucosal sites and TGF-β, IL-10 and IL-2 are key cytokines implicated in their induction [150-152]. Of these, TGF-β signaling is critical for lineage commitment of pTreg, and activates the master transcription factor Foxp3 [92,153]. IL-10 and IL-2 signaling activate STAT3 and STAT5, respectively, which in turn enhances Foxp3 expression and Treg stability [92,152]. Of note, both TGF-β and STAT3 are also involved in Th17 differentiation, but in a setting of IL-6 signaling [145,152]. Indeed, several factors determine pTreg lineage commitment, and commensal bacteria-derived molecules, granulocyte-macrophage colony-stimulating factor (GM-CSF) and retinoic-acid, have all been associated with pTreg induction [107]. pTreg in the intestinal mucosa may also be dependent on CTLA-4, as proven in CTLA-deficient mice which had an impaired accumulation of commensal bacteria-specific pTreg [154]. Furthermore, Treg also display plasticity and may acquire traits of other Th cell subsets or altogether convert into another Th cell subset upon alterations of peripheral signaling cues [136,146,155-157]. In addition, while Foxp3 is a key transcription for both nTreg and pTreg, it is not

specific to Treg and may also be expressed in human nonregulatory $CD4^+CD25^-$ T cells upon stimulation *in vitro* [158]. To date, the conventional markers used to identify Treg does not allow for differentiation between thymus-derived and periphal Treg [159].

Several conventional cell markers are used in combination to identify Treg, and apart from the markers already mentioned (Foxp3, CD3 and CD4), additional basic Treg markers are CD25 (alpha chain of the IL-2 receptor) and CD127 (IL-7 receptor) [160]. Also the expression level of these markers is important and more specifically, Treg are identified as $CD3^+CD4^+CD25^{hi}CD127^{low}Foxp3^+$ cells. Other characteristic Treg markers are TGF-β, IL-10, CTLA-4, GITR, and neuropilin [147,161,162], but these molecules are not specific to Treg. Importantly in this context, $CD4^+Foxp3^+$ T cells isolated from peripheral blood has been shown to be a heterogenous cell population when characterized by their combined expression of Foxp3, CD25 and CD45RA [163]. Of the isolated cell fractions, two fractions were immunosuppressive putative thymic-derived Treg; $CD4^+CD45RA^+CD25^{++}Foxp3^{lo}$ resting naïve Treg (Fr. I) and $CD4^+CD45RA^-CD25^{+++}Foxp3^{hi}$ activated or memory Treg (Fr. II), and one fraction was $CD4^+CD45RA^-CD25^{++}Foxp3^{lo}$ non-suppressive putative effector T cells [163]. This confirms previous *in vitro* findings, which suggests that Foxp3 expression in combination with a high expression of CD25 is also associated with the activation state of T cells and not only confined to Treg [158].

In order for Treg to impose regulatory function on other immune cells, Treg are equipped with homing molecules and specific chemokine receptors, which direct them to the appropriate site in the tissue [164]. Importantly, Treg exert regulatory function both in the tissue by suppressing proliferation and function of effector immune cells such as T cells, NK cells and NKT cells [165], and in peripheral lymphoid organs by regulating T cell activation and differentiation [164,165]. Treg also interact with DCs and macrophages to induce tolerogenic properties in these cells [166]. In addition, despite an ability of Treg to perform bystander suppression on neighboring immune cells *in vitro*, Treg are primarily designed to exert antigen-specific immunosuppression and operate close at hand to effector T cells of the same antigen-specificity [151,167]. The effector functions of Treg consist of a wide range of suppressive mechanisms, basically including two major modes of action, i.e. cell-cell contact independent and cell-cell contact dependent mechanisms. These mechanisms can also be classified into different functional modes of suppression (*Figure 5*), which are production of anti-inflammatory cytokines

(TGF-β, IL-10 and IL-35) [166,168-170], cell-cell contact dependent T cell inhibition (CTLA-4, PD-1 and PD-L1) [161,171,172], cytolytic killing of target cells (perforin and granzyme B) [161], modulation of antigen presenting cell maturation and function (CTLA-4) [161,173], and disruption of metabolic pathways (IL-2 deprivation and generation of immunosuppressive adenosine) [161,174].

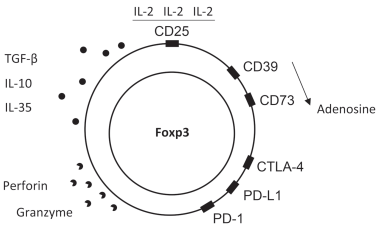

Figure 5. Effector functions by Treg.

While the specifics of some of these mechanisms will be explained more in detail in the coming sections, i.e. cytolytic killing of target cells and generation of immunosuppressive adenosine by Treg, others will be briefly overviewed in this section. CTLA-4 and PD-1 are commonly expressed by Treg but instead of propagating an inhibitory signal to Treg upon binding to its respective ligands, as is the case for other effector T cells upon binding of these co-inhibitory surface molecules, CTLA-4 and PD-1 signaling by Treg propagate an inhibitory signal in the opposite direction, acting on DCs and effector T cells, respectively [161,171,173]. Treg can also suppress effector T cells via PD-L1 by binding to PD-1 on effector T cells in a more conventional manner [171,172].

$CD8^+$ T cells

$CD8^+$ T cells or CTLs are already lineage committed naïve T cells upon their release from the thymus and undergo a faster differentiation upon antigen-specific encounter in the peripheral tissue compared to Th cells [175]. Upon clonal expansion and differentiation, effector $CD8^+$ T cells migrate to the appropriate site, where they similar to Th1 cells, mediate type I immunity as previously described [141]. Accordingly, $CD8^+$ T cells and Th1 cells often work in synergy to provide an effective and long-lasting response against typically intracellular microbes but also cancer [132,135,176,177]. Like Th1 cells, conventional $CD8^+$ T cells also express T-bet and IFN-γ, and T-bet is induced in $CD8^+$ T cells by pro-inflammatory cytokines such as IL-12 [141,178]. In addition, conventional $CD8^+$ T cells are specialized at

mediating cytotoxicity and thereby kill target cells in a cell-cell contact dependent manner inducing apoptosis in target cells [132]. Upon release by CTLs of cytolytic granules containing perforin and granzyme A and B, perforin causes a pore-formation in the target cell membrane enabling passage of caspase-activating granzymes into the cytosol [179,180]. The death receptor pathway, which involves interactions between the cell surface death receptor Fas on target cells and Fas ligand (FasL) on CTLs, is an additional cytolytic pathway by CTLs also leading to caspase dependent apoptosis in the target cell [181].

1.4 Specific properties of CD39$^+$ Treg and MAIT cells

CD39$^+$ Treg

CD39, also called ENTPD1 (ectonucleoside triphosphate diphosphohydrolase-1), is an ectoenzyme expressed on the surface of various immune cells but also on several other cells such as mesenchymal stem cells (MSCs) and endothelial cells [182,183]. It is an important regulator of purinergic signaling, and responsible for the first and rate-limiting step in the conversion of extracellular ATP (eATP) into adenosine. Upon hydrolysis of eATP into ADP and AMP by CD39, another ectoenzyme, CD73 in turn hydrolyzes AMP into adenosine [182,184]. Levels of extracellular ATP and adenosine are tightly regulated, but in response to increased levels of eATP, which commonly follows upon tissue damage, an increased activity by CD39 and CD73 is required to shift the balance from a highly proinflammatory state to a new equilibrium. In high concentrations, eATP thus serves as a danger signal to stimulate the immune system to an appropriate response [185,186]. However, also during normal conditions ATP is constantly present in the extracellular space. This is also true for adenosine, and purinergic signaling, via adenosine and ATP-receptors, is part of an important signaling system highly involved in the regulation of various immune cell functions, e.g. migration, activation, differentiation and suppression [185,187]. The adenosine receptors are a family of G protein-coupled receptors (GPCRs), and includes the A1, A2A, A2B, and A3 receptors [188]. For example, adenosine signaling via the A2BR receptor induces tolerogenic properties in APCs by alternative activation (*Figure 6*) [182]. ATP-receptors, on the other hand, can be either GPCRs (P2Y receptors) or ligand-gated ion channels (P2X receptors) [189].

Treg frequently express CD39 on their surface, and commonly CD39 expression is enriched among Treg both in various tissues of healthy individuals and different settings of disease [182,190-192]. On the other side of the spectra, CD39 expression by Treg can also be reduced, as has been shown in peripheral blood of multiple sclerosis (MS) patients [186,193,194]. In mice, CD39 is expressed by a fraction of thymic-derived Treg already upon their release from the thymus [190], but an upregulation of CD39 during disease in different T cell subsets, which lack CD39 expression under normal conditions, also suggests that CD39 can be upregulated in the tissue in response to different stimuli [195,196]. Also in mice, CD39 expression was shown to be driven by upregulated Foxp3 expression and enhanced by TCR-ligation [186], and interestingly in this context a recent study in humans provide

evidence of a stronger stability and function in $CD39^+$ Treg compared to $CD39^-$ Treg during inflammatory conditions [197]. $CD39^+$ Treg are also enriched in colorectal tumors [198-200], but their specific function in this setting remains to be elucidated. However, a high immunosuppressive potential of $CD39^+$ Treg have been shown both in healthy individuals and several diseases [191,196,201,202]. In this context, $CD39^+$ Treg are believed to exert immunosuppressive function via adenosine (*Figure 6*) but also via other more conventional Treg effector functions, such as secretion of TGF-β and IL-10 [172,174,196,199]. Also tumor stroma and tumor cells can express CD39, and in a recent study a CD39-inhibitor alleviated immunosuppression by $CD39^+CD73^+$ cancer cells and restored proliferation of both $CD4^+$ and $CD8^+$ T cells [203]. In addition, purinergic signaling also has direct effects on tumor growth in colon cancer, but a defined role of adenosine and ATP-signaling in this context remains to be elucidated [204-207].

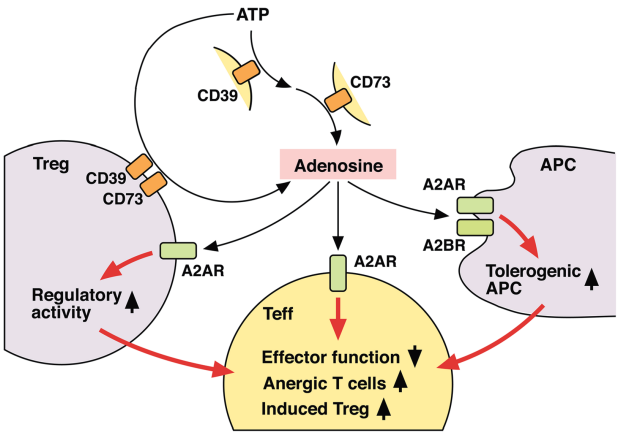

Figure 6. Generation and signaling effects of adenosine.
Ohta et al. Front Immunol 2014;5:304.

MAIT cells

Mucosal-associated invariant T (MAIT) cells are innate-like T cells, and in healthy individuals MAIT cells are predominantly located in peripheral blood, the intestine, and in the liver [208-211]. At these sites, and especially in the intestine, they are suggested to provide a first line defense against microbial pathogens [212,213]. However, MAIT cells have also been implicated both in autoimmune diseases [214,215] and inflammatory bowel disease [216,217]. MAIT cells express a semi-invariant TCR consisting of one invariant α chain (Vα7.2 joined to Jα33), combined with a

limited number of Vβ chains [208,218,219]. Apart from Vα7.2, also IL-18R and the NK receptor CD161 are commonly used to identify MAIT cells [209,219]. The MAIT cell TCR recognizes microbially derived vitamin B (riboflavin) metabolites bound to the invariant and highly conserved HLA-Ib major histocompatibility complex-related protein 1 (MR1) [220,221], which is expressed on both APCs and epithelial cells [208,222]. Activation of MAIT cells occur primarily in the peripheral tissue and importantly, to become activated, MAIT cells do not need to pass the PLNs prior to entering the tissue [223]. Once in the tissue, MAIT cells are readily activated by cognate antigen, but can also respond to antigen-independent stimuli, such as microbially induced cytokines [212,224]. Effector functions of MAIT cells are partly context dependent but generally comprise cytotoxic ability and secretion of both Th1 and Th17 type cytokines [221,225-228].

1.5 Cancer immunology

The link between cancer and the immune system is complex and needs to be assessed from different perspectives. On the one hand, inflammation itself may promote cancer, as clearly demonstrated by a higher risk of cancer development in several types of inflammatory diseases [110,229,230], and on the other hand the immune system responds to the cancer by initiating an anti-tumor immune response in order to eliminate the cancer [231]. In addition, cancers may also induce an inflammatory or tolerogenic microenvironment that promotes further cancer growth [110,232,233]. The process by which the immune system responds to and shapes the cancer is referred to as cancer immunoediting and involves three phases, i.e. elimination, equilibrium, and escape [231]. Interestingly, this interaction goes both ways, and if a cancer clone reaches the later phases of cancer immunoediting it has transformed and will no longer be detected by the immune system. The cancer will continue to grow in an uncontrolled manner and is out of reach from the immune system. Cancer immunosurveillance is a related process to cancer immunoediting, but refers mostly to the elimination phase and involves cooperate forces of innate and adaptive immunity to eradicate the cancer [234-236].

Classification of the immune contexture in human tumors, which include the immune cell compartment, microenvironment of the tumor, and the profile of expressed cytokines and chemokines, is a recent approach to determine the pro- and anti-tumorigenic properties of the immune infiltrate present in the tumors [237]. These studies have led to the introduction of the "immunoscore", a scoring system to assess the importance of the intratumoral immune landscape in human cancers [238,239], and the immunoscore was recently validated in patients with colon cancer and provides clues with regard to the risk of disease recurrence [240]. In another study, it was shown that immunoscore in CRC is a stronger predictor of patient survival than microsatellite instability [29,241]. Despite the obvious importance of tumor-infiltrating immune cells in CRC, only MSI-H colorectal tumors, a minority of all CRC, have so far responded to treatment with immune checkpoint inhibitors [242,243].

2 AIMS

Both $CD39^+$ Treg and MAIT cells accumulate in colon adenocarcinomas but their independent contribution to patient outcome remains unclear. The overall aim of this thesis was to investigate the specific functions of these immune cells in order to determine their potential as therapeutic targets in colon cancer.

Specific aims were as follows:

1. Determine the phenotype and function of intratumoral $CD39^+$ and $CD39^-$ Treg in colon adenocarcinomas *ex vivo*.

2. Investigate the suppressive ability of intratumoral $CD39^+$ and $CD39^-$ Treg in colon adenocarcinomas *in vitro*.

3. Assess the impact of intratumoral $CD39^+$ Treg on cumulative relapse-free survival and cumulative cancer-specific survival in colon adenocarcinoma patients.

4. Determine the presence and effector functions of MAIT cells in colon adenocarcinomas.

5. Assess the impact of intratumoral MAIT cell infiltration on patient outcome in colon adenocarcinoma.

3 MATERIAL AND METHODS

Patients, volunteers, and sample collection

All studies included in the thesis were performed with the permission of the Regional Research Committee of West Sweden, and informed consent was given by all included patients. Only patients that had not received radiotherapy or chemotherapy for 3 years prior to surgery, and had no autoimmune diseases were included. Tissue material was obtained from patients undergoing curative resection of colon adenocarcinomas, and in part also from healthy blood donors. Heparinized venous blood was collected from the patients during surgery and biopsies of colon tumors and macroscopically normal appearing colon mucosa (unaffected colon tissue) was collected from the tumor resectate post-surgery. Tumor biopsies were representative of both central and peripheral parts of the tumor and unaffected colon tissue was taken > 10 cm from the tumor margin. Smaller biopsies were placed in RNA-later for 24 hours before freezing in -80 °C and subsequent RNA-extraction. Remaining tissue was placed in ice-cold PBS for less than 2 hours before cell isolation. Information of tumor stage, tumor differentiation grade, localization and metastases, was retrieved from the pathology report post-surgery.

Isolation and stimulation of lymphocytes

Pre-washed biopsies of colon tumor and unaffected colon tissue were cut into 5-mm small tissue pieces and subjected to EDTA-buffer four times to remove the intraepithelial cell fraction. Intraepithelial lymphocytes (iELs) were saved from the second and third fractions, and the remaining tissue was further processed by enzymatic digestion (with Liberase and DNase) to obtain lamina propria lymphocytes (LPLs). Peripheral blood mononuclear cells (PBMCs), on the other hand, were isolated by Ficoll-Paque density-gradient centrifugation. Isolated lymphocytes were stimulated with PMA and ionomycin for approximately 10 hours, i.e. a polyclonal stimulation which activates lymphocytes independently of TCR engagement, or with manufactured IL-12 and IL-18, two cytokines commonly secreted by macrophages in response to antigen and capable of inducing cell-mediated immunity. Golgi-stop was added 4-12 hours before harvesting, in order to halt the cytokine secretion of stimulated cells in due time prior to flow cytometric analysis.

Flow cytometry analyses and flow cytometry cell sorting

Single cells isolated from peripheral blood, tumor, and unaffected colon tissue of colon adenocarcinoma patients, and PBMCs isolated from buffycoats of healthy donors, were stained with Live/dead aqua to exclude dead cells and at a later stage with a cocktail of antibody-fluorochrome conjugates in a one (extracellular staining) or two step (intracellular staining) process to detect marker expression. Foxp3 Staining Buffer Set was used for intracellular staining. FMO (fluorescence minus one) and isotype controls were run for reference to detect marker expression. Samples were run on an LSRII equipped with FACS DIVA software, which has three lasers (red, blue, violet) and allows for marker detection of up to approximately ten markers. Data was analyzed using Flow Jo software. Treg were identified as $CD4^{+}CD25^{hi}Foxp3^{+}$ Treg (for phenotype experiments) and $CD4^{+}CD127^{low}CD25^{hi}$ Treg (for functional experiments). Treg and conventional $CD4^{+}$ T cells were sorted using a FACSAria. Sorted cells were manually counted with trypan blue and viability was 94% for Treg and 92% for conventional T cells. MAIT cells were identified as $CD45^{+}CD8^{+}CD4^{-}TCRyd^{-}Va7.2^{+}CD161^{high}$ or as $CD45^{+}CD8^{+}CD4^{-}$ stained with MR1 tetramer loaded with 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU).

Suppression assay and cytokine analysis

Single cells were isolated from buffycoat or tissue from colon adenocarcinoma patients. Buffycoats were pre-enriched for $CD4^{+}$ T cells. Monocytes were enriched from $CD4^{+}$ T cells using bead based separation, and seeded onto the 96-well plate (20000/well). Remaning isolated cells were stained and sorted. Conventional Treg (10000) and $CD4^{+}$ T cells (10000), were seeded onto the plate, in different ratios of Treg:responder T cells, and stimulated with anti-CD3, and cells were harvested after four days in culture. In some experiments Treg were pre-stained with Far Red prior to culture. Upon harvesting cell proliferation was assessed by thymidine incorporation or analyzed by flow cytometry (experiments with Far Red stain). In addition, supernatants were collected after 2 days of culture and cytokine secretion was analyzed using a luminescence bead-based technique (the MAGPIX system).

MSS/MSI analysis

The microsatellite status of each colon tumor was analyzed through detection and qualitative assessment of amplified and fluorescently labeled microsatellite markers using the MSI Analysis System, Version 1.2 (ProMega). In total seven markers were co-amplified using PCR-based technique, including five

mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two pentanucleotide repeat markers (Penta C and Penta D). Upon completion of the PCR-part of the assay, co-amplicons were detected on the ABI PRISM® 3730 using PowerPlex 4C Matrix Standard (Cat.# DG4800), according to the manufacturer's instructions. Microsatellite instability, defined as peak alterations in the dinucleotide repeats of each microsatellite marker, was assessed using the marker electropherogram of the tumor compared to corresponding unaffected colon tissue. Tumors were defined as MSI-H tumors (if > 1 of the 5 markers showed instability), MSI-L tumors (if only 1 marker showed instability) or MSS tumors (if no MSI was detected), and were analyzed using Peak scanner Software 2.

Survival analysis

Of patients selected to participate in the two survival analyses studies, the same basal inclusion criteria applied as for the patients included in the other studies. In addition, patients with non-radical surgery, non-primary tumors, or diagnosed rectal cancer, were also excluded from the studies. Patients characterized as stage IV were excluded from the relapse-free survival analysis but included in the cancer-specific survival analysis. Several patients were censored due to non-cancer related death but remained in the study until they were censored. Estimated survivor curves were performed using the Kaplan-Meier method.

Statistical methods

Wilcoxon matched-pairs signed rank test was used to compare data obtained from the same patients. Mann-Whitney test and Kruskal-Wallis test were used to compare data obtained from different patients, between two groups and more than two groups, respectively. Correlation analysis was performed using nonparametric Spearman correlation. The Log-Rank test was used to compare Kaplan-Meier curves. P values of < 0.05 were considered significant.

4 KEY FINDINGS AND DISCUSSION

4.1 PAPER I AND II

An enrichment of CD39$^+$ Treg have previously been observed in tumors of colon adenocarcinoma patients [198-200], and based on the attributed functions of CD39 expression by Treg in both cancer and other settings *in vitro* [196,201], we speculated that CD39$^+$ Treg in colon tumors may be a superior immunosuppressive subset compared to other intratumoral Treg subsets. If so, CD39$^+$ Treg would potentially be a major regulator of tumor immunity in colon cancer, due to their high numbers in the tumors. Further substantiating this hypothesis, a recent study by our group showed that Treg from colon cancer patients were able to inhibit the transendothelial migration of conventional effector T cells *in vitro* [198], accounting for a potential additional mechanism of suppression by CD39$^+$ Treg in colon tumors. This effect was dependent on CD39-activity by Treg and an effect of adenosine on monocytes which in turn acted on endothelial cells.

To test our hypothesis, we first characterized CD39$^+$ Treg in colon cancer patients using flow cytometry. Also in our material Treg were enriched in the tumors (Paper I), and the majority of Treg also expressed CD39 on their surface. Furthermore, CD39 expression was higher among intratumoral Treg compared to Treg from both unaffected colon tissue and peripheral blood (*Figure 7, left graph*).

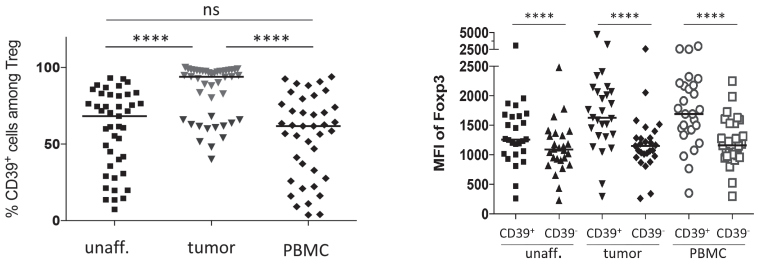

Figure 7. Frequencies of CD39$^+$ Treg in colon adenocarcinoma patients, and MFI of Foxp3 expression by CD39$^+$ and CD39$^-$ Treg.

At this stage, after having confirmed previous findings [198-200], we wanted to further characterize intratumoral CD39$^+$ Treg *ex vivo* with regard to established and functionally important Treg effector molecules in other settings and diseases. We did this by comparing marker expression between CD39$^+$ and CD39$^-$ Treg in the tissues; in Treg isolated from colon tumors, unaffected colon tissue and peripheral blood. CD39 expression by Treg had previously been correlated

with an upregulated FoxP3 expression [186], and we therefore first analyzed Foxp3-expression within the respective subsets. Interestingly, mean fluoresence intensity (MFI) of Foxp3, i.e. mean level of Foxp3 expression per cell, was significantly higher in $CD39^+$ Treg compared to $CD39^-$ Treg in all three tissues. (*Figure 7, right graph*).

In light of this finding we were curious to investigate if a high expression of Foxp3 would potentially drive further upregulation of other established Treg markers and effector molecules in the $CD39^+$ Treg subset. Indeed, using the same methodology to compare marker expression between $CD39^+$ and $CD39^-$ Treg intra tissue, we found a higher expression of several immunosuppressive effector molecules, such as PD-L1 and CTLA-4, in the intratumoral $CD39^+$ Treg subset (Paper I, Figure 3). Furthermore, $CD39^+$ Treg in the tumor proliferated more compared to $CD39^-$ Treg as measured by Ki67 expression, and $CD39^+$ Treg also had a higher expression of the co-stimulatory marker ICOS indicating a higher level of activation in this subset (Paper 1). In conclusion, this phenotypic data suggests that $CD39^+$ Treg are a highly immunosuppressive Treg subset in colon tumors.

So far, the phenotypic data on intratumoral $CD39^+$ Treg supported our stated hypothesis, but we had not yet confirmed this in functional experiments. Although unlikely, $CD39^-$ Treg could for example constitute a more stable subset or have a higher expression of other suppressive markers which we had not included in our phenotypic analysis, thus balancing the presumed difference in suppressive ability between the subsets. To investigate the function of $CD39^+$ Treg we first performed suppression assay experiments in vitro using peripheral blood of healthy donors. While this suppression assay system, further explained in the methodology section of the thesis, was optimized to measure proliferative responses, it also allowed us to assess cytokine production in the co-cultures. However, detected cytokine levels were low and, in cultures of peripheral blood, only IFN-γ production by conventional $CD4^+$ T cells (responder T cells), was reproducibly suppressed by both $CD39^+$ and $CD39^-$ autologous Treg, when these were added to the cultures (Paper I, Figure 6). In the same cultures, suppressive effect by Treg on responder T cell proliferation was however easier detected, and both Treg subsets were clearly suppressive in this regard (*Figure 8, left graph*). In the cultures where $CD39^+$ Treg were added to the responder T cells we found lower levels of remaining proliferation compared to the corresponding cultures with $CD39^-$ Treg (*Figure 8, left graph*). However, these experiments were based on thymidine incorporation measurements and consequently reflected total proliferation levels in the cultures. Since also the different Treg subsets potentially could proliferate in the presence of IL-2 produced by responder T cells, we could not be certain that these initial findings accounted for an isolated suppressive effect by Treg on autologous responder T cells. This effect is presumably not dependent on our *in vitro* system and also previous studies that have measured suppression by Treg using thymidine incorporation should

have encountered similar problems. Consequently, both CD39$^+$ and CD39$^-$ Treg isolated from peripheral blood of healthy donors, potently suppress autologous responder T cells *in vitro* (*Figure 8, left graph*), but we could not determine difference in suppressive ability between the two subsets at this stage. Also, in this *in vitro* system, Treg isolated from colon tumors were highly immunosuppressive, but independent of CD39 expression, which confirms previous findings (Paper I, Suppl. Figure 7).

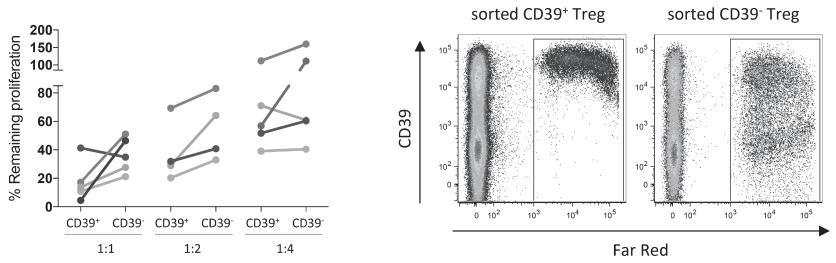

Figure 8. Effect of CD39$^+$ and CD39$^-$ Treg on conventional CD4$^+$ T cell proliferation, and dynamics of CD39$^+$ and CD39$^-$ Treg during culture.

To determine the dynamics of CD39$^+$ and CD39$^-$ Treg in the co-cultures, we stained both Treg subsets with Celltrace Far Red prior to adding them to the responder T cells. Upon harvesting, we were thus able to differentiate between responder T cells and Far Red$^+$ Treg using flow cytometry, and Celltrace Far Red also allowed us to determine proliferative response in each Treg subset (*Figure 8, right graph*). Somewhat surprisingly, we observed a significant upregulation of CD39 expression in the sorted original CD39$^-$ Treg subset (*Figure 8, right graph*). This was in contrast to previous control experiments when we stimulated total PBMCs with PMA/Ionomycin and detected only a modest upregulation of CD39 by Treg. Consequently, an upregulation of CD39 in the original CD39$^-$ Treg subset during culture, led us to conclude that it would be almost impossible to compare suppressive ability between CD39$^+$ and CD39$^-$ Treg *in vitro*. However, these experiments allowed us to compare proliferative response of the different Treg subsets during culture; on the one hand sorted CD39$^+$ Treg and on the other hand sorted original CD39$^-$ Treg which either remained CD39$^-$ or upregulated CD39 during culture (converted CD39$^+$ Treg). By clustering Far Red$^+$ cells into different populations based on fluorescence intensity (a proportionally lower intensity the more times a cell has divided), we were able to determine degree of cell division within each Treg subsets. Interestingly, the replication index, i.e. the fold

expansion of only the responding cells, showed that converted CD39$^+$ Treg had the highest proliferation of all three subsets (Paper I).

In Paper II we wanted to correlate CD39 expression by intratumoral Treg in colon cancer with patient outcome. A correlation between high levels of intratumoral Treg and a less favorable patient outcome, has been found in some studies [244-246], and since intratumoral Treg generally are believed to suppress anti-tumor immunity by inhibiting effector T cells, this would be an alternative approach to test the hypothesis that intratumoral CD39$^+$ Treg have superior suppressive ability and thus presumably a negative impact on patient outcome.
To broaden our knowledge of intratumoral CD39$^+$ Treg, we first investigated the inter tissue relationship of CD39 expression by Treg in colon cancer patients. Interestingly, there was a positive correlation between frequencies of intratumoral CD39$^+$ Treg and CD39$^+$ Treg frequencies in both peripheral blood and unaffected colon tissue (*Figure 9*). In addition to the potential interest from a prognostic biomarker perspective, this suggests that intratumoral CD39$^+$ Treg levels are in part dependent on peripheral blood CD39$^+$ Treg which may supply the tumor site with new CD39$^+$ Treg.

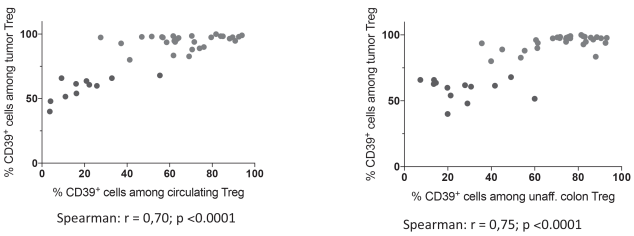

Figure 9. Correlation between CD39 expression by Treg in different tissues.

It was clear from the data obtained in paper I that colon tumors divide into two populations based on the frequency of CD39-expressing Treg among total intratumoral Treg (*Figure 7, left graph*). We suggest that this division reflects differences in either tumor characteristics or individual differences in the immune response towards the tumor. Consequently, we used this already existing difference between patient groups to stratify colon cancer tumors into two types, i.e. group I and II tumors with high (> 75%) and low (< 75%) frequencies of CD39-expressing Treg among total intratumoral Treg, respectively (*Figure 10, FACS-plots*). We also referred to these two patient groups as population I (group I tumors) and population II (group II tumors).

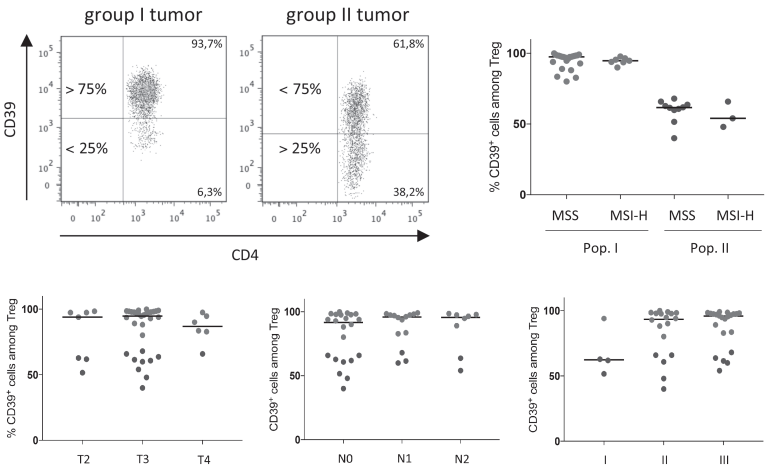

Figure 10. CD39 expression by Treg from colon adenocarcinoma patients, and frequencies of intratumoral CD39$^+$ Treg in relation to microsatellite status and clinical parameters.

As a first step to investigate the contribution of intratumoral CD39$^+$ Treg to patient prognosis, we analyzed group I and II tumors in relation to prognostically important clinical parameters in colon cancer [4,40,55]. Since MSI-H tumors are associated with a stronger immune activation compared to MSS tumors, and also a more favorable patient outcome [44], we were particularly interested in determining a role of intratumoral CD39$^+$ Treg in this context. However, there was no relationship between tumor microsatellite status and CD39 expression by intratumoral Treg (*Figure 10, above graph*), and presumably a strong immune activation within MSI-H tumors cannot be explained by a low number of CD39$^+$ Treg in those tumors. Also, there was no significant relationship between CD39 expression by intratumoral Treg and any of the other variables analyzed, and group I and II tumors seemed evenly distributed between groups when analyzed in relation to T stage, lymph node spread and tumor stage (*Figure 10, lower graphs*).

We then compared cumulative cancer-specific survival and cumulative relapse-free survival between group I and II colon tumors (population I and II, respectively). This survival analysis is explained further in the methodology section of the thesis. Based on previous findings with regard to phenotype and presumed function of intratumoral CD39$^+$ Treg in colon tumors, we speculated that patients belonging to population I would have a higher degree of immunosuppression in the tumors and consequently a less favorable patient

outcome. Preliminary survival data also pointed in this direction, and especially with regard to cumulative cancer-specific survival, as cumulative relapse-free survival was more similar between groups when comparing Kaplan-Meier curves (*Figure 11*).

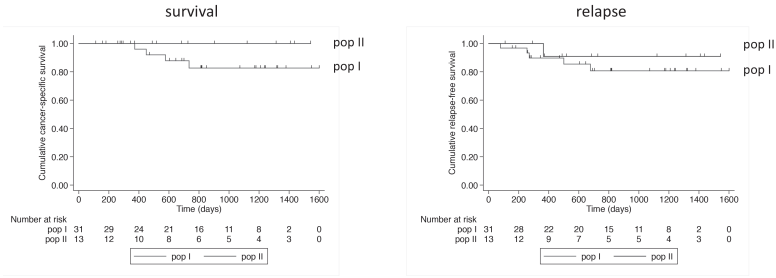

Figure 11. Frequencies of intratumoral CD39$^+$ Treg and patient outcome.

With the relatively low number of events detected so far in the sample this difference was however not significant but a lower cumulative cancer-specific survival in population I compared to population II, may possibly substantiate the presence of functionally diverse intratumoral Treg subsets in colon cancer. Additional patient events (from death or relapse), which will likely follow upon added follow-up time, are required in order to conduct multivariate analysis to investigate if CD39 expression by intratumoral Treg, also is an independent predictor of survival in colon cancer patients.

4.2 PAPER III AND APPENDIX

In paper III and Appendix, we studied MAIT cells in colon adenocarcinomas, a subset of innate-like T cells, present at the tumor site. MAIT cells are believed to provide a first line defense towards intestinal microbes and since colon tumors are associated with both epithelial barrier breach and an increased infiltration of microbes [111], MAIT cell presence at the tumor site should be expected. MAIT cells were identified as $CD45^+CD3^+CD4^-TCR\gamma\delta^-CD161^{high}V\alpha7.2^+$ cells or by tetramer staining of $CD45^+CD3^+CD4^-$ cells using MR1 tetramer loaded with 5-OP-RU. In paper III we were one of the first to show an accumulation of MAIT cells in colon tumors compared to unaffected colon tissue (Paper III, Figure 2), and we also confirm this in a separate patient cohort (*Figure 12, left graph*), where we studied the impact of intratumoral MAIT cells on patient survival in colon cancer (Appendix). In the same patient cohort (Appendix), we also detected a positive correlation ($p < 0.0001$) between frequencies of circulating MAIT cells and intratumoral MAIT cell frequencies (*Figure 12, right graph*). This suggests that frequencies of circulating MAIT cells in colon cancer patients may serve as a predictor of intratumoral MAIT cell frequencies, but it may also suggest that colon cancer affects the circulating pool of MAIT cells to a lower degree compared to inflammatory bowel disease (IBD) and autoimmune diseases [215,216]. Indeed, frequencies of MAIT cells isolated from peripheral blood of colon cancer patients were equal to those of healthy controls (Paper III, Figure 1B), while frequencies of MAIT cells in peripheral blood of multiple sclerosis and IBD patients were lower in affected patients [215,216].

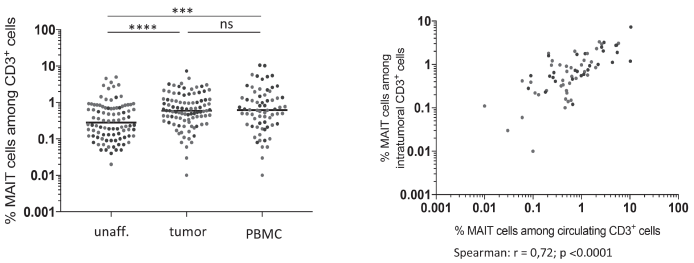

Figure 12. Frequencies of MAIT cells in colon adenocarcinoma patients, and correlation between circulating and intratumoral MAIT cell frequencies.

It has been shown that MAIT cells in the colonic lamina propria are dominated by a Th1 phenotype, but in other contexts MAIT cells can also produce Th17 associated cytokines. To investigate the context dependent function of intratumoral MAIT cells in colon cancer, with regard to cytokine secretion, we

stimulated MAIT cells with PMA and ionomycin *ex vivo*. Also intratumoral MAIT cells in colon cancer were dominated by a Th1 phenotype (with IFN-γ and TNF-α production; Paper III, Figure 3). Interestingly, the frequencies of IFN-γ-producing MAIT cells was lower in colon tumors compared to unaffected colon tissue (*Figure 13, left graph*), while IL-17 production in this context was produced by few cells irrespective of tissue origin (*Figure 13, right graph*).

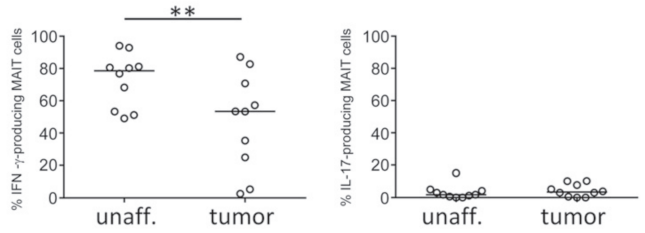

Figure 13. Cytokine production of IFN-γ and IL-17 by MAIT cells in colon adenocarcinoma patients.

High infiltration of MAIT cells in colon tumors have previously been associated with a less favorable prognosis in colon cancer [247] and we performed a similar survival analysis also in our patient cohort to examine this further. However, in contrast to the result obtained by Zabijak *et al.*, we found the opposite result in our preliminary survival data, with a higher cumulative relapse-free survival in the patient group with high MAIT cell infiltration in colon tumors when the sample was stratified according to the above and lower quartile values of intratumoral MAIT cell frequencies (*Figure 14, left graph*). Cumulative cancer-specific survival was more similar between groups (*Figure 14, right graph*).

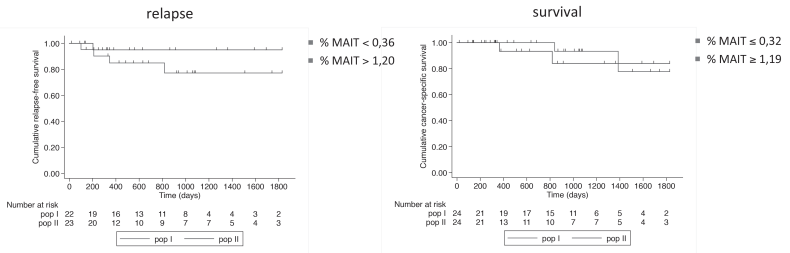

Figure 14. Frequencies of intratumoral MAIT cells and patient outcome.

5 CONCLUSION AND FUTURE PERSPECTIVES

To conclude from Paper I and II, we confirm an accumulation of Treg in colon adenocarcinomas and a high immunosuppressive ability of this subset on responder T cell proliferation *in vitro* is potentially a result of high levels of $CD39^+$ Treg among total intratumoral Treg. Indeed, similar to $CD39^+$ Treg isolated from peripheral blood, which are highly immunosuppressive *in vitro*, $CD39^+$ Treg in colon tumors express markers of increased suppressive function. In addition, intratumoral $CD39^+$ Treg also proliferated more compared to $CD39^-$ Treg as measured by Ki67 expression *ex vivo*, and a similar observation was made by flow cytometry in Treg isolated from peripheral blood with regard to converted $CD39^+$ Treg which displayed a superior proliferative response. These findings suggest that $CD39^+$ Treg are a highly immunosuppressive Treg subset in colon tumors and interestingly, $CD39^+$ Treg possess several phenotypic similarities with putative $CD4^+CD45RA^-CD25^{+++}Foxp3^{hi}$ activated or memory Treg, as characterized by Miyara *et al.* in peripheral blood [163], and more recently correlated to a less favorable patient outcome in CRC patients [248]. So far, immunosuppressive ability of intratumoral $CD39^+$ Treg in colon cancer has not been directly linked to CD39-activity, as it has in breast cancer where an anti-CD39 antibody released the suppressive effect of Treg on IFN-γ production by Th1 and $CD8^+$ T cells [196]. The survival analysis in paper II is at this stage somewhat preliminary but at least there is a clear trend towards a lower cancer-specific survival in patients with high accumulation of intratumoral $CD39^+$ Treg. This is promising data and it remains to be established if $CD39^+$ Treg could serve as a potential future target of cancer immunotherapy in colon cancer.

To conclude from paper III and appendix, a high MAIT cell infiltration in colon tumors has previously been correlated to a less favorable patient prognosis which is in direct contrast to our preliminary survival data. It is curious why MAIT cells, dominated by a Th1 phenotype in colon cancer, would be associated with a less favorable patient prognosis. This is in contrast to colon tumors with high infiltration of Th 1 cells. Additional studies are needed to clarify the impact of intratumoral MAIT cells in colon cancer, especially with regard to their effector functions.

ACKNOWLEDGEMENTS

Marianne, thank you for good supervising and your motivating spirit, also in times of challenge. I have greatly appreciated our work together and the confidence you have invested in me during these years.

Sukanya, I have enjoyed having you as my co-supervisor and our scientific small talks at the 6th floor.

Bengt, Yvonne, Elinor, Ann-Louise and Hillevi, thank you for our translational collaboration which have made my studies both possible and fun.

Patrik, you are a great friend and have been a great travel companion during conferences.

Paulina, Louis, William and Ahmad, thank you for all support and all the great company late evenings in the lab.

Department colleagues, thank you for all the positive spirit and all the nice talks.

Family and friends, if it hadn't been for you this endeavor would have been so much harder. Thanks for all the nice times together.

REFERENCES

1. Vogelstein, B.*, et al.* Cancer genome landscapes. *Science* **339**, 1546-1558 (2013).
2. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
3. Vesely, M.D., Kershaw, M.H., Schreiber, R.D. & Smyth, M.J. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* **29**, 235-271 (2011).
4. Brenner, H., Kloor, M. & Pox, C.P. Colorectal cancer. *Lancet* **383**, 1490-1502 (2014).
5. Coussens, L.M., Zitvogel, L. & Palucka, A.K. Neutralizing tumor-promoting chronic inflammation: a magic bullet? *Science* **339**, 286-291 (2013).
6. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
7. Hui, L. & Chen, Y. Tumor microenvironment: Sanctuary of the devil. *Cancer Lett* **368**, 7-13 (2015).
8. Fleming, M., Ravula, S., Tatishchev, S.F. & Wang, H.L. Colorectal carcinoma: Pathologic aspects. *J Gastrointest Oncol* **3**, 153-173 (2012).
9. Tamas, K.*, et al.* Rectal and colon cancer: Not just a different anatomic site. *Cancer Treat Rev* **41**, 671-679 (2015).
10. Cancer Genome Atlas, N. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **487**, 330-337 (2012).
11. Liu, Y.*, et al.* Comparative Molecular Analysis of Gastrointestinal Adenocarcinomas. *Cancer Cell* **33**, 721-735 e728 (2018).
12. Bray, F.*, et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* **68**, 394-424 (2018).
13. Siegel, R.L., Miller, K.D. & Jemal, A. Cancer statistics, 2019. *CA Cancer J Clin* (2019).
14. Kanth, P., Grimmett, J., Champine, M., Burt, R. & Samadder, N.J. Hereditary Colorectal Polyposis and Cancer Syndromes: A Primer on Diagnosis and Management. *Am J Gastroenterol* **112**, 1509-1525 (2017).
15. Yurgelun, M.B.*, et al.* Cancer Susceptibility Gene Mutations in Individuals With Colorectal Cancer. *J Clin Oncol* **35**, 1086-1095 (2017).
16. Taylor, D.P., Burt, R.W., Williams, M.S., Haug, P.J. & Cannon-Albright, L.A. Population-based family history-specific risks for colorectal cancer: a constellation approach. *Gastroenterology* **138**, 877-885 (2010).
17. Boland, P.M., Yurgelun, M.B. & Boland, C.R. Recent progress in Lynch syndrome and other familial colorectal cancer syndromes. *CA Cancer J Clin* **68**, 217-231 (2018).
18. Ma, H.*, et al.* Pathology and genetics of hereditary colorectal cancer. *Pathology* **50**, 49-59 (2018).
19. Muller, A. & Fishel, R. Mismatch repair and the hereditary non-polyposis colorectal cancer syndrome (HNPCC). *Cancer Invest* **20**, 102-109 (2002).
20. Nusse, R. Wnt signaling in disease and in development. *Cell Res* **15**, 28-32 (2005).
21. Kinzler, K.W. & Vogelstein, B. Lessons from hereditary colorectal cancer. *Cell* **87**, 159-170 (1996).
22. Jess, T., Rungoe, C. & Peyrin-Biroulet, L. Risk of colorectal cancer in patients with ulcerative colitis: a meta-analysis of population-based cohort studies. *Clin Gastroenterol Hepatol* **10**, 639-645 (2012).
23. Risio, M. The natural history of colorectal adenomas and early cancer. *Pathologe* **33 Suppl 2**, 206-210 (2012).
24. Jass, J.R. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* **50**, 113-130 (2007).
25. Fearon, E.R. Molecular genetics of colorectal cancer. *Annu Rev Pathol* **6**, 479-507 (2011).
26. To, K.K., Tong, C.W., Wu, M. & Cho, W.C. MicroRNAs in the prognosis and therapy of colorectal cancer: From bench to bedside. *World J Gastroenterol* **24**, 2949-2973 (2018).
27. Pino, M.S. & Chung, D.C. The chromosomal instability pathway in colon cancer. *Gastroenterology* **138**, 2059-2072 (2010).
28. Fodde, R.*, et al.* Mutations in the APC tumour suppressor gene cause chromosomal instability. *Nat Cell Biol* **3**, 433-438 (2001).
29. Boland, C.R. & Goel, A. Microsatellite instability in colorectal cancer. *Gastroenterology* **138**, 2073-2087 e2073 (2010).

30. Battaglin, F., Naseem, M., Lenz, H.J. & Salem, M.E. Microsatellite instability in colorectal cancer: overview of its clinical significance and novel perspectives. *Clin Adv Hematol Oncol* **16**, 735-745 (2018).
31. Issa, J.P. CpG island methylator phenotype in cancer. *Nat Rev Cancer* **4**, 988-993 (2004).
32. Weisenberger, D.J*., et al*. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* **38**, 787-793 (2006).
33. Guinney, J*., et al.* The consensus molecular subtypes of colorectal cancer. *Nat Med* **21**, 1350-1356 (2015).
34. Dienstmann, R*., et al.* Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer. *Nat Rev Cancer* **17**, 79-92 (2017).
35. Linnekamp, J.F*., et al.* Consensus molecular subtypes of colorectal cancer are recapitulated in in vitro and in vivo models. *Cell Death Differ* **25**, 616-633 (2018).
36. Prasetyanti, P.R*., et al.* Capturing colorectal cancer inter-tumor heterogeneity in patient-derived xenograft (PDX) models. *Int J Cancer* **144**, 366-371 (2019).
37. Sveen, A*., et al*. Colorectal Cancer Consensus Molecular Subtypes Translated to Preclinical Models Uncover Potentially Targetable Cancer Cell Dependencies. *Clin Cancer Res* **24**, 794-806 (2018).
38. Venkatesan, S., Swanton, C., Taylor, B.S. & Costello, J.F. Treatment-Induced Mutagenesis and Selective Pressures Sculpt Cancer Evolution. *Cold Spring Harb Perspect Med* **7**(2017).
39. TNM Classification, 8th edition of the uicc tnm classification of malignant tumors. (2017).
40. Saha, S*., et al.* Tumor size predicts long-term survival in colon cancer: an analysis of the National Cancer Data Base. *Am J Surg* **209**, 570-574 (2015).
41. Bertero, L*., et al*. Eighth Edition of the UICC Classification of Malignant Tumours: an overview of the changes in the pathological TNM classification criteria-What has changed and why? *Virchows Arch* **472**, 519-531 (2018).
42. Dienstmann, R. Tumor Side as Model of Integrative Molecular Classification of Colorectal Cancer. *Clin Cancer Res* **24**, 989-990 (2018).
43. Komor, M.A*., et al*. Consensus molecular subtype classification of colorectal adenomas. *J Pathol* **246**, 266-276 (2018).
44. Germano, G., Amirouchene-Angelozzi, N., Rospo, G. & Bardelli, A. The Clinical Impact of the Genomic Landscape of Mismatch Repair-Deficient Cancers. *Cancer Discov* **8**, 1518-1528 (2018).
45. Raut, C.P., Pawlik, T.M. & Rodriguez-Bigas, M.A. Clinicopathologic features in colorectal cancer patients with microsatellite instability. *Mutat Res* **568**, 275-282 (2004).
46. Loree, J.M*., et al.* Classifying Colorectal Cancer by Tumor Location Rather than Sidedness Highlights a Continuum in Mutation Profiles and Consensus Molecular Subtypes. *Clin Cancer Res* **24**, 1062-1072 (2018).
47. Lam, A.K., Chan, S.S. & Leung, M. Synchronous colorectal cancer: clinical, pathological and molecular implications. *World J Gastroenterol* **20**, 6815-6820 (2014).
48. Kijima, S*., et al.* Preoperative evaluation of colorectal cancer using CT colonography, MRI, and PET/CT. *World J Gastroenterol* **20**, 16964-16975 (2014).
49. Das, V., Kalita, J. & Pal, M. Predictive and prognostic biomarkers in colorectal cancer: A systematic review of recent advances and challenges. *Biomed Pharmacother* **87**, 8-19 (2017).
50. Roberts, B.S*., et al.* Discovery and Validation of Circulating Biomarkers of Colorectal Adenoma by High-Depth Small RNA Sequencing. *Clin Cancer Res* **24**, 2092-2099 (2018).
51. Chang, K*., et al.* Colorectal premalignancy is associated with consensus molecular subtypes 1 and 2. *Ann Oncol* **29**, 2061-2067 (2018).
52. Socialstyrelsen. Nationella riktlinjer för tjock- och ändtarmscancer, vetenskapligt underlag. (2014).
53. Samverkan Rci. Tjock- och ändtarmscancer, Nationellt vårdprogram. (2016).
54. Yamaoka, Y*., et al.* The distribution of lymph node metastases and their size in colon cancer. *Langenbecks Arch Surg* **402**, 1213-1221 (2017).
55. Miller, K.D*., et al*. Cancer treatment and survivorship statistics, 2016. *CA Cancer J Clin* **66**, 271-289 (2016).
56. Benson, A.B., 3rd*, et al*. Colon Cancer, Version 1.2017, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw* **15**, 370-398 (2017).
57. Saridaki, Z., Souglakos, J. & Georgoulias, V. Prognostic and predictive significance of MSI in stages II/III colon cancer. *World J Gastroenterol* **20**, 6809-6814 (2014).

58. Koopman, M., *et al.* Deficient mismatch repair system in patients with sporadic advanced colorectal cancer. *Br J Cancer* **100**, 266-273 (2009).
59. Fujiyoshi, K., *et al.* Metastatic Pattern of Stage IV Colorectal Cancer with High-Frequency Microsatellite Instability as a Prognostic Factor. *Anticancer Res* **37**, 239-247 (2017).
60. Domingo, E., *et al.* Somatic POLE proofreading domain mutation, immune response, and prognosis in colorectal cancer: a retrospective, pooled biomarker study. *Lancet Gastroenterol Hepatol* **1**, 207-216 (2016).
61. Sinicrope, F.A., *et al.* Molecular markers identify subtypes of stage III colon cancer associated with patient outcomes. *Gastroenterology* **148**, 88-99 (2015).
62. Samowitz, W.S., *et al.* Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. *Cancer Res* **65**, 6063-6069 (2005).
63. French, A.J., *et al.* Prognostic significance of defective mismatch repair and BRAF V600E in patients with colon cancer. *Clin Cancer Res* **14**, 3408-3415 (2008).
64. De Sousa, E.M.F., *et al.* Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat Med* **19**, 614-618 (2013).
65. Lamprecht, S., *et al.* PBX3 Is Part of an EMT Regulatory Network and Indicates Poor Outcome in Colorectal Cancer. *Clin Cancer Res* **24**, 1974-1986 (2018).
66. Kandimalla, R., *et al.* Genome-wide Discovery and Identification of a Novel miRNA Signature for Recurrence Prediction in Stage II and III Colorectal Cancer. *Clin Cancer Res* **24**, 3867-3877 (2018).
67. Noda, M., *et al.* Glycosyltransferase Gene Expression Identifies a Poor Prognostic Colorectal Cancer Subtype Associated with Mismatch Repair Deficiency and Incomplete Glycan Synthesis. *Clin Cancer Res* **24**, 4468-4481 (2018).
68. Liang, Q., *et al.* RING-Finger Protein 6 Amplification Activates JAK/STAT3 Pathway by Modifying SHP-1 Ubiquitylation and Associates with Poor Outcome in Colorectal Cancer. *Clin Cancer Res* **24**, 1473-1485 (2018).
69. Sinicrope, F.A., *et al.* Prognostic impact of deficient DNA mismatch repair in patients with stage III colon cancer from a randomized trial of FOLFOX-based adjuvant chemotherapy. *J Clin Oncol* **31**, 3664-3672 (2013).
70. Arnold, D., *et al.* Prognostic and predictive value of primary tumour side in patients with RAS wild-type metastatic colorectal cancer treated with chemotherapy and EGFR directed antibodies in six randomized trials. *Ann Oncol* **28**, 1713-1729 (2017).
71. Missiaglia, E., *et al.* Distal and proximal colon cancers differ in terms of molecular, pathological, and clinical features. *Ann Oncol* **25**, 1995-2001 (2014).
72. Franko, J., *et al.* Prognosis of patients with peritoneal metastatic colorectal cancer given systemic therapy: an analysis of individual patient data from prospective randomised trials from the Analysis and Research in Cancers of the Digestive System (ARCAD) database. *Lancet Oncol* **17**, 1709-1719 (2016).
73. Sargent, D.J., *et al.* Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol* **28**, 3219-3226 (2010).
74. Dienstmann, R., Salazar, R. & Tabernero, J. Overcoming Resistance to Anti-EGFR Therapy in Colorectal Cancer. *Am Soc Clin Oncol Educ Book*, e149-156 (2015).
75. Hsu, H.C., *et al.* Mutations of KRAS/NRAS/BRAF predict cetuximab resistance in metastatic colorectal cancer patients. *Oncotarget* **7**, 22257-22270 (2016).
76. De Roock, W., *et al.* Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* **11**, 753-762 (2010).
77. Toledo, R.A., *et al.* Exome Sequencing of Plasma DNA Portrays the Mutation Landscape of Colorectal Cancer and Discovers Mutated VEGFR2 Receptors as Modulators of Antiangiogenic Therapies. *Clin Cancer Res* **24**, 3550-3559 (2018).
78. Berger, M.D., *et al.* A Polymorphism within the Vitamin D Transporter Gene Predicts Outcome in Metastatic Colorectal Cancer Patients Treated with FOLFIRI/Bevacizumab or FOLFIRI/Cetuximab. *Clin Cancer Res* **24**, 784-793 (2018).
79. Iwasaki, A. & Medzhitov, R. Control of adaptive immunity by the innate immune system. *Nat Immunol* **16**, 343-353 (2015).
80. Landsverk, O.J., Bakke, O. & Gregers, T.F. MHC II and the endocytic pathway: regulation by invariant chain. *Scand J Immunol* **70**, 184-193 (2009).
81. Hammad, H. & Lambrecht, B.N. Barrier Epithelial Cells and the Control of Type 2 Immunity. *Immunity* **43**, 29-40 (2015).

82. Chen, G., Shaw, M.H., Kim, Y.G. & Nunez, G. NOD-like receptors: role in innate immunity and inflammatory disease. *Annu Rev Pathol* **4**, 365-398 (2009).
83. Medzhitov, R. Toll-like receptors and innate immunity. *Nat Rev Immunol* **1**, 135-145 (2001).
84. Loo, Y.M. & Gale, M., Jr. Immune signaling by RIG-I-like receptors. *Immunity* **34**, 680-692 (2011).
85. Blaser, H., Dostert, C., Mak, T.W. & Brenner, D. TNF and ROS Crosstalk in Inflammation. *Trends Cell Biol* **26**, 249-261 (2016).
86. Patel, S. Danger-Associated Molecular Patterns (DAMPs): the Derivatives and Triggers of Inflammation. *Curr Allergy Asthma Rep* **18**, 63 (2018).
87. Mathur, A., Hayward, J.A. & Man, S.M. Molecular mechanisms of inflammasome signaling. *J Leukoc Biol* **103**, 233-257 (2018).
88. Hajishengallis, G., Reis, E.S., Mastellos, D.C., Ricklin, D. & Lambris, J.D. Novel mechanisms and functions of complement. *Nat Immunol* **18**, 1288-1298 (2017).
89. Marnell, L., Mold, C. & Du Clos, T.W. C-reactive protein: ligands, receptors and role in inflammation. *Clin Immunol* **117**, 104-111 (2005).
90. Dinarello, C.A. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev* **281**, 8-27 (2018).
91. Deguine, J. & Barton, G.M. MyD88: a central player in innate immune signaling. *F1000Prime Rep* **6**, 97 (2014).
92. Luckheeram, R.V., Zhou, R., Verma, A.D. & Xia, B. CD4(+)T cells: differentiation and functions. *Clin Dev Immunol* **2012**, 925135 (2012).
93. Pieper, K., Grimbacher, B. & Eibel, H. B-cell biology and development. *J Allergy Clin Immunol* **131**, 959-971 (2013).
94. MacLeod, M.K., Kappler, J.W. & Marrack, P. Memory CD4 T cells: generation, reactivation and re-assignment. *Immunology* **130**, 10-15 (2010).
95. Landsverk, O.J.*, et al.* Antibody-secreting plasma cells persist for decades in human intestine. *J Exp Med* **214**, 309-317 (2017).
96. Zhang, K., Hornef, M.W. & Dupont, A. The intestinal epithelium as guardian of gut barrier integrity. *Cell Microbiol* **17**, 1561-1569 (2015).
97. Watson, A.J. Claudins and barrier dysfunction in intestinal inflammation: cause or consequence? *Gut* **64**, 1501-1502 (2015).
98. Mowat, A.M. & Agace, W.W. Regional specialization within the intestinal immune system. *Nat Rev Immunol* **14**, 667-685 (2014).
99. Pabst, O. & Mowat, A.M. Oral tolerance to food protein. *Mucosal Immunol* **5**, 232-239 (2012).
100. Zhou, Z.*, et al.* Toll-like receptor-mediated immune responses in intestinal macrophages; implications for mucosal immunity and autoimmune diseases. *Clin Immunol* **173**, 81-86 (2016).
101. Paul, G., Khare, V. & Gasche, C. Inflamed gut mucosa: downstream of interleukin-10. *Eur J Clin Invest* **42**, 95-109 (2012).
102. Ohno, H. Intestinal M cells. *J Biochem* **159**, 151-160 (2016).
103. Jung, C., Hugot, J.P. & Barreau, F. Peyer's Patches: The Immune Sensors of the Intestine. *Int J Inflam* **2010**, 823710 (2010).
104. Macpherson, A.J. & Smith, K. Mesenteric lymph nodes at the center of immune anatomy. *J Exp Med* **203**, 497-500 (2006).
105. Houston, S.A.*, et al.* The lymph nodes draining the small intestine and colon are anatomically separate and immunologically distinct. *Mucosal Immunol* **9**, 468-478 (2016).
106. Sorini, C., Cardoso, R.F., Gagliani, N. & Villablanca, E.J. Commensal Bacteria-Specific CD4(+) T Cell Responses in Health and Disease. *Front Immunol* **9**, 2667 (2018).
107. Caballero, S. & Pamer, E.G. Microbiota-mediated inflammation and antimicrobial defense in the intestine. *Annu Rev Immunol* **33**, 227-256 (2015).
108. Lycke, N.Y. & Bemark, M. The regulation of gut mucosal IgA B-cell responses: recent developments. *Mucosal Immunol* **10**, 1361-1374 (2017).
109. Yu, L.C. Microbiota dysbiosis and barrier dysfunction in inflammatory bowel disease and colorectal cancers: exploring a common ground hypothesis. *J Biomed Sci* **25**, 79 (2018).
110. Mantovani, A., Allavena, P., Sica, A. & Balkwill, F. Cancer-related inflammation. *Nature* **454**, 436-444 (2008).
111. Grivennikov, S.I.*, et al.* Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* **491**, 254-258 (2012).
112. Ackermann, M., Liebhaber, S., Klusmann, J.H. & Lachmann, N. Lost in translation: pluripotent stem cell-derived hematopoiesis. *EMBO Mol Med* **7**, 1388-1402 (2015).

113. Krueger, A., Zietara, N. & Lyszkiewicz, M. T Cell Development by the Numbers. *Trends Immunol* **38**, 128-139 (2017).
114. Melief, C.J. Mini-review: Regulation of cytotoxic T lymphocyte responses by dendritic cells: peaceful coexistence of cross-priming and direct priming? *Eur J Immunol* **33**, 2645-2654 (2003).
115. Xu, M.M.*, et al.* Dendritic Cells but Not Macrophages Sense Tumor Mitochondrial DNA for Cross-priming through Signal Regulatory Protein alpha Signaling. *Immunity* **47**, 363-373 e365 (2017).
116. del Rio, M.L., Bernhardt, G., Rodriguez-Barbosa, J.I. & Forster, R. Development and functional specialization of CD103+ dendritic cells. *Immunol Rev* **234**, 268-281 (2010).
117. Bern, M.D.*, et al.* Inducible down-regulation of MHC class I results in natural killer cell tolerance. *J Exp Med* **216**, 99-116 (2019).
118. Stagg, A.J. Intestinal Dendritic Cells in Health and Gut Inflammation. *Front Immunol* **9**, 2883 (2018).
119. Corthay, A. A three-cell model for activation of naive T helper cells. *Scand J Immunol* **64**, 93-96 (2006).
120. Smith-Garvin, J.E., Koretzky, G.A. & Jordan, M.S. T cell activation. *Annu Rev Immunol* **27**, 591-619 (2009).
121. Gardell, J.L. & Parker, D.C. CD40L is transferred to antigen-presenting B cells during delivery of T-cell help. *Eur J Immunol* **47**, 41-50 (2017).
122. Johnson-Leger, C., Aurrand-Lions, M. & Imhof, B.A. The parting of the endothelium: miracle, or simply a junctional affair? *J Cell Sci* **113 (Pt 6)**, 921-933 (2000).
123. Salmi, M. & Jalkanen, S. Lymphocyte homing to the gut: attraction, adhesion, and commitment. *Immunol Rev* **206**, 100-113 (2005).
124. Koboziev, I., Karlsson, F. & Grisham, M.B. Gut-associated lymphoid tissue, T cell trafficking, and chronic intestinal inflammation. *Ann N Y Acad Sci* **1207 Suppl 1**, E86-93 (2010).
125. Habtezion, A., Nguyen, L.P., Hadeiba, H. & Butcher, E.C. Leukocyte Trafficking to the Small Intestine and Colon. *Gastroenterology* **150**, 340-354 (2016).
126. Mora, J.R.*, et al.* Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* **314**, 1157-1160 (2006).
127. Griffith, J.W., Sokol, C.L. & Luster, A.D. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu Rev Immunol* **32**, 659-702 (2014).
128. Cheroutre, H. & Kronenberg, M. Mucosal T lymphocytes--peacekeepers and warriors. *Springer Semin Immunopathol* **27**, 147-165 (2005).
129. Pepper, M. & Jenkins, M.K. Origins of CD4(+) effector and central memory T cells. *Nat Immunol* **12**, 467-471 (2011).
130. Fan, X. & Rudensky, A.Y. Hallmarks of Tissue-Resident Lymphocytes. *Cell* **164**, 1198-1211 (2016).
131. Mayassi, T. & Jabri, B. Human intraepithelial lymphocytes. *Mucosal Immunol* **11**, 1281-1289 (2018).
132. Mittrucker, H.W., Visekruna, A. & Huber, M. Heterogeneity in the differentiation and function of CD8(+) T cells. *Arch Immunol Ther Exp (Warsz)* **62**, 449-458 (2014).
133. Raphael, I., Nalawade, S., Eagar, T.N. & Forsthuber, T.G. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine* **74**, 5-17 (2015).
134. Licona-Limon, P., Kim, L.K., Palm, N.W. & Flavell, R.A. TH2, allergy and group 2 innate lymphoid cells. *Nat Immunol* **14**, 536-542 (2013).
135. Tosolini, M.*, et al.* Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res* **71**, 1263-1271 (2011).
136. Brucklacher-Waldert, V., Carr, E.J., Linterman, M.A. & Veldhoen, M. Cellular Plasticity of CD4+ T Cells in the Intestine. *Front Immunol* **5**, 488 (2014).
137. Corthay, A. How do regulatory T cells work? *Scand J Immunol* **70**, 326-336 (2009).
138. Ivanova, E.A. & Orekhov, A.N. T Helper Lymphocyte Subsets and Plasticity in Autoimmunity and Cancer: An Overview. *Biomed Res Int* **2015**, 327470 (2015).
139. Kallies, A. & Good-Jacobson, K.L. Transcription Factor T-bet Orchestrates Lineage Development and Function in the Immune System. *Trends Immunol* **38**, 287-297 (2017).
140. Wang, C., Collins, M. & Kuchroo, V.K. Effector T cell differentiation: are master regulators of effector T cells still the masters? *Curr Opin Immunol* **37**, 6-10 (2015).
141. Annunziato, F., Romagnani, C. & Romagnani, S. The 3 major types of innate and adaptive cell-mediated effector immunity. *J Allergy Clin Immunol* **135**, 626-635 (2015).

142. Schroder, K., Hertzog, P.J., Ravasi, T. & Hume, D.A. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**, 163-189 (2004).
143. Bettelli, E., Oukka, M. & Kuchroo, V.K. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* **8**, 345-350 (2007).
144. Symons, A., Budelsky, A.L. & Towne, J.E. Are Th17 cells in the gut pathogenic or protective? *Mucosal Immunol* **5**, 4-6 (2012).
145. Kimura, A. & Kishimoto, T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol* **40**, 1830-1835 (2010).
146. Xu, L., Kitani, A., Fuss, I. & Strober, W. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol* **178**, 6725-6729 (2007).
147. Piccirillo, C.A. Regulatory T cells in health and disease. *Cytokine* **43**, 395-401 (2008).
148. Venuprasad, K., Kong, Y.C. & Farrar, M.A. Control of Th2-mediated inflammation by regulatory T cells. *Am J Pathol* **177**, 525-531 (2010).
149. Abbas, A.K.*, et al.* Regulatory T cells: recommendations to simplify the nomenclature. *Nat Immunol* **14**, 307-308 (2013).
150. Yadav, M., Stephan, S. & Bluestone, J.A. Peripherally induced tregs - role in immune homeostasis and autoimmunity. *Front Immunol* **4**, 232 (2013).
151. Ohkura, N. & Sakaguchi, S. Regulatory T cells: roles of T cell receptor for their development and function. *Semin Immunopathol* **32**, 95-106 (2010).
152. Hsu, P.*, et al.* IL-10 Potentiates Differentiation of Human Induced Regulatory T Cells via STAT3 and Foxo1. *J Immunol* **195**, 3665-3674 (2015).
153. Curiel, T.J. Regulatory T-cell development: is Foxp3 the decider? *Nat Med* **13**, 250-253 (2007).
154. Barnes, M.J.*, et al.* CTLA-4 promotes Foxp3 induction and regulatory T cell accumulation in the intestinal lamina propria. *Mucosal Immunol* **6**, 324-334 (2013).
155. Voo, K.S.*, et al.* Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci U S A* **106**, 4793-4798 (2009).
156. Levine, A.G.*, et al.* Stability and function of regulatory T cells expressing the transcription factor T-bet. *Nature* **546**, 421-425 (2017).
157. Vocanson, M.*, et al.* Inducible costimulator (ICOS) is a marker for highly suppressive antigen-specific T cells sharing features of TH17/TH1 and regulatory T cells. *J Allergy Clin Immunol* **126**, 280-289, 289 e281-287 (2010).
158. Wang, J., Ioan-Facsinay, A., van der Voort, E.I., Huizinga, T.W. & Toes, R.E. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* **37**, 129-138 (2007).
159. Gottschalk, R.A., Corse, E. & Allison, J.P. Expression of Helios in peripherally induced Foxp3+ regulatory T cells. *J Immunol* **188**, 976-980 (2012).
160. Santegoets, S.J.*, et al.* Monitoring regulatory T cells in clinical samples: consensus on an essential marker set and gating strategy for regulatory T cell analysis by flow cytometry. *Cancer Immunol Immunother* **64**, 1271-1286 (2015).
161. Wing, J.B. & Sakaguchi, S. Multiple treg suppressive modules and their adaptability. *Front Immunol* **3**, 178 (2012).
162. Sakaguchi, S., Miyara, M., Costantino, C.M. & Hafler, D.A. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* **10**, 490-500 (2010).
163. Miyara, M.*, et al.* Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* **30**, 899-911 (2009).
164. Ding, Y., Xu, J. & Bromberg, J.S. Regulatory T cell migration during an immune response. *Trends Immunol* **33**, 174-180 (2012).
165. Raghavan, S. & Quiding-Jarbrink, M. Regulatory T cells in gastrointestinal tumors. *Expert Rev Gastroenterol Hepatol* **5**, 489-501 (2011).
166. Olson, B.M., Sullivan, J.A. & Burlingham, W.J. Interleukin 35: a key mediator of suppression and the propagation of infectious tolerance. *Front Immunol* **4**, 315 (2013).
167. von Boehmer, H. Mechanisms of suppression by suppressor T cells. *Nat Immunol* **6**, 338-344 (2005).
168. Ouyang, W., Rutz, S., Crellin, N.K., Valdez, P.A. & Hymowitz, S.G. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* **29**, 71-109 (2011).

169. Li, M.O., Sanjabi, S. & Flavell, R.A. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* **25**, 455-471 (2006).
170. Huber, S.*, et al.* Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. *Immunity* **34**, 554-565 (2011).
171. Park, H.J.*, et al*. PD-1 upregulated on regulatory T cells during chronic virus infection enhances the suppression of CD8+ T cell immune response via the interaction with PD-L1 expressed on CD8+ T cells. *J Immunol* **194**, 5801-5811 (2015).
172. De Simone, M.*, et al*. Transcriptional Landscape of Human Tissue Lymphocytes Unveils Uniqueness of Tumor-Infiltrating T Regulatory Cells. *Immunity* **45**, 1135-1147 (2016).
173. Fallarino, F.*, et al.* Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* **4**, 1206-1212 (2003).
174. Ernst, P.B., Garrison, J.C. & Thompson, L.F. Much ado about adenosine: adenosine synthesis and function in regulatory T cell biology. *J Immunol* **185**, 1993-1998 (2010).
175. Seder, R.A. & Ahmed, R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat Immunol* **4**, 835-842 (2003).
176. Ekkens, M.J.*, et al.* Th1 and Th2 cells help CD8 T-cell responses. *Infect Immun* **75**, 2291-2296 (2007).
177. Wong, P. & Pamer, E.G. CD8 T cell responses to infectious pathogens. *Annu Rev Immunol* **21**, 29-70 (2003).
178. Zhang, N. & Bevan, M.J. CD8(+) T cells: foot soldiers of the immune system. *Immunity* **35**, 161-168 (2011).
179. Catalfamo, M. & Henkart, P.A. Perforin and the granule exocytosis cytotoxicity pathway. *Curr Opin Immunol* **15**, 522-527 (2003).
180. Lieberman, J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* **3**, 361-370 (2003).
181. Hassin, D., Garber, O.G., Meiraz, A., Schiffenbauer, Y.S. & Berke, G. Cytotoxic T lymphocyte perforin and Fas ligand working in concert even when Fas ligand lytic action is still not detectable. *Immunology* **133**, 190-196 (2011).
182. Ohta, A. & Sitkovsky, M. Extracellular adenosine-mediated modulation of regulatory T cells. *Front Immunol* **5**, 304 (2014).
183. de Oliveira Bravo, M., Carvalho, J.L. & Saldanha-Araujo, F. Adenosine production: a common path for mesenchymal stem-cell and regulatory T-cell-mediated immunosuppression. *Purinergic Signal* **12**, 595-609 (2016).
184. Whiteside, T.L., Mandapathil, M. & Schuler, P. The role of the adenosinergic pathway in immunosuppression mediated by human regulatory T cells (Treg). *Curr Med Chem* **18**, 5217-5223 (2011).
185. Burnstock, G. & Boeynaems, J.M. Purinergic signalling and immune cells. *Purinergic Signal* **10**, 529-564 (2014).
186. Borsellino, G.*, et al*. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* **110**, 1225-1232 (2007).
187. Di Virgilio, F. Purinergic signalling in the immune system. A brief update. *Purinergic Signal* **3**, 1-3 (2007).
188. Kazemi, M.H.*, et al.* Adenosine and adenosine receptors in the immunopathogenesis and treatment of cancer. *J Cell Physiol* **233**, 2032-2057 (2018).
189. Adinolfi, E.*, et al.* The P2X7 receptor: A main player in inflammation. *Biochem Pharmacol* **151**, 234-244 (2018).
190. Rissiek, A.*, et al.* The expression of CD39 on regulatory T cells is genetically driven and further upregulated at sites of inflammation. *J Autoimmun* **58**, 12-20 (2015).
191. Herrath, J., Chemin, K., Albrecht, I., Catrina, A.I. & Malmstrom, V. Surface expression of CD39 identifies an enriched Treg-cell subset in the rheumatic joint, which does not suppress IL-17A secretion. *Eur J Immunol* **44**, 2979-2989 (2014).
192. Jie, H.B.*, et al.* Intratumoral regulatory T cells upregulate immunosuppressive molecules in head and neck cancer patients. *Br J Cancer* **109**, 2629-2635 (2013).
193. Dalla Libera, D.*, et al.* T regulatory cells are markers of disease activity in multiple sclerosis patients. *PLoS One* **6**, e21386 (2011).
194. Fletcher, J.M.*, et al.* CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *J Immunol* **183**, 7602-7610 (2009).

195. Noble, A., Mehta, H., Lovell, A., Papaioannou, E. & Fairbanks, L. IL-12 and IL-4 activate a CD39-dependent intrinsic peripheral tolerance mechanism in CD8(+) T cells. *Eur J Immunol* **46**, 1438-1448 (2016).
196. Thibaudin, M.*, et al.* Human ectonucleotidase-expressing CD25(high) Th17 cells accumulate in breast cancer tumors and exert immunosuppressive functions. *Oncoimmunology* **5**, e1055444 (2016).
197. Gu, J.*, et al.* Human CD39(hi) regulatory T cells present stronger stability and function under inflammatory conditions. *Cell Mol Immunol* **14**, 521-528 (2017).
198. Sundstrom, P.*, et al.* Regulatory T Cells from Colon Cancer Patients Inhibit Effector T-cell Migration through an Adenosine-Dependent Mechanism. *Cancer Immunol Res* **4**, 183-193 (2016).
199. Scurr, M.*, et al.* Highly prevalent colorectal cancer-infiltrating LAP(+) Foxp3(-) T cells exhibit more potent immunosuppressive activity than Foxp3(+) regulatory T cells. *Mucosal Immunol* **7**, 428-439 (2014).
200. Dunne, M.R.*, et al.* Enrichment of Inflammatory IL-17 and TNF-alpha Secreting CD4(+) T Cells within Colorectal Tumors despite the Presence of Elevated CD39(+) T Regulatory Cells and Increased Expression of the Immune Checkpoint Molecule, PD-1. *Front Oncol* **6**, 50 (2016).
201. Mandapathil, M.*, et al.* Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells. *J Biol Chem* **285**, 7176-7186 (2010).
202. Hua, J., Davis, S.P., Hill, J.A. & Yamagata, T. Diverse Gene Expression in Human Regulatory T Cell Subsets Uncovers Connection between Regulatory T Cell Genes and Suppressive Function. *J Immunol* **195**, 3642-3653 (2015).
203. Bastid, J.*, et al.* Inhibition of CD39 enzymatic function at the surface of tumor cells alleviates their immunosuppressive activity. *Cancer Immunol Res* **3**, 254-265 (2015).
204. Burnstock, G. & Di Virgilio, F. Purinergic signalling and cancer. *Purinergic Signal* **9**, 491-540 (2013).
205. Di Virgilio, F. & Adinolfi, E. Extracellular purines, purinergic receptors and tumor growth. *Oncogene* **36**, 293-303 (2017).
206. Hofman, P.*, et al.* Genetic and pharmacological inactivation of the purinergic P2RX7 receptor dampens inflammation but increases tumor incidence in a mouse model of colitis-associated cancer. *Cancer Res* **75**, 835-845 (2015).
207. Qian, F.*, et al.* High expression of P2X7R is an independent postoperative indicator of poor prognosis in colorectal cancer. *Hum Pathol* **64**, 61-68 (2017).
208. Treiner, E.*, et al.* Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* **422**, 164-169 (2003).
209. Dusseaux, M.*, et al.* Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood* **117**, 1250-1259 (2011).
210. Billerbeck, E.*, et al.* Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. *Proc Natl Acad Sci U S A* **107**, 3006-3011 (2010).
211. Tang, X.Z.*, et al.* IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J Immunol* **190**, 3142-3152 (2013).
212. van Wilgenburg, B.*, et al.* MAIT cells are activated during human viral infections. *Nat Commun* **7**, 11653 (2016).
213. Salou, M., Franciszkiewicz, K. & Lantz, O. MAIT cells in infectious diseases. *Curr Opin Immunol* **48**, 7-14 (2017).
214. Treiner, E. & Liblau, R.S. Mucosal-Associated Invariant T Cells in Multiple Sclerosis: The Jury is Still Out. *Front Immunol* **6**, 503 (2015).
215. Gracey, E.*, et al.* IL-7 primes IL-17 in mucosal-associated invariant T (MAIT) cells, which contribute to the Th17-axis in ankylosing spondylitis. *Ann Rheum Dis* **75**, 2124-2132 (2016).
216. Serriari, N.E.*, et al.* Innate mucosal-associated invariant T (MAIT) cells are activated in inflammatory bowel diseases. *Clin Exp Immunol* **176**, 266-274 (2014).
217. Haga, K.*, et al.* MAIT cells are activated and accumulated in the inflamed mucosa of ulcerative colitis. *J Gastroenterol Hepatol* **31**, 965-972 (2016).
218. Porcelli, S., Yockey, C.E., Brenner, M.B. & Balk, S.P. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med* **178**, 1-16 (1993).

219. Tilloy, F., *et al.* An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. *J Exp Med* **189**, 1907-1921 (1999).
220. Kjer-Nielsen, L., *et al.* MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* **491**, 717-723 (2012).
221. Le Bourhis, L., *et al.* MAIT cells detect and efficiently lyse bacterially-infected epithelial cells. *PLoS Pathog* **9**, e1003681 (2013).
222. Keller, A.N., Corbett, A.J., Wubben, J.M., McCluskey, J. & Rossjohn, J. MAIT cells and MR1-antigen recognition. *Curr Opin Immunol* **46**, 66-74 (2017).
223. Lee, C.H., *et al.* C/EBPdelta drives interactions between human MAIT cells and endothelial cells that are important for extravasation. *Elife* **7**(2018).
224. Ussher, J.E., *et al.* CD161++ CD8+ T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner. *Eur J Immunol* **44**, 195-203 (2014).
225. Kurioka, A., *et al.* MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. *Mucosal Immunol* **8**, 429-440 (2015).
226. Leeansyah, E., *et al.* Arming of MAIT Cell Cytolytic Antimicrobial Activity Is Induced by IL-7 and Defective in HIV-1 Infection. *PLoS Pathog* **11**, e1005072 (2015).
227. Sundstrom, P., *et al.* Human Mucosa-Associated Invariant T Cells Accumulate in Colon Adenocarcinomas but Produce Reduced Amounts of IFN-gamma. *J Immunol* **195**, 3472-3481 (2015).
228. Dias, J., *et al.* Factors Influencing Functional Heterogeneity in Human Mucosa-Associated Invariant T Cells. *Front Immunol* **9**, 1602 (2018).
229. Coussens, L.M. & Werb, Z. Inflammation and cancer. *Nature* **420**, 860-867 (2002).
230. Terzic, J., Grivennikov, S., Karin, E. & Karin, M. Inflammation and colon cancer. *Gastroenterology* **138**, 2101-2114 e2105 (2010).
231. Fouad, Y.A. & Aanei, C. Revisiting the hallmarks of cancer. *Am J Cancer Res* **7**, 1016-1036 (2017).
232. Pandolfi, F., Altamura, S., Frosali, S. & Conti, P. Key Role of DAMP in Inflammation, Cancer, and Tissue Repair. *Clin Ther* **38**, 1017-1028 (2016).
233. Ruffell, B., Affara, N.I. & Coussens, L.M. Differential macrophage programming in the tumor microenvironment. *Trends Immunol* **33**, 119-126 (2012).
234. Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J. & Schreiber, R.D. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* **3**, 991-998 (2002).
235. Patidar, A., *et al.* DAMP-TLR-cytokine axis dictates the fate of tumor. *Cytokine* **104**, 114-123 (2018).
236. Scarpa, M., *et al.* Mismatch repair gene defects in sporadic colorectal cancer enhance immune surveillance. *Oncotarget* **6**, 43472-43482 (2015).
237. Fridman, W.H., Pages, F., Sautes-Fridman, C. & Galon, J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* **12**, 298-306 (2012).
238. Galon, J., *et al.* Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol* **232**, 199-209 (2014).
239. Bindea, G., *et al.* Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* **39**, 782-795 (2013).
240. Pages, F., *et al.* International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* **391**, 2128-2139 (2018).
241. Mlecnik, B., *et al.* Integrative Analyses of Colorectal Cancer Show Immunoscore Is a Stronger Predictor of Patient Survival Than Microsatellite Instability. *Immunity* **44**, 698-711 (2016).
242. Le, D.T., *et al.* Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* **357**, 409-413 (2017).
243. Kroemer, G., Galluzzi, L., Zitvogel, L. & Fridman, W.H. Colorectal cancer: the first neoplasia found to be under immunosurveillance and the last one to respond to immunotherapy? *Oncoimmunology* **4**, e1058597 (2015).
244. Salama, P., *et al.* Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* **27**, 186-192 (2009).
245. Frey, D.M., *et al.* High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. *Int J Cancer* **126**, 2635-2643 (2010).

246. Ling, A., Edin, S., Wikberg, M.L., Oberg, A. & Palmqvist, R. The intratumoural subsite and relation of CD8(+) and FOXP3(+) T lymphocytes in colorectal cancer provide important prognostic clues. *Br J Cancer* **110**, 2551-2559 (2014).
247. Zabijak, L.*, et al.* Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients. *Cancer Immunol Immunother* **64**, 1601-1608 (2015).
248. Saito, T.*, et al.* Two FOXP3(+)CD4(+) T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat Med* **22**, 679-684 (2016).