T cells in colon cancer; migration and effector functions

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Cover illustration: Colon cancer with immune cell infiltration; white - nuclei, blue - tumor cells, green - CD8 T cells; other colours - myeloid cells and PD-L1. ©2022 Louis Szeponik

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"Sometimes science is more art than science"

Rick and Morty

ABSTRACT

Immune cells are recognised as one of the main players in the tumor microenvironment and targeted by many new cancer therapies. Regulatory T cells (Treg) can suppress tumor infiltrating lymphocytes which are associated with a better patient outcome. Additionally, unconventional T cells have the potential to kill tumor cells through T cell receptor-independent mechanisms and also in a non- major histocompatibility complex restricted manner. The aim of this thesis was to investigate how Treg can supress the T cell migration into intestinal tumors and what type of T cell populations are affected. Furthermore, we characterized different unconventional T cell populations in human colon cancer samples using mass cytometry. We used $APC^{Min/+}$/DEREG mice to deplete Treg in intestinal tumors. We demonstrated that Treg inhibit the transendothelial migration of T cells into tumors dependent on the interaction of CXCR3 with its ligand CXCL10. Endothelial cells increased expression of CXCL10 when Treg were depleted in tumors. Furthermore, Treg inhibited the expression of endothelial neutral sphingomyelinase 2 (nSMase2) through TGF-β and other unknown soluble factors which resulted in reduced expression of adhesion molecules and chemokines, and decreased tumor infiltration of T cells. CD8αβ T cells were specifically affected by Treg depletion which increased their expression of Th1 related molecules, activation, and proliferation, while CD8αα T cells and γδ T cells were unaffected. In human tumors, exhausted mucosal associated invariant T (MAIT) cells were increased compared to unaffected tissue and none of the MAIT cell populations expressed high levels of activating natural killer cell receptors. In addition, γδ T cell subpopulations showed a great diversity, and some populations were patient specific.

In conclusions, this thesis demonstrates that Treg depletion increases the migration of tumor infiltrating T cells associated with a Th1 response. This is partly mediated by nSMase2 inhibition in endothelial cells. Treg depletion could be a viable option to increase beneficial effector T cells in colorectal cancer patients.

Keywords: Treg, CRC, TIL, nSMase2, Transendothelial migration, Unconventional T cells

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SAMMANFATTNING PÅ SVENSKA

Tjocktarmscancer är en av de vanligaste cancerformerna i världen, särskilt i höginkomstländer och fallen ökar konstant. De kända riskfaktorerna är alla kopplade till en västerländsk stillasittande livsstil, och innefattar alkoholbruk, rökning, fetma, brist på motion, konsumtion av rött kött och brist på kostfiber. T celler, en typ av vita blodkroppar, är viktiga för att eliminera mikroorganismer, men även tumörer. Effektor-T-celler som producerar cytokiner och cytotoxiska T-celler som kan döda andra celler är avgörande i denna process, medan en annan typ, s.k. regulatoriska T-celler (Treg) hjälper till att balansera immunsvaret för att undvika vävnadsskador. Okonventionella T-celler kan också döda tumörceller, men känner igen dem på andra sätt än cytotoxiska T-celler. Kunskapen om okonventionella T-celler och den roll de spelar i försvaret mot tjocktarmscancer är dock fortfarande låg. Tumörer kan utveckla strategier för att undvika immunförsvaret eller till och med använda det till sin fördel. Ett exempel på detta är att Treg ackumulerar i tjocktarmscancer och kan då hindra andra immunceller från att attackera tumören. Treg kan också verka på endotelceller, vilka bygger upp blodkärlen och reglerar vilka celler som kan komma in i tumören från blodet. I denna avhandling har vi undersökt hur Treg i tumören påverkar andra typer av T-celler och genom vilka mekanismer de kan reglera migrationen av celler från blodet in i tumören. Dessutom karakteriserade vi okonventionella T-celler i humana kolontumörer.

Vi använde genetiskt modifierade möss ($APC^{Min/+}$ möss) som får spontana tarmtumörer. Detta orsakas av en mutation i apc-genen, vilken ofta är muterad även i humana tumörer. Vi korsade sedan $APC^{Min/+}$ möss med DEREG-möss, som har en difteritoxinreceptor på ytan av sina Treg, men inga andra celler. Detta gör det möjligt att specifikt eliminera Treg genom injektion av difteritoxin, och vi kunde på detta sätt studera de hämmande effekterna av Treg på effektor- och cytotoxiska T-celler. Dessutom använde vi endotelcellinjer för att studera effekterna av Treg på endotelceller i laboratoriet och färska endotelceller från tumörer. Masscytometri är en metod som kan identifiera 40-50 proteiner på ytan av en cell och den användes för att karakterisera okonventionella T-celler i kolontumörer från patienter direkt efter kirurgi.

Kemokiner är små protener som ger viktiga signaler för migration av celler från blod till vävnader. Vi fann att T-celler är beroende av kemokinreceptorn CXCR3 för att migrera in i tarmtumörer i vår musmodell. Kemokinerna CXCL9 och CXCL10, vilka binder CXCR3, ökade också i tumören efter eliminering av Treg. Effektor-T-celler och cytotoxiska T-celler migrerade mer effektivt in i tumörerna när Treg var borta. Dessutom var tumörendotelceller den enda celltyp som ökade sin produktion av CXCL10 efter Treg eliminering. Detta gjorde oss intresserade av hur Treg kan påverka endotelceller att ändra kemokinproduktionen, och därmed migrationen av T-celler in i tumören. Vi identifierade ett enzym i endotelceller som kallas neutralt sfingomyelinas 2 (nSMase2) som är viktigt för de hämmande effekterna av Treg på T-cells migration. Adhesionsmolekyler är avgörande för att celler ska fästa vid endotelceller och migrera in i vävnaden. Flera av dessa adhesionsmolekyler minskade på ytan av endotelceller när nSMase2 hämmas, och även kemokinen CXCL10 minskade. Treg minskade uttrycket av nSMase2 i endotelceller genom att utsöndra lösliga mediatorer, vilka dock behöver karakteriseras bättre i framtiden. Vi kunde också se att Treg hämmade förmågan hos cytotoxiska T-celler att utsöndra cytokiner och cytotoxiska molekyler, som är viktiga för att attrahera andra fördelaktiga T-celler eller döda tumörceller. Däremot påverkade inte Treg-minskningen antalet eller aktiviteten hos de okonventionella T-cellerna i tumörerna, vilket också skulle kunna förklara varför vi inte hittade någon minskning av tumörvolymen hos våra möss. Vi dokumenterade också olika okonventionella T-cells populationer i humana kolontumörer. Vissa av dessa populationer hade minskad funktion i tumörer och andra visade en stor mångfald med specifik anrikning hos vissa patienter.

Sammanfattningsvis visar denna avhandling att om Treg elimineras från tumörer ökar migrationen av effektor-T-celler till tumörerna genom kemokinen CXCL10 och dess receptor CXCR3. Detta förmedlas delvis av att ensymet nSMase2 hämmas i endotelceller. Därför kan Treg eliminering vara ett genomförbart alternativ för att öka mängden fördelaktiga effektor- och cytotoxiska T-celler i tumören hos patienter med tjocktarmscancer.

LIST OF PAPERS

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

I. Akeus P, Szeponik L, Ahlmanner F, Sundstrom P, Alsen S, Gustavsson B, Sparwasser T, Raghavan S, Quiding-Järbrink M (2018) **Regulatory T cells control endothelial chemokine production and migration of T cells into intestinal tumors of APC$^{Min/+}$ mice.** Cancer Immunology, Immunotherapy 67 (7):1067-1077. doi:10.1007/s00262-018-2161-9

II. Akeus P, Szeponik L, Langenes V, Karlsson V, Sundström P, Bexe-Lindskog E, Tallon C, Slusher BS, Quiding-Järbrink M (2021) **Regulatory T cells reduce endothelial neutral sphingomyelinase 2 to prevent T-cell migration into tumors.** European Journal of Immunology 51 (9):2317-2329. doi:10.1002/eji.202149208

III. Szeponik L, Akeus P, Rodin W, Raghavan S, Quiding-Järbrink M (2020) **Regulatory T cells specifically suppress conventional CD8αβ T cells in intestinal tumors of APC$^{Min/+}$ mice.** Cancer Immunology, Immunotherapy 69 (7):1279-1292. doi:10.1007/s00262-020-02540-9

IV. Szeponik L, Rodin W, Sundström P, Raghavan S, Lindskog E, Wettergren Y, Cosma A, Quiding-Järbrink M. **Unconventional T cells in colon cancer – phenotypic characterization.** Manuscript

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CONTENT

ABBREVIATIONS

AMP	Antimicrobial peptides
APC	Adenomatous polyposis coli
APCs	Antigen presenting cells
APD	Avalanche photodiode
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CIMP	CpG island methylator type
CIN	Chromosomal instability
CLA	Cutaneous lymphocyte antigen
CRC	Colorectal cancer
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DCs	Dendritic cells
DEREG	DEpletion of REGulatory T cells
dNKT cells	Diverse NKT cells
DPTIP	2,6-Dimethoxy-4-(5-Phenyl-4-Thiophen-2-yl-1H-Imidazol-2-yl)-Phenol
DT	Diphtheria toxin
EDTA	Ethylenediaminetetraacetic acid
FAP	Familial adenomatous polyposis
FFPE	Formalin-fixed paraffin-embedded
Foxp3	Forkhead box P3
GALT	Gut-associated lymphoid tissues
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GrzB	Granyzme B
HEV	High endothelial venules
HNPCC	Hereditary nonpolyposis colorectal cancer
i.p.	Intraperitoneal
ICAM-1	Intercellular adhesion molecule 1
ICOS	Inducible T-cell co-stimulator
IDO	Indoleamine-2,3-dioxygenase
IEL	Intraepithelial lymphocytes

IFN-γ	Interferon gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IL2-R	High-affinity IL2 receptor
iNKT cell	Invariant NKT cell
LAG-3	Lymphocyte-activation gene 3
LFA-1	Leukocyte function-associated antigen type 1
LP	Lamina propria
LT-α	Lymphotoxin-alpha
M cells	Microfold cells
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MAIT cells	Mucosal associated invariant T cells
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MR1	MHC class I-related
mRNA	Messenger RNA
MSI	Microsatellite instability
MSS	Microsatellite stability
Mφ	Macrophage
NK cells	Natural killer cells
NKT cells	Natural killer T cells
nSMase2	Neutral sphingomyelinase 2
PD-1	Programmed cell death protein 1
PDDC	Phenyl (*R*)-(1-(3-(3,4-dimethoxyphenyl)-2,6-dimethylimidazo[1,2-*b*]pyridazin-8-yl)pyrrolidin-3-yl)carbamate
PD-L1	Programmed death-ligand 1
PMA	Phorbol-12-myristate 13-acetate
PMT	Photon multiplier tube
PNAd	Peripheral lymph node addressin
PP	Peyer's patches
PSGL-1	P-selectin glycoprotein ligand 1
pTreg	Peripheral Treg

RNAseq	RNA sequencing
S1P	Sphingosine-1-phosphate
TAM	Tumor-associated macrophages
TCR	T cell receptor
T_{EFF} cells	Effector T cells
TGF-β	Transforming growth factor beta
Th cells	T helper cells
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptors
TNF	Tumor necrosis factor
Treg	Regulatory T cells
tTreg	Thymic-derived Treg
VCAM-1	Vascular cell adhesion molecule 1

INTRODUCTION

Gastrointestinal tract and intestinal barrier function

The mucosal surfaces are the parts of the body most vulnerable to infections, and pathogens commonly enter the body through these sites. The main mucosal tissues are vital for the exchange of gas, digestion of food, and reproduction. The immune system has an intricate role in protecting these sites from infections. At the same time, many unharmful substances and the commensal bacteria are in contact with the mucosal layers and should not cause any adverse immune reactions. This requires a delegate balance between immune response and tolerance [1].

The gastrointestinal tract consists of the oral cavity, oesophagus, stomach, small intestine, large intestine, and anus. The small intestine's main function is to digest and absorb nutrients while maintaining a barrier to the sterile inside of the body. The first cellular barrier is a monolayer of mainly epithelial cells (absorptive enterocytes) but also Paneth cells, goblet cells and neuroendocrine cells [2]. These cells form a finger-like projection (villi) that extends into the lumen of the small intestine [3]. The lining cells derive from a common stem cell precursor that is originating in the crypts of Lieberkühn [4]. The epithelial cells are constantly pushed outwards to the tip of the villi and into the lumen, so the epithelial lining is renewed every 4-5 days [5]. Paneth cells sit in the bottom of the crypts and produce antimicrobial molecules which are released into the mucus layer [6]. Goblet cells are secreting mucins to form this protective layer (also called glycocalyx) which forms above the epithelial lining [7]. Furthermore, the so-called intraepithelial lymphocytes (IEL) reside between the epithelial cells and play an important role in maintaining the epithelial barrier integrity but can also be involved in inflammation [8]. The lamina propria (LP) is a connective tissue that lies below the epithelial lining and contains a large number of immune cells of the innate and adaptive branch. Innate immune cells include dendritic cells (DCs), macrophages (Mφs), eosinophils, mast cells, natural killer (NK) cells and unconventional T cells while conventional B and T cells derive form the adaptive immunity. The lamina muscularis mucosae (a thin layer of muscle) separates the mucosa

(epithelium, lamina propria and muscularis mucosae) form the submucosa [3].

The mucosa of the large intestine does not have villi but a flatter epithelial surface structure with straight tubular glands that harbour the crypts [9]. The main function is to absorb water and maintain commensal bacteria that are essential for health [2]. Commensal bacteria are symbionts that help humans to synthesise essential vitamins and digest complex polysaccharides [10]. They are more numerous than cells in a human body. The role of commensal bacteria is being increasingly recognized and the effects on human health are just emerging [11]. Even psychological health may be influenced by the microbiome through the gut-brain axis [12]. Mucin secreting goblet cells are much more abundant in the large intestine which leads to a thicker mucus layer with an outer and inner component. The outer layer is looser and functions as a physical barrier to keep the commensal bacteria away from the mucosa [13]. The glycocalyx is also a matrix for protective molecules like secreted Immunoglobulin (Ig)A antibodies and antimicrobial peptides (AMP) [14]. The number of Paneth cells and IEL in the large intestine are few in comparison to the small intestine.

The immune system of the gut is a complex environment (Figure 1) with different cells residing in the epithelial layer, lamina propria and the highly organized lymphoid structures called gut-associated lymphoid tissues (GALT). Although there seems to be some confusion, Peyer's patches (PP), the appendix, and isolated lymphoid follicles are considered the main GALT in the intestine whereas mesenteric lymph nodes (MLN) are not considered as GALT but rather a separate entity with afferent lymph connection to the gut [15]. GALT have similar structures to lymph nodes with B and T cell zones, antigen presenting cells (APCs) like DCs and Mφs , but they lack afferent lymphatics [16]. Instead, they use specialized epithelial cells called microfold cells (M cells) that actively sample the lumen and transport exogenous antigen to the subepithelial side for presentation on APCs and B cells [17]. APCs from the LP and GALT can either induce an immune response or oral tolerance which is dependent on the type of APCs and additional signals from the tissue environment [18].

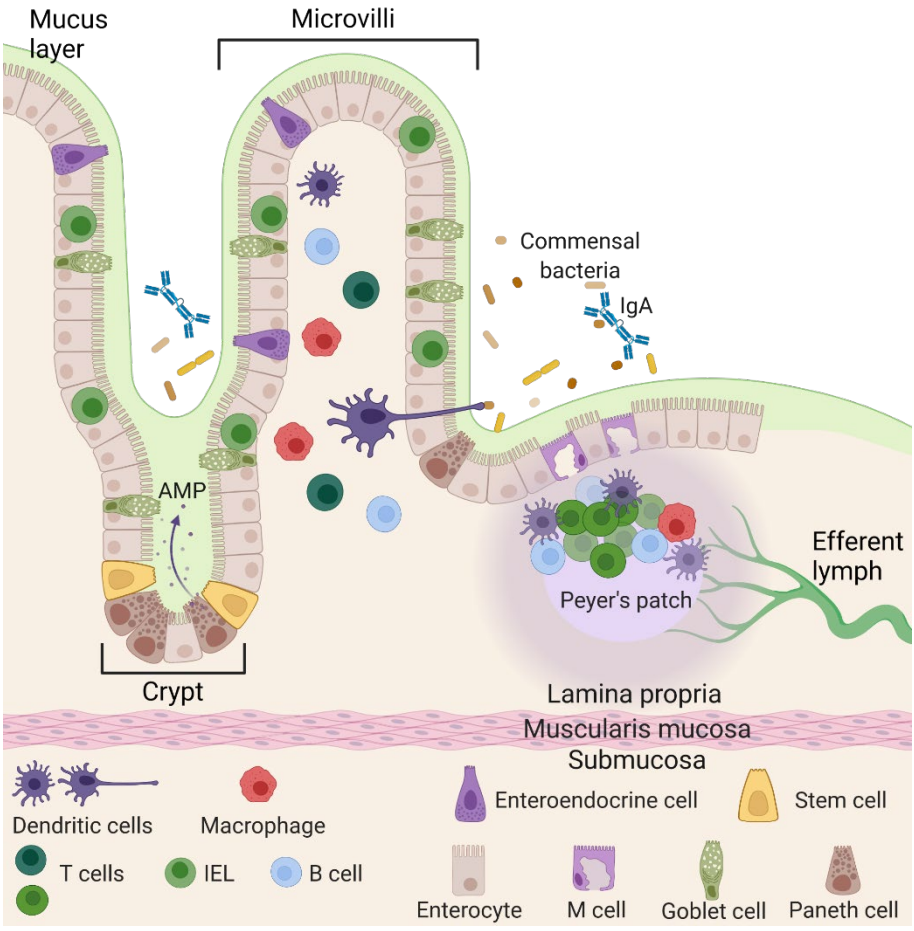

Figure 1: Components of the intestinal barrier in the small and large intestine. Different immune cells reside in the lamina propria and intraepithelial layer (granulocytes not shown here). Paneth cells secrete AMP, goblets cells secrete mucus that creates a protective layer, and B cells secrete IgA antibodies that are transported to the lumen. All these components contribute to the protection of the host and simultaneously allow commensal bacteria to reside in the intestine and nutrients to be absorbed. Adapted from "Intestinal Immune System (Small Intestine)" by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates. [2]

Furthermore, APCs can travel to MLN through the afferent lymph vessels where they can prime T and B cells [19]. After priming, T and B cells are released through efferent lymph into the circulation and home back to the

gut tissues by the expression of specific gut homing receptors [20, 21]. These cells are then contributing to the pool of effector T (T_{EFF}) cells and antibody secreting B cells residing in the intestinal tissues.

Intestinal T cells

T cells are abundant in lymphoid tissue and the circulation but they also reside in barrier tissues like the intestine in large numbers [22]. They are constantly required to discriminate between friend and foe by being in contact with commensal bacteria, food, and pathogens. The IEL compartment harbours innate like γδ and CD8αα T cells but also adaptively induced T cells. In contrast, the LP compartment contains many conventional T cells and more specialised subsets like Regulatory T cells (Treg), Mucosal associated invariant T (MAIT) cells and natural killer T (NKT) cells that are not present in the IEL compartment. IEL and T cells from the LP are mainly antigen experienced effector memory T cells that are retained in the tissue and do usually not recirculate [23].

Conventional T cells from GALT and MLN exhibit a naïve phenotype and enter the specific sites from circulation through high endothelial venules (HEV)[24]. They are generated in the thymus and can recognize a broad range of antigens in peripheral tissue through their αβ T cell receptor (TCR) [25]. They re-circulate through the body via the blood and lymph system to be able to encounter their cognate antigen presented on the major histocompatibility complex (MHC) by APCs in lymphoid tissue [26]. Once naïve T cells are stimulated by TCR engagement, the binding of the co-stimulatory molecule CD28 to CD80/CD86 on APCs is required for proper activation [27]. The lineage fate of the T cell is then defined by cytokines secreted by APCs and other innate immune cells [28]. Antigens presented on the MHC class II molecules can activate $CD4^+$ T cells that can then develop into T helper (Th) cells with different functional properties whereas cytotoxic $CD8^+$ T cells are recognizing antigens presented on MHC class I [29]. Th1, Th2, Th9, Th17, Th22, Treg and T follicular helper cells are the Th subsets which are generally recognised [30]. All subsets are defined by their profile of cytokine secretion and the

expression of certain transcription factors (Figure 2)[30]. It is now well-known that these Th lineages are a simple model which does not represent the full picture of the complex immune environment. There is a great plasticity between certain subsets which seems to be important for reoccurring immune challenges and homeostasis [31]. Furthermore, innate-like T cells can have the same properties as e.g. Th1 cells and are therefore referred as cells exerting a Th1 response. We will later focus on the canonical subsets (Th1, Th2, Th17) and Treg as they play a major role in tumor immunity. In contrast to the immune environment shaping properties of Th cells, cytotoxic $CD8^+$ cells are directly killing infected cells through release of cytotoxic molecules and Fas-FasL interaction [32, 33].

Unconventional T cells like MAIT cells, γδ T cells and NKT cells commonly develop in the thymus and directly become T_{EFF} cells without circulating as naïve T cells [34]. They can recognize antigens like lipids, metabolites and modified peptides which are presented on non-MHC molecules [35]. They can also bind non-MHC molecules without loaded antigen which can be upregulated in stressed cells and act as a danger signal. Additionally, IEL represent a tissue specific population of both conventional and unconventional T cell that is further discussed below.

Intraepithelial lymphocytes

IEL are the largest T cell population in the intestine with an estimated 1 cell per 10-20 intestinal epithelial cells in the small intestine but lower numbers in the large intestine [36, 37]. IEL can be divided into two groups of natural IEL and induced IEL [38]. Natural or also innate like IEL originate in the thymus like other T cells but they additionally undergo a self-antigen-based maturation process at the same site [39]. The precursor of IEL evade the negative selection process but go through agonist selection, where clonal deviation instead of clonal deletion is induced by TCR signalling [40]. This results in mature αβ T cells or γδ T cells that are functional differentiated, lack expression of both CD4 and CD8αβ, and directly home to the intestinal epithelium [41, 42]. In mice, an additional natural αβ T cell subset exists which expresses the CD8αα homodimer and

shows a similar developmental process [43]. A recent study in human tissue also suggested a subset of TCRαβ$^+$CD8αβ$^+$ cells that co-express the CD8αα homodimer and have therefore escaped detection, but further studies are required [44]. Conversely, one study in mice shows that γδ IEL can mature extrathymically by the activation through butyrophilin heteromers of the cognate receptor [45].

In contrast to natural IEL, induced IEL are originating from conventional CD4$^+$ or CD8αβ$^+$ TCRαβ$^+$ T cells that are activated in GALT or MLN by antigen exposure and co-stimulation through APCs. They as well migrate to the gut where they reside as T_{EM} [38]. Induced IEL are more common in the human intestine where they make up about 80-90 % of the IEL compartment whereas in mice IEL are mainly from the innate like type with up to 80% [46].

It is still difficult to assign specific functions to the different IEL subsets as they share many properties and functional studies are rare and mainly available for mice. Additionally, many studies do not separate IEL and T cells from the LP in their analysis and treat them as one entity. A recent study by Brenes et al. also shows that natural and induced IEL have redundant functions and are rather imprinted by tissue location than their ontogeny [47]. The cytokines interleukin (IL)-15 and IL-18 that are secreted by intestinal epithelial cells or APCs from the LP are essential for maintenance and maturation of IEL as well as maintaining the barrier function [48, 49]. IELs can exert potent cytolytic and Th1 type responses [38]. It has been shown that they are important in responding to intestinal infections and prevent pathogen dissemination [50, 51]. In mice, all natural IEL (except CD8αβ$^+$TCRαβ$^+$) express activating NK cell receptors that can mediate cell killing without previous TCR activation [52]. Little is known about the ligands of IEL but butyrophilins seem to be one type of self-antigen that is expressed by enterocytes and important for the barrier integrity [45, 53]. γδ T cells can recognize lipid antigens through the MHC-like I molecule CD1d but this is not specific for IEL although most γδ T cells from the intestine can be found in the epithelial layer.

Few studies have separated the IEL compartment from the LP/tumor stroma from unaffected and tumor tissue. We isolated the IEL

compartment in paper III to investigate the different T cell populations (supplementary data) but analysing the cytokine secretion by stimulating with PMA/ionomycin was unfortunately not feasible due to low cell numbers. We also started out investigating the different T cell populations among IEL with mass cytometry in paper IV but we could not reach a sample purity where we could acquire samples with sufficient data quality.

T helper cells

Th1 cells are defined by their expression of the transcription factor Tbet and their secretion of the proinflammatory cytokine interferon gamma (IFN-γ) but they can also produce other cytokines like tumor necrosis factor (TNF), IL-2, lymphotoxin-alpha (LT-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [30]. Tbet is considered the master regulator for Th1 development and is controlled by TCR engagement and the presence of IFN-γ which induces the activation of the transcription factor STAT1 [54]. Furthermore, IL-12 secreted by activated Mφs and DCs drives the activation of the transcription factor STAT4 which is necessary to enhance IFN-γ production [55]. IFN-γ secreted by Th1 cells acts in an autocrine fashion to enhance the Th1 development further [56]. Th1 cells are important for the immune response against intracellular pathogens (intracellular bacteria, viruses, and protozoans). IFN-γ increases the capability of Mφs to phagocytose and kill intracellular pathogens by increasing microbicidal activity [57]. Furthermore, IFN-γ promotes IgG class switching [58], expression of toll-like receptors (TLR) on innate immune cells [59], and increased processing of antigens presented on MHC-I and MHC-II [60]. Other Th1 cytokines like TNF and LT-α act on the tissue endothelium to increase migration of Mφs, and GM-CSF further activates bone marrow–derived Mφ differentiation [61, 62].

Th2 cells are characterized by their expression of the transcription factor GATA3 and secretion of the cytokines IL-4, IL-5, IL-9 and IL-13 [63, 64]. Activation of the IL-2 receptor by IL-4 is required for Th2 development which activates the transcription factor STAT6 and subsequently the master regulator GATA3. Mast cells, basophils, eosinophils and NKT cells

do all have the ability to secret IL-4 and induce the Th2 commitment. The differentiation is further stabilized by the autocrine feedback loop of IL-4 acting on Th2 cells, similar to IFN-γ in Th1 cells [65]. Th2 are establishing immunity against extracellular parasites like helminth which can be recognised by their structural polysaccharide chitin [66]. IL-4 promotes IgE production that binds to the Fcε receptor on granulocytes like basophils and mast cells. These activated cells secrete several mediators that lead to vascular permeability and increased recruitment of inflammatory cells [64]. Eosinophil recruitment to the tissue is induced by IL-5 secretion whereas mast cell recruitment is induced by IL-9 [67]. This leads to tissue eosinophilia and mast cell hyperplasia which contributes to helminth killing [68]. Mucus production by goblet cells is promoted through IL-13 and is a common driver for the symptoms of asthma [69]. Th2 responses that are mistargeted to non-infectious environmental stimuli lead to an allergic response and can have detrimental effects on health [67].

Th17 cells express the transcription factor RORγt and are generally known for their secretion of the proinflammatory cytokine IL-17 [70]. The differentiation of Th17 cells is induced by IL-1β, IL-6 and transforming growth factor beta (TGF-β) [71, 72] while IL-23 is required for the maturation and expansion of these cells [73]. IL-6 induces expression of the transcription factor STAT3 which is essential for Th17 commitment [74]. Th17 cells mainly express the cytokines IL-17A, IL-17F, IL-22, and IL-21 [75] but expression of TNF and IL-6 have also been reported though not conclusively [76]. Th17 cells are playing a crucial role in the response to infections of extracellular bacteria or fungi. The secretion of IL17A and IL-17F increases granulopoiesis, the recruitment of neutrophils to tissues through chemokine induction and their survival [77]. Furthermore, IL-17 stimulates the secretion of the proinflammatory cytokines TNF, GM-CSF and IL-1ß by Mφs and contributes to sustain the inflammatory repsonse [78]. IL-17 and IL-22 enhance expression of AMP at mucosa sites [79] , and IL-22 alone is important for maintaining the epithelial barrier function [80]. Additionally, Th17 cells help B cells by inducing proliferation and class switching to opsonizing IgG2 and IgG3 antibodies that enhance phagocytosis of extracellular pathogens [81]. On the other hand, Th17 cells

are one of the major contributors to autoimmunity and chronic inflammation [82].

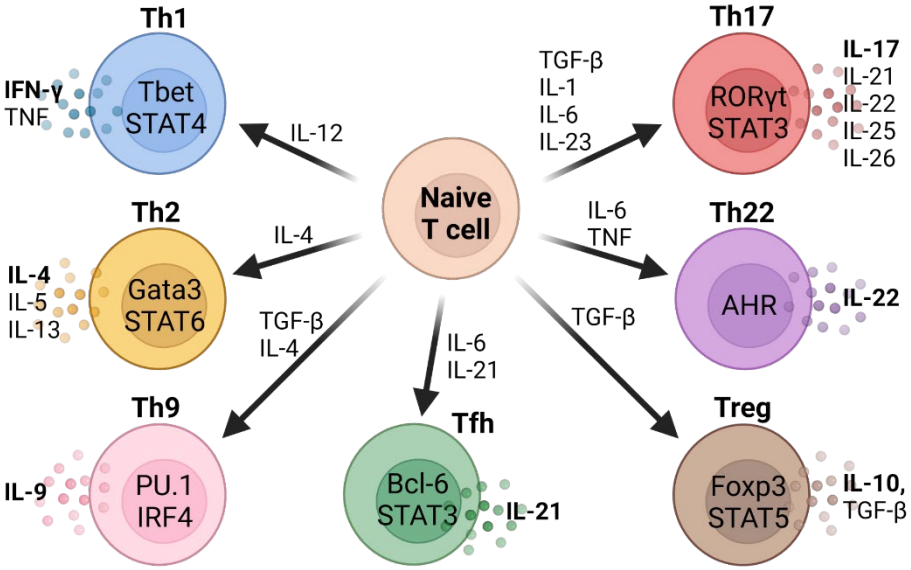

Figure 2: The currently recognized Th subsets and the T follicular helper T cell (Tfh) subset. Cytokines that are important for the development of a lineage are shown next to the arrow and transcription factors within the cell. Cytokines which are released by a specific subset are shown next to the cell with the signature cytokine highlighted in bold. Created with BioRender.com. [30, 83]

The fate of the three lineages of Th1, Th2 and Th17 cells have long been considered irreversible but recent research has shown a lot of plasticity between certain subsets [84]. Especially Th17 cells seem to be able to change their phenotype whereas the Th1 and Th2 cells are rather stable. The phenotypes are stabilized by the repression of transcription factors of the opposite lineages and reinforcement of the linage commitment through autocrine cytokine signalling [85]. Th17 cells have been described to secrete IFN-γ in inflammatory bowel disease or IL-4 in allergy which are the signature cytokines for Th1 and Th2, respectively, but further research is

necessary to strengthen these findings [86, 87]. Treg and γδ T cells which can also acquire a Th17 like phenotype are discussed separately.

Cytotoxic T lymphocytes

Cytotoxic T lymphocytes (CTL) are an essential part of the cell-mediated adaptive immunity that generally protects against intracellular pathogens which reside inside the cytosol of infected cells (mainly viruses) [88]. Naïve $CD8^+$ T cells are initially activated in lymphoid tissues where they encounter their cognate antigen which starts a developmental programme to become CTL [89]. In contrast to $CD4^+$ Th cells which interact with MHC-II, $CD8^+$ T cells/CTL recognise peptides presented by APCs on MHC-I [90]. These peptides originate from the cytosol and are transported into the endoplasmic reticulum to be loaded onto the MHC-I complex [91]. Additionally, DCs can present antigens from extracellular sources like endocytosed material or exosomes which is important for certain viruses that do not effectively infect DCs, or tumor cells of non-hematopoietic origin [92]. This cross-presentation of self-antigens by DCs does also play an important role in the maintenance of peripheral tolerance. [93]. Once activated, CTL migrate to the tissues and recognise the cognate peptide presented by MHC-I on the surface of infected cells. The most effective way of killing is through release of granules which accumulate at the immunological synapse between the effector and target cell [94]. Three cytotoxic proteins act in concert to induce apoptosis of the target cell. First, perforin creates pore-like structures in the cell membrane which enables granzymes and granulysin to enter the cell. Second, granzymes act on pro-caspases and trigger mitochondrial permeabilization to induce the controlled cell death while granulysin (not present in mice) has antimicrobial properties and can kill intracellular bacteria but is only apoptotic at high concentrations which might not be physiological [95-97]. Another less important pathway is the binding of Fas ligand on the surface of effector cells with Fas on the target cell [98]. Furthermore, CTL secrete IFN-γ, TNF and LT-α that shape the immune environment but can also have direct effects on target cells [99]. IFN-γ increases MHC-I expression and peptide processing, inhibits viral replication, and activates Mφs [33,

100]. TNF can bind to the tumor necrosis factor receptor 1 on target cells and lead to apoptosis through caspase activation [101]. Cytotoxic T cells have great implications in anti-tumor immunity due to their effective means of directly killing target cells.

Regulatory T cells

Treg are critical in maintaining peripheral tolerance, preventing autoimmune diseases, and limiting chronic inflammatory processes [102, 103]. Though, they can also hamper the clearance of pathogens or the anti-tumor response by supressing effector cells [104, 105]. They exert their functions both in inflammatory sites and lymph nodes by suppressing T_{EFF} cells and NK cells and inducing tolerogenic properties of APCs [106-108]. Two main subsets of Treg with a distinct TCR repertoire have been identified: thymic-derived Treg (tTreg) and peripheral Treg (pTreg). tTreg derive from the thymus, are largely reactive to self-antigen, and predominant in blood and lymph nodes [108, 109] whereas pTreg are induced from Th naïve or effector cells in the peripheral tissues and recognize exogenous antigens [110]. Both subsets are nonredundant and required for regulatory responses and homeostasis [111].

In immunofluorescence experiments, Treg are commonly identified by surface expression of $CD4^{+}CD25^{hi}CD127^{low}$ and intracellular detection of forkhead box P3 (Foxp3) [112]. It has become clear that especially in humans, Treg populations are very heterogenous and the conventional Treg markers are not sufficient [113]. The transcription factor Helios and the surface molecule Neuropilin-1 have been suggested to define a thymic origin of Treg [114, 115] but the findings have been controversial and not conclusive [116, 117]. Furthermore, in a study by Miyara et al. human suppressive Treg were divided into resting Treg ($CD45RA^{+}Foxp3^{low}$) and activated Treg (aTreg, $CD45RA^{-}Foxp3^{high}$) with the first being able to proliferate and differentiate into aTreg cells after TCR activation and the latter being anergic and prone to apoptosis [118]. The transcription factor Foxp3 is considered the master regulator of Treg development and required for their maintenance and function [119]. However, there seem to

be a lot of heterogeneity as Foxp3 expression is mainly stable in tTreg due to demethylation of promotor regions, while pTreg show a more transient expression [120]. There is also evidence that tTreg can loose Foxp3 expression and become exTreg ($CD4^+$ $CD25^{low}$ $Foxp3^{low}$) with Th phenotype [121]. Specifically, the plasticity between Treg and Th17 cells has been observed in multiple studies and seems to have important implications in diseases [122-124]. Additionally, Treg have been shown to upregulate the Th1 specific transcription factor Tbet co-expressed with Foxp3 and therefore supress the Th1 response *in vivo* [125]. In humans, Foxp3 has been detected in activated T cells without suppressive activity suggesting that Foxp3 is not enough to induce regulatory functions [126, 127]. It is still unclear how this discrepancy between mice and humans translates into functional differences *in vivo*. Treg express other cellular markers that are important for their function like OX-40, inducible T-cell co-stimulator (ICOS), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) , Programmed cell death protein 1 (PD-1), glucocorticoid-induced TNFR-related protein, CD39 or CD73 (mainly in mice) but none of these is reliably stable and changes with inflammatory conditions and disease states [112, 128].

The development and maintenance of tTreg and pTreg are dependent on the cytokine IL-2 which binds to the high-affinity IL2 receptor (IL2-R) and is indispensable [108, 129]. CD25 constitutes the alpha subunit of the IL2-R and is highly expressed on Treg as mentioned before [130]. tTreg require mainly TCR stimulation and IL-2 for their development whereas pTreg seem to develop in the presence of TGF-β and retinoic acid [131]. The latter can be produced by DCs after uptake of vitamin A and leads to priming of Treg that are gut tropic which suggests that pTreg are mainly derived from GALT [132]. Retinoic acid and IL-2 inhibit the Th17 polarization of Treg and induce Foxp3 expression [133, 134].

There are four basic mechanisms by which Treg control immune responses (Figure 3)[135]. Treg secrete the inhibitory cytokines IL-10, IL-35 and TGF-β that act on other immune cells, but their effect differs according to the pathogenic or homeostatic setting and can be both beneficial and detrimental for the host [136].

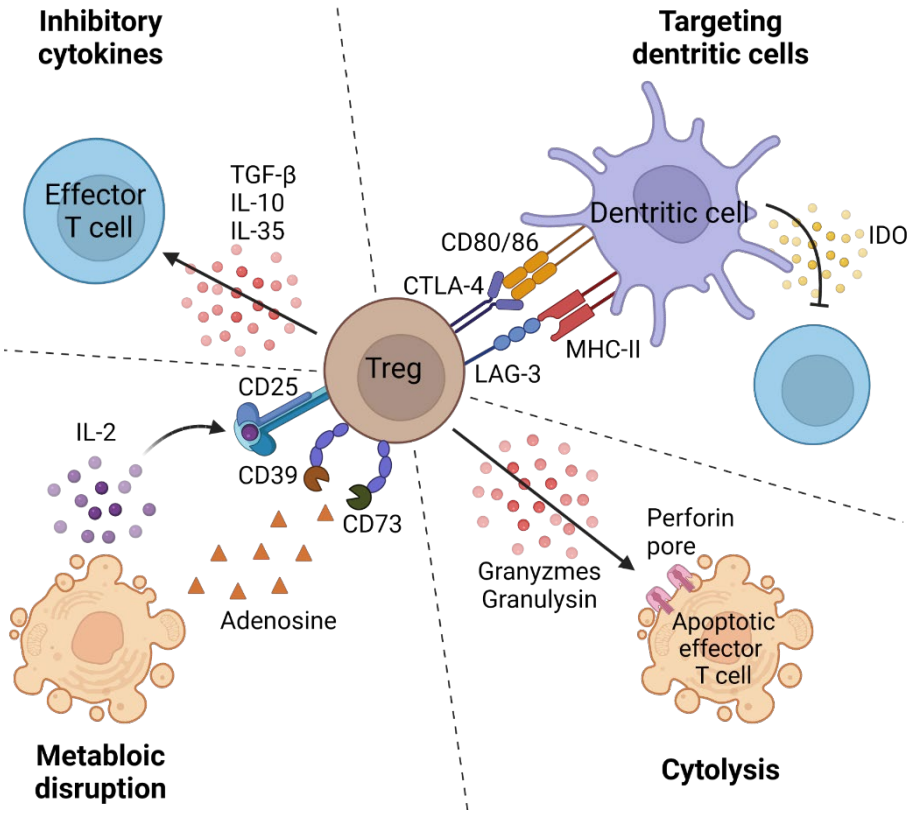

Figure 3: Four known mechanisms that Treg can use to suppress an immune response: Secrete inhibitory cytokines, cytolysis of immune cells through cytotoxic molecules, metabolic disruption by scavenging IL-2 or producing adenosine, and inhibiting dendritic cell maturation and function. Created with BioRender.com. [135]

The second way Treg can supress immune responses is the direct cytolytic activity towards other immune cells like NK cells or CTL [137-139]. Some Treg are capable of secreting cytotoxic molecules like granzyme B in the same way as CTL and thereby kill other immune cells to regulate the immune response [135]. Metabolic disruption is the third way of suppression by which Treg use surface molecules to modify their direct environment. The high expression of the IL-2R can act as a scavenger on

Treg and deplete IL-2 for other T cells which can lead to suppression of proliferation and apoptosis [140]. Treg also express the ectoenzymes CD39 and CD73 which can generate pericellular adenosine that binds to the adenosine receptor 2A on T cells and inhibits their function [141]. Lastly, Treg can act on the activating functions of DCs through CTLA-4 that binds to CD80 and/or CD86 on DCs and is the inhibitory counterpart of CD28 [142]. This can lead to the expression of indoleamine-2,3-dioxygenase (IDO) that generates pro-apoptotic metabolites and results in the suppression of T_{EFF} cells [143]. Furthermore, Treg express the CD4-homologue LAG-3 that can bind to MHCII molecules and transmit an inhibitory signal to DCs [144]. A recent study by Tekguc et al. has also shown that Treg can deplete CD80/CD86 from the surface of DCs by trogocytosis and in turn the expression of the inhibitory ligand programmed death-ligand 1 (PD-L1) increases [145].

Mucosal associated invariant T cells

MAIT cells belong to the group of unconventional T cells with innate-like properties. They have a specific semi-invariant TCR receptor that recognises metabolites from the riboflavin synthesis (vitamin B2) bound to the MHC class I-related (MR1) molecule [146]. The development of MAIT cells and their tissue homing properties are already established in the thymus and require MR1 expression on a nonhematopoietic cell [147]. After leaving the thymus, peripheral B cells and the mucosal commensal flora control the expansion and development into effector/memory T cells that reside in tissues [148]. MAIT cells are the most abundant T cell population with the same TCR specificity and make up to 45% of liver lymphocytes, up to 10 % of blood T cells and 0.13 to 16.1% of colonic T cells [149, 150]. MAIT cells can be identified by their expression of $CD161^{hi}$, TCR $V\alpha7.2^{+}$ and binding to the MR-1 tetramer loaded with 5-OP-RU, a derivate of the microbial riboflavin precursor 5-A-RU [151]. The majority of MAIT cells express the co-receptor CD8αα (a small fraction expresses CD8αβ) whereas $CD4^{+}$ and DN cells are rare but have been reported in different tissues [152, 153]. The TCR repertoire of MAIT cells is very limited and the Vα7.2 chain most often pairs with the Jα33 chain,

although coupling with Jα12 or Jα20 has also been observed. The Vβ chain diversity is also significantly less than in unconventional T cells [154]. MR-1 dependent activation of MAIT cells can be triggered by a large variety of bacteria and some fungi [155, 156]. Additionally, MAIT cells can be activated in an TCR-independent manner by the cytokines IL-7, IL-12, IL-15, IL-18 and type I interferons [157-159]. MAIT cells are one of the first cells to rapidly respond to pathogens with secretion of IFN-γ, TNF and IL-17 which contributes to the inflammatory immune environment and development of an adaptive immunity [149, 160, 161]. Furthermore, they have the ability to directly kill target cells through cell-mediated killing by releasing cytotoxic effector molecules as described for CTL before [162, 163]. Therefore, MAIT cells may play an important role in different types of infections in multiple tissues as well as in the maintenance and integrity of the mucosal barrier [164-166]. The effects of MAIT cells in tumor tissues and their capability of killing target cells through NK cell receptors is still under investigation.

Natural killer T cells

NKT cells are another subset of innate-like T cells that is thought to bridge the gap between innate and adaptive immunity [167]. NKT cells can be generally divided into two types: invariant NKT (iNKT) cells which recognize the lipid antigen α-galactosylceramide (α-GalCer), and diverse NKT (dNKT) cells which share a more diverse TCR repertoire and can recognize other lipid antigens [168, 169]. NKT cells have a semi-invariant αβ TCR (Vα24-Jα18 and a variable β chain) that recognizes lipid antigens that are presented on the MHC class-I like molecule CD1d [170]. iNKT cells are abundant in mice whereas dNKT cells seem to be more specific to humans [34]. NKT cells are expanding and acquiring effector functions before they exit the thymus [171]. Once activated, NKT cells can produce a variety of Th1, Th2 and Th17 related cytokines like IFN-γ, IL-4 and IL-17 [172-174] that support immune responses before adaptive cells have developed. They have also been reported to respond to innate activating signals through TLR [175]. Furthermore, stimulation through the NK cell receptor NKG2D induces NK cell like cytolytic activity [176]. In general,

iNKT cells play a protective role in the defence against bacteria, viruses, fungi and parasites [177]. Most studies on NKT cells are focused on mice or human blood. NKT cells are rather rare in human tissues and therefore challenging to study.

γδ T cells

γδ T cells are a conserved subset of T cells that undergoes somatic recombination of the TCR (like conventional T cells) and is present in all animals with an adaptive immune system [178]. They are having both innate and adaptive like features and straddle a unique classification [179]. Their rearrangement of the antigen receptor is an adaptive like feature although it is limited in diversity [180]. On the other hand, most of these cells are exiting the thymus as developed T_{EFF} cells with the ability to immediately respond to stress signals [181]. γδ T cells have also been shown to act as APCs for αβ T cells through cross-representation [182, 183].

There is some evidence that γδ T cells can exit the thymus both as naïve or T_{EFF} cells with either innate-like or adaptive-like characteristics, respectively [179]. The majority of γδ T cells are homing to peripheral tissues rather than lymphoid organs which highlights their difference from conventional αβ T cells [184]. As mentioned earlier, the γ and δ chain form a heterodimeric TCR which is generated by somatic recombination of variable (V), diversity (D), joining (J) and constant (C) regions [185]. γδ T cells are more limited in the number of V, D and J segments than αβ T cells and expression of specific γ and δ-chain pairings is favoured [186]. It is important to point out that the γδ T cell populations and their development differ greatly between mice and humans, and they share no homologous subsets [187]. γδ T cell subsets acquire their name according to the expressed V gene segment on the γ-chain (mice) and δ-chain (humans). Based on this nomenclature, there are seven populations of γδ T cells in mice (γ1- γ7) and eight in humans (δ1- δ8) which are then subcategorized by their pairing δ- or γ-chain, respectively [188]. However,

the δ1- δ3 chains are the most commonly found δ-chains in humans which can be paired with seven γ-chains [187].

In mice, the first γδ T cells are emerging from the fetal thymus at day 15 after gestation . Several waves of γδ T cells, corresponding to different functional subsets, are released to populate the peripheral tissues at distinct time points already before birth [189, 190]. It demonstrates that there are developmental molecular programmes in place that give rise to different γδ T-cell subsets from gestation to adult hood [191]. For example, Vγ5Vδ1 T cell are the first to migrate to the dermis as precursors of dendritic epidermal T cells which are specific for mice [192] followed by Vγ6Vδ1 T cells that populate multiple peripheral locations a few days later (tongue, dermis, uterus, testis, peritoneal cavity, adipose tissue, and brain meninges) [189]. Most γδ T cells are already committed in the thymus to secrete either IFN-γ or IL-17 and they maintain these effector functions in peripheral tissues [193].

In contrast to mice, human γδ T cells show less diversity with Vγ9Vδ2 cells being the most abundant subset in fetal and adult blood [194, 195]. This subset already emerges in the fetal liver before the thymus has developed and suggest a similar importance as in mice for the early protection before the development of any adaptive immunity [196]. Vδ1 and Vδ3 cells are predominantly present in tissues but studies have been focusing mainly on Vδ2 cells as they are very abundant and easier to isolate from blood [188]. Vδ2 cells are not only present in blood but also in the intestinal mucosa [197]. Not much is known about Vδ3 cells because antibodies for immunological methods are not available.

There has been a long search for possible TCRγδ ligands and to this date there is no clear consensus about which antigens can be recognized and how they are presented. Most of the ligands which have been reported are non-polymorphic in contrast to αβ T cell that recognize peptides on polymorphic MHC class I and class II complexes. There is a vast range of molecular structures that can present different antigens that have been proposed or confirmed as ligands for γδ T cells supporting the notion that no single antigen presentation mode can explain all γδ T-cell responses [198-200]. In fact, there are several known MHC-like ligands e.g., CD1c,

CD1d, ULBP, MICA or EPCR that are recognized by the γδ TCR without the presence of any antigen [201-205]. MICA, ULBP and EPCR are good examples of self-ligands that play an important role in the recognition of stressed or dysregulated cells. Several other co-stimulatory receptors like NKG2D can complement or even replace the activation of the TCR [206, 207] . Furthermore, there is a subset of γδ T cells that can even recognize soluble phycoerythrin without any antigen presenting cell [208].

One of the best studied γδ T cells population in humans are Vγ9Vδ2 cells. In healthy individuals Vγ9Vδ2 are making up to 1–5% of T cells from the blood but frequencies can increase to 50% upon infection [209]. They recognize phosphoantigens (phosphorylated intermediates of the non-mevalonate pathway of isoprenoid biosynthesis) that are present in a broad range of bacteria and cancers [210]. Recently, it has been shown that the activated butyrophilin 2A1 is essential for the reactivity of γδ T cells to phosphor antigens and that co-binding to the TCR is required [211]. This highlights the complex nature of the γδ TCR binding and the challenges in shining light on the potential ligands and their interaction.

γδ T cells can rapidly secrete large amounts of cytokines of either the Th1, Th2 or Th17 type depending on the subpopulation and immune environment [212, 213]. Additionally, they are possessing the same cytotoxic mechanism as CTL which were described in detail earlier [214].

Taken together, γδ T cells can recognize an immense range of molecules and danger signals that lead to a broad contribution in the immune response to infections and tumor cells. Furthermore, they have many functions in the homeostasis and surveillance of tissues [189, 215].

Migration of T cells into intestinal tissue and tumors

T cells can express several homing receptors that bind ligands on endothelial cells to migrate into specific tissues. Additionally, the homing receptors play a crucial role in the retention of T cells in these sites. Naive T cells recirculate from the blood to lymphoid tissues and back in search

for their cognate antigen presented on APCs. They express L-selectin which reversibly binds to peripheral lymph node addressin (PNAd) on HEVs and starts a rolling process on the wall of the blood vessel [24]. The homeostatic chemokines CCL19 and CCL21 are secreted in lymphoid tissues and bind to the chemokine receptor CCR7 expressed by T cells [216]. This triggers the activation of the integrin leukocyte function-associated antigen type 1 (LFA-1) on the T cell which binds to intercellular adhesion molecule 1 (ICAM-1) or ICAM-2 on the endothelial cell and leads to firm adhesion and transmigration into the lymph node [217]. Naive T cells homing to PP and MLN are not dependent on PNAd [218] but instead they use their receptor α4β7 or L-selectin with the ligand Mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) on HEV [219, 220].

T_{EFF} cells and effector memory T cells downregulate CCR7 and L-selectin which excludes them from homing to lymph tissues whereas central memory T cells retain CCR7 expression [221, 222]. Additionally, T_{EFF} become gut tropic by upregulation of α4β7 [223] and CCR9 due to the priming of gut associated DCs that produce retinoic acid from retinol [21, 224]. Intestinal effector T cells effectively migrate to the LP and epithelia layer of the small and large intestine through the interaction of α4β7 with MAdCAM-1 [225]. However, CCR9 expression is only required for homing to the small intestine especially the epithelial layer but not for homing to the large intestine [226]. The chemokine CCL25 is constitutively expressed by epithelial cells of the small intestine and interacts with CCR9 [227]. Furthermore, IEL express the integrin αEβ7 (CD103) which interacts with the adhesion molecule E-cadherin on epithelial cells to retain IEL in the epithelial layer [228].

Selectins and their ligands

Selectins build a family of three cell adhesion molecules which are important in leukocyte migration and homing to tissues. The role of L-selectin in lymphocyte adhesion to blood vessels and subsequent transendothelial migration has been discussed before. E- and P-selectin are

expressed by activated endothelial cells to increase immune cell migration into inflamed non-lymphoid tissue [229, 230]. P-selectin is stored in secretory granules (Weibel-Palade bodies) and therefore surface expression on endothelial cells can be rapidly increased in inflammation. In contrast, E-selectin expression is delayed because de novo synthesis is required but it is sustained for longer periods [231]. The ligands for selectins are carbohydrate structures (sialofucosylated glycans) that are usually presented on specialized protein scaffolds [232]. P-selectin glycoprotein ligand 1 (PSGL-1) is the best characterized ligand for P- and E-selectin although not the major ligand for the latter and also a ligand for L-selectin [233]. PSGL-1 plays an important role in the tethering/rolling process of T_{EFF} cells and myeloid cells on activated endothelial cells expressing P-selectin [234]. There is some evidence that CD44 on activated T cells can also act as a ligand for P-selectin [235]. Apart from PSGL-1, there are no reports about specific E-selectin ligands on T cells, but neutrophils do express CD44 and ESL-1 to migrate into inflamed tissues [236]. Both E- and P-selectin expression can be induced by inflammatory cytokines like TNF or IL-1 but is reduced by TGF-β [229, 237]. Selectin ligands on T cells can be induced by several cytokines that affect T cell differentiation like IL-9, IL-12, IL-18, IL-25, IL-27 and TGF-β [238].

Integrins and their ligands

Integrins are important for the tighter adhesion of leukocytes to the endothelium after the initial binding of selectins to their ligands. The integrins α4β7, αEβ7 and LFA-1 have been mentioned earlier in the role of intestinal migration so here we want to focus on some of the ligands which are expressed by endothelial cells and have been investigated in paper II of this thesis. These are namely ICAM-1 and ICAM-2 as well as vascular cell adhesion molecule 1 (VCAM-1) which are all involved in transendothelial migration during inflammation [239]. ICAM-2 is constitutively expressed on endothelial cells whereas ICAM-1 and VCAM-1 are almost absent in homeostasis but increased by proinflammatory stimulators like TNF, IFN-γ , IL-1β or IL-4 [240]. ICAM-1 and ICAM-2 are both known to bind to the integrin LFA-1 and ICAM-1 additionally

binds to the macrophage antigen 1 [241]. VCAM-1 on the other hand is associated with the integrins α4β1 (VLA-4) and α4β7 [242].

Chemokines and their receptors

Chemokines (a group of chemotactic cytokines) are mostly known for their role in the migration of leukocytes mediated by chemotaxis, but they can also affect cytokine production, proliferation, survival, degranulation, differentiation and respiratory burst [243]. They are small secreted proteins and signal through surface G protein-coupled heptahelical chemokine receptors [244]. There are four subfamilies of chemokines which are defined by their primary amino acid sequence and the position of certain cysteine residues closest to the N-terminus: XC, CC, CXC and CX3C [244, 245]. The function of the chemokine ligand CCL25 and its receptor CCR9 for intestinal homing have been already discussed earlier but other chemokines (about 50) and their receptors (about 13) are known, and many regulate the migration of T cells.

CXCL9 (MIG), CXCL10 (IP-10) and CXCL 11 (I-TAC) are ligands for the chemokine receptor CXCR3 which is known for its expression on Th1 and CTL [246]. There are three isoforms of CXCR3 (CXCR3-A, CXCR3-B and CXCR3-alt) which are linked to different functions. In general, CXCR3-A is the first one discovered and linked to proliferation, chemotaxis, cell migration and invasion whereas CXCR3-B seems to facilitate antiproliferative, angiostatic, and pro-apoptotic effects of the CXCR3 ligands. Mice do only have the CXCR3-A variant [247]. CXCR3 is not present on naïve T cells but DC-induced antigen-specific T cell activation leads to rapid upregulation [248, 249]. T cells expressing CXCR3 are enriched in inflamed tissues of autoimmune diseases indicating that CXCR3 and its ligands are important in Th1 T cell recruitment [250, 251]. Furthermore, blocking of the CXCR3 receptor with antibodies can reduce T cell recruitment to tissues and inhibit the delayed-type hypersensitivity response [252]. The Th1 master regulator Tbet is essential for the expression of CXCR3 and regulates the Th1 mediated trafficking to inflammatory sites [253]. CXCR3 expression and its role in the recruitment has been shown in other cell types like NK cells, NKT cells, Treg, B cells and plasmacytoid DCs not only to inflamed tissues [254-258].

The chemokine ligands for CXCR3 are expressed by different cells e.g. endothelial cells, epithelial cells, activated neutrophils, stromal cells and keratinocytes in response to IFN-γ [259]. The affinity of the CXCR3 ligands differ with CXCL11 having the highest, CXCL10 intermediate, and CXCL9 the lowest affinity [260, 261]. C57BL/6 mice used in this thesis do not express CXCL11 due to a point mutation that inserts a stop codon into the gene [262]. Both CXCL9 and CXCL10 induction is strongly induced by IFN-γ while CXCL10 induction is also sensitive to innate stimuli from the type I interferons (IFNα/β). Moreover, TNF does not stimulate the expression of CXCL9 and CXCL10 alone but synergizes with IFN-γ [263-265]. Little is known on how the three ligands work together and influence each other but different studies came to the result that they can be sometimes work redundantly, collaboratively, synergistically, and antagonistically [266].

CX3CL1 (also known as fractalkine) is the only member of the CX3C chemokine family and binds to the G protein-coupled, 7-transmembrane receptor CX3CR1 [267]. CX3CL1 is larger than other chemokines and is synthesized as a membrane-anchored protein (chemokine domain on top of an extended mucin-like stalk) that can function as an adhesion molecule on epithelial cells for the attachment of lymphocytes [268]. The chemokine component can be cleaved from the surface by the tumor necrosis factor-α-converting enzyme and acts as a potent chemoattractant for T cells, NK cells and monocytes [269]. CX3CL1 expression on endothelial cells increases through pro-inflammatory stimuli like IFN-γ, IL-1β and TNF [270, 271]. There is evidence that p53 activation induces CX3CL1 on the surface of stressed or damaged cells which could lead to attraction and/or binding of NK cells or CTL to eliminate the target cell [272].

Neutral sphingomyelinase 2

The bioactive sphingolipid ceramide is involved in many cellular processes like apoptosis, differentiation, growth inhibition and senescence [273]. The Neutral sphingomyelinase 2 (nSMase2) enzyme catalyses the hydrolysis of sphingomyelin to ceramide (see Figure 4 for synthesis and metabolism of

ceramide) and therefore regulates cell growth and cell signalling [274]. It is considered the major pathway for stress induced ceramide production and most likely mediates cytokine effects on ceramide [275]. The inflammatory cytokine TNF induces nSMase2 activity probably through post-transcriptional mechanisms [275]. Additionally, a study of Clarke et al. showed that the adhesion molecules VCAM-1 and ICAM-1 on lung epithelial cells were upregulated by TNF stimulation, highlighting the role of nSMase2 in pro-inflammatory immune responses [276]. Overexpression of nSMase2 in a melanoma mouse model increased $CD8^+$ tumor infiltrating T cells and the efficacy of PD-1 therapy which indicates a role of the ceramide signalling pathways in anti-tumor immunity [277]. On the other hand, the release of exosomal PD-L1 can be an immune evasion strategy of tumors and is inhibited by knocking out nSMase2 in cancer cell lines [278].

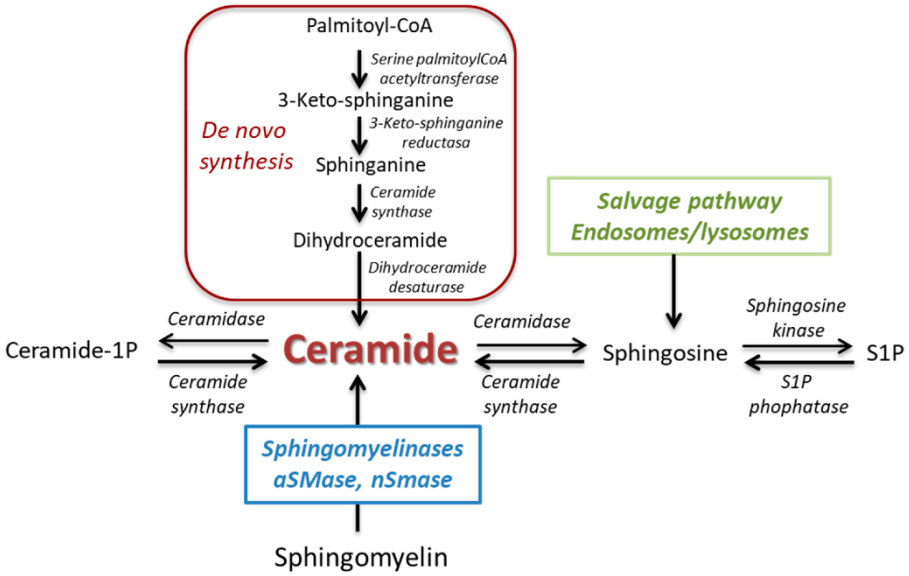

Figure 4: Synthesis and metabolism of ceramide. ©2019 Cogolludo, Villamor, Perez-Vizcaino, Moreno (Licensed under CC BY 4.0)[279]

Otherwise, nSMase2 has been studied very little in tumor immune responses. Using a tumor mouse model, we found an effect of Treg depletion on nSMase2 in endothelial cells by using RNA sequencing (RNAseq) in paper II. This led us to investigate the role of endothelial nSMase2 in the migration of T cells into tumors. We could expand the knowledge through which mechanisms Treg might suppress T cell migration into tumors and confirmed the results with *in vivo* studies.

Colorectal cancer

Epidemiology

Colorectal cancer (CRC) is a disease of the lower intestinal tract and is the third most common (1,9 million cases) and second deadliest (935'000 cases) cancer in the world representing about 10% of all cancer cases and deaths [280]. It is most diagnosed in transitioned countries with a 4-fold higher incidence compared to transitioning countries (Figure 5). The Human Development Index reflects the CRC rates worldwide which is an indicator that the disease is linked to environmental and lifestyle risk factors including smoking, alcohol consumption, processed meat consumption, lack of physical activity, a western dietary pattern, and obesity [281-285]. Countries with increasing Human Development Index experience a shift from cancers caused by infection towards cancers caused by a western lifestyle like CRC [286]. The global burden of CRC is expected to increase by about 50% in transitioned and 80% in transitioning countries by 2040 [287] which highlights the importance of better prevention and the development of effective treatments.

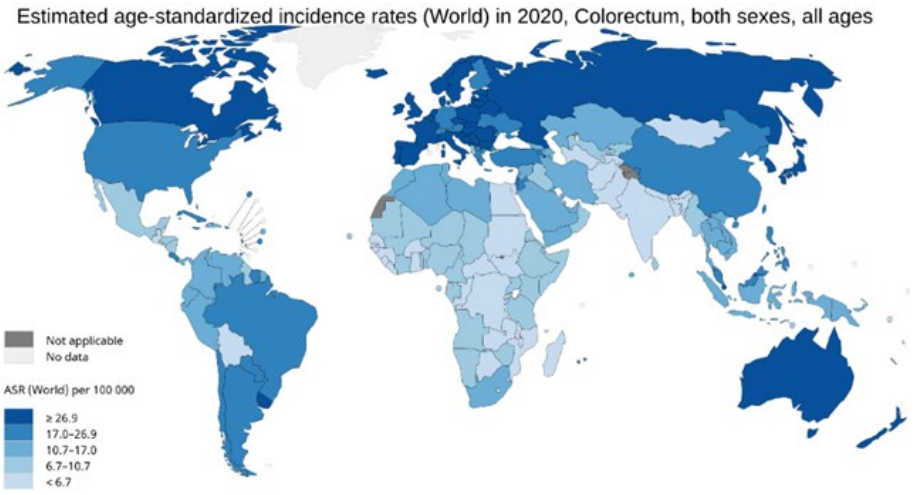

Figure 5: Estimated age-standardized incidence rates of CRC (both sexes and all ages) in 2020 (5, 13, 14)

Carcinogenesis

The largest part of about 60-65 % CRC cases is originating from sporadic somatic gene alterations while the remaining cases are thought to have an inherited CRC component with either a family history of CRC (25%), hereditary cancer syndromes like hereditary nonpolyposis colorectal cancer (HNPCC, 2–4%) and familial adenomatous polyposis (FAP, <1%), or unknown inherited genomic alterations [288].

CRC is believed to originate from mutations in the stem cell niche in the crypts of the epithelial lining [289] but there has also been some evidence for the development from committed progenitor cells [290]. The series of mutations leading to CRC has been termed the " adenoma–carcinoma sequence" and was established 1992 by Fearon and Vogelstein [291]. It is thought that most lesions start with a mutation in the adenomatous polyposis coli (APC) gene as 80% of adenomatous polyps carry such mutation [292]. The loss of the APC gene leads to a constitutive activation of the Wnt signal transduction pathway which leads to the accumulation of β-Catenin and subsequently the adenoma cells maintain their progenitor

status with proliferative and renewal abilities [293]. Further mutations in oncogenes (e.g. KRAS, PIK3CA) and tumor suppressor genes (e.g. SMAD4 and TP53) lead to the growth of adenomatous polyps and subsequently to the progression into malignant carcinoma (Figure 6)[294]. The disease can proceed with further spread to local lymph nodes and distant organs which are mostly the liver and lung.

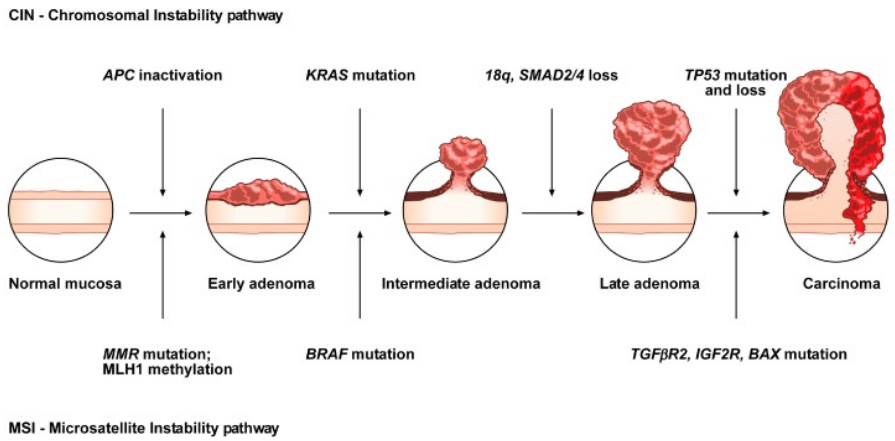

Figure 6: Development from benign adenoma to malignant carcinoma. The CIN and MSI pathway and common associated mutations that are acquired by tumor cells during the development. ©2019 De Palma, D'Argenio, Pol, Kroemer, Maiuri and Salvatore (Licensed under CC BY 4.0)[295]

Molecular subtypes

In the past, CRC has been divided into three different subtypes based on their anatomical location of the tumor. This has been the proximal colon (caecum, ascending colon, hepatic flexure and transverse colon), distal colon (splenic flexure, descending colon and sigmoid colon) and rectum [296]. The knowledge of CRC has increased immensely over the last two decades and let to a more heterogenous picture of CRC development and its molecular features. However, these features are somewhat linked to the anatomical sites which shows that location of the tumor is still important [297]. One of the first molecular classifications was discovered through the

genetic studies of HNPCC where the role of mutated genes in the DNA mismatch repair system was identified [298-300]. This led to the classification of microsatellite instability (MSI) and microsatellite stability (MSS). MSS tumors (60-70% of CRCs) were shown to carry many chromosomal changes like rearrangements and aneuploidy, and were hence called chromosomally unstable or short CIN (chromosomal instability)[301]. MSI tumors make up about 15-20% of all CRCs (10-15% of sporadic CRCs) and they usually have a different immune landscape compared to MSS tumors which has been linked to a better clinical outcome in the first [302]. A third molecular subtype was recognized as the CpG island methylator type (CIMP) which is a distinct epigenetic phenotype characterized by methylation of certain promotor regions of tumor suppressor genes [303, 304]. In recent years, it has been recognized that a subgroup of CRC (10-15 % of sporadic CRCs) called serrated adenoma ("saw-toothed" epithelial glandular crypts) shows different molecular features with an early BRAF mutation as a key component [305]. Both the MSI status and the serrated phenotype are commonly associated with DNA methylation ($CIMP^+$) and it is believed that most of the MSI tumors originate from the "serrated pathway" [306]. Apart from the classification based on solely mutational parameters, a new classification of four molecular consensus subtypes (CMS) was created by a consortium of six different research groups (Figure 7)[307]. These CMS also include an assessment of gene expression patterns to assess the type of immune response, mesenchymal changes, and metabolic features of the tumor. These recent findings have sparked some excitement about a more focused therapy for patients but it is yet unknown what clinical role this will play in the near future as the translation is a complex task and even the implementation of the MSI/MSS subtyping has taken a long time [308].

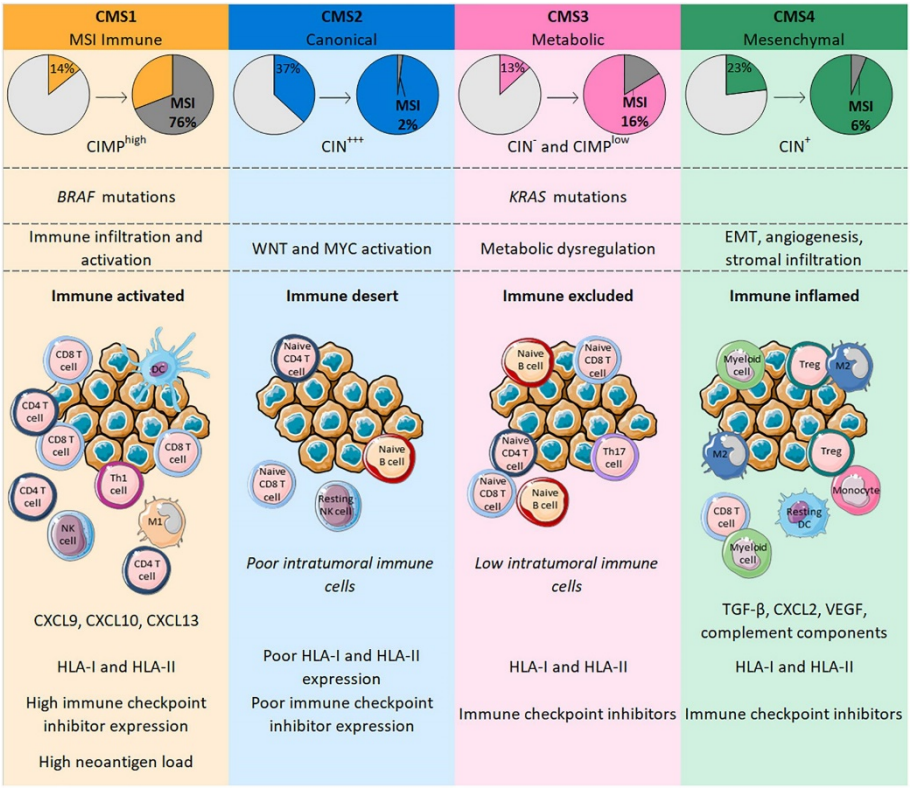

Figure 7: CMS subtypes in CRC are classified by distinct genetic profiles. CMS1 is highly enriched in MSI tumors and shows active T cell infiltration and expression of checkpoint inhibitors. CMS2 and CMS3 type tumors are infiltrate by few immune cells and are mainly associated with the CIN tumor type but CMS3 tumors show metabolic dysregulation and retain expression of human leukocyte antigen (HLA) molecules. CMS4 is associated with a strong inflammatory immune environment where suppressive cells are accumulated and secrete soluble factors to shape the TME. ©2020 Picard, Verschoor, Ma and Pawelec (Licensed under CC BY 4.0)[309]

Conventional treatment

The ideal treatment of CRC aims for a complete removal of the tumor and all metastases which usually requires surgical intervention [310]. Although there are many screening programs in place to diagnose CRC early enough, almost a quarter of patients are diagnosed at an advanced stage with metastases. This leads to unresectable tumors which can only be controlled

but not removed and subsequent tumor-related deaths. Radiotherapy and chemotherapy are the common therapies to control the tumor growth, but they are also applied before and after surgery to either reduce tumor size or prevent reoccurrence [311]. However, the application of new therapies with monoclonal antibodies and combined treatments has not been a breakthrough as in melanoma. One major problem is the development of drug resistance and escape mechanism of the tumors. The molecular mechanisms need further investigation to improve therapies [312].

Mouse models of colorectal cancer

We utilized the $APC^{Min/+}$ mouse as a model for colorectal cancer [313]. These mice carry a nonsense mutation in the tumor suppressor gene APC leading to spontaneous tumor development in the small and large intestine. $APC^{Min/+}$ mice develop about 15-30 adenomas (see histology in Figure 8) along the small and large intestine together without progressing to invasive carcinoma [314]. As described in the chapter "Carcinogenesis", mutations of the APC gene are found in more than 80 % of human sporadic CRCs and it is considered one of the first mutations driving the tumorigenesis [315]. Additionally, the inherited autosomal CRC form of FAP is mostly caused by a truncation of the APC gene [316] and a corresponding mouse model was already developed 1990 by Moser et al. [317]. Therefore, the $APC^{Min/+}$ model is commonly used to research the development and immune infiltration of intestinal tumors. However, there are other models with different APC alterations leading to slight changes in histopathology and tumor numbers: $APC^{+/1638}$ [318], $APC^{\Delta 716}$ [319] and $APC^{\Delta 242/+}$ [320]. One potential problem of all these models is the predominant development of tumors in the small intestine instead of the colon. Along with tumorigenesis, the APC mutation also causes developmental impairments which lead to enlarged spleens and anaemia as tumors progress. Furthermore, numbers of lymphoid follicles in the spleen, and PP in the intestine are reduced [321].

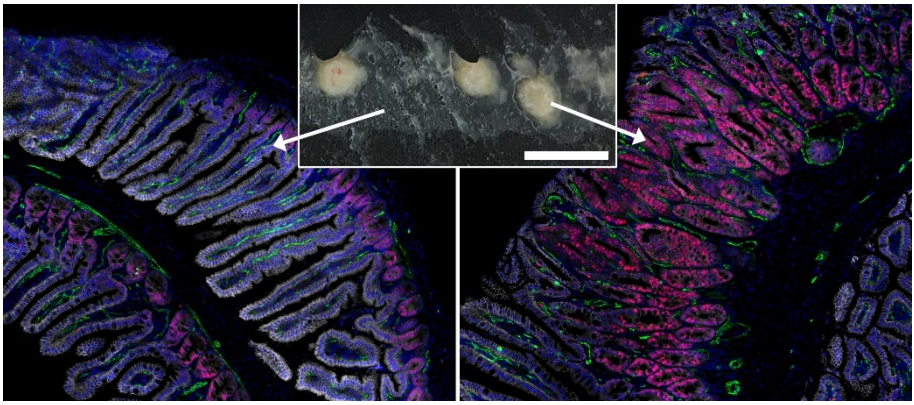

Figure 8: Macroscopical picture of the small intestine of $APC^{Min/+}$ mice (middle, white bar 1 cm). Representative immunofluorescence images show the unaffected (left) and tumor tissue (right) stained with DAPI (blue, nuclei), EpCAM (white, epithelial cells), CD31 (green, endothelial cells) and Ki-67 (red, proliferating cells).

There are several other genes (*P53*, *KRAS*, *PIK3CA*, *FBXW7*, *SMAD4*, and *TCF7L2*) that are commonly mutated in CRC and are considered to contribute to further tumor development [322]. Therefore, some of these mutations have been introduced into the $APC^{Min/+}$ model which has led to invasive tumors and metastases in many [323, 324] but the number of combined mutations has led to a drastically reduced life span of the animals [325]. Interestingly, restoration of APC does lead to spontaneous tumor regression in KRAS-mutated and p53-deficient mice suggesting that APC plays a significant role in the development of adenocarcinomas [326]. Moreover, other mouse models with chemically induced CRC or models based on the Cre-loxP-system (e.g. Villin-Cre*)* have been used to study tumor development but are not widely applied in tumor immunology.

The transfer of faeces from CRC patients into $APC^{Min/+}$ has shown that the microbiota can promote tumor growth, lead to impairment of the barrier function, and increase inflammation [327]. Otherwise, tumors in $APC^{Min/+}$ mice show reduced numbers and cytotoxicity of $CD3^+$ IEL as well as expansion of IL-17 producing Treg [328, 329]. IL-17 seems to

promote tumorigenesis while IFN-γ inhibits the development of tumors as shown in an IFN-γ knockout model [330, 331].

The increasing negative public about animal models and the high costs of genetically modified mice might lead to further application of the 3R-principle (replacement, reduction and refinement) in cancer research. Additionally, recent research has shown the importance of the microbiota in intestinal inflammatory diseases and CRC. Inbred mice in an animal facility hardly represent the diversity of microbes in humans and the comparison between facilities is also hampered.

Tumor immunology

The first time the immune system was considered to repress cancers was 1908 by Paul Ehrlich [332]. The idea could not be supported by any evidence also because of the lack of available inbred mouse strains and it took another 50 years that the existence of tumor-specific antigens was established [333, 334]. This supported the idea of the cancer immunosurveillance hypothesis which was proposed earlier by Frank Macfarlane Burnet and Lewis Thomas [335]. The concept that nascent transformed cells could be destroyed by the immune system which therefore protects from the development of tumors. However, the principle could not be supported by animal experiments and thus little interest emerged and the theory was abandoned [336]. Two key finding in the 90s revived the immunosurveillance hypothesis: the chemokine IFN-γ showed protective effects against tumors, and mice lacking perforin which is important for cell mediated cytotoxicity were prone to get more tumors [337, 338]. Further research in the field and the emergence of new mouse models finally led to the recognition of the immunosurveillance theory. Dunn and Schreiber introduced an extended concept of immunoediting with three components (the three Es): elimination, evasion and escape (Figure 9)[339]. The elimination phase corresponds to immunosurveillance where the tumor is recognized by the immune system and elicits a tumor specific response. This can lead to tumor elimination or to the equilibrium phase. Tumor cells surviving the elimination enter a dynamic process by

which certain clones mutate and survive because of the selective pressure by the immune system. These clones acquire mechanisms to escape the immune system through genetic or epigenetic changes and expand uncontrollably into clinically observable malignant tumors. It has also become clear that tumors can shape the tumor microenvironment (TME) and recruit immune cells that support tumor growth and metastasis. The success of new antibody immunotherapies like anti-PD-1 treatment has put the immune system into focus in cancer research.

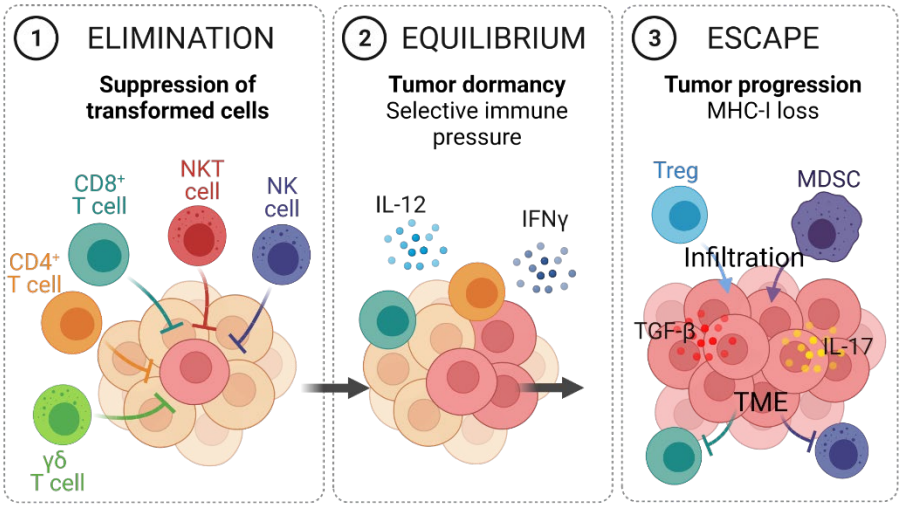

Figure 9: Simplified view of the three phases of cancer immune editing [340]. Modified from "Cancer Immunoediting" by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

Tumor microenvironment

There are several mechanisms tumors use to evade the elimination by the immune system. They can exhibit a low immunogenicity by not presenting peptide-MHC ligands and downregulate adhesion or costimulatory molecules [341]. Tumor antigens could also be seen as self-antigen and induce tolerance because of the lack of co-stimulatory signals to APCs [342]. Furthermore, tumors can lose their antigens over time that were

previously recognized, making them invisible to the adaptive immune system [343]. Tumor cells can secrete immunosuppressive cytokines like IL-10 or TGF-β but also exhibit PD-L1 expression on their surface to inhibit T cells directly and avoid killing [344, 345]. All these mechanisms are rather a modulation of the tumor cell itself, but it can also secrete certain factors to shape the whole tissue environment to its favour.

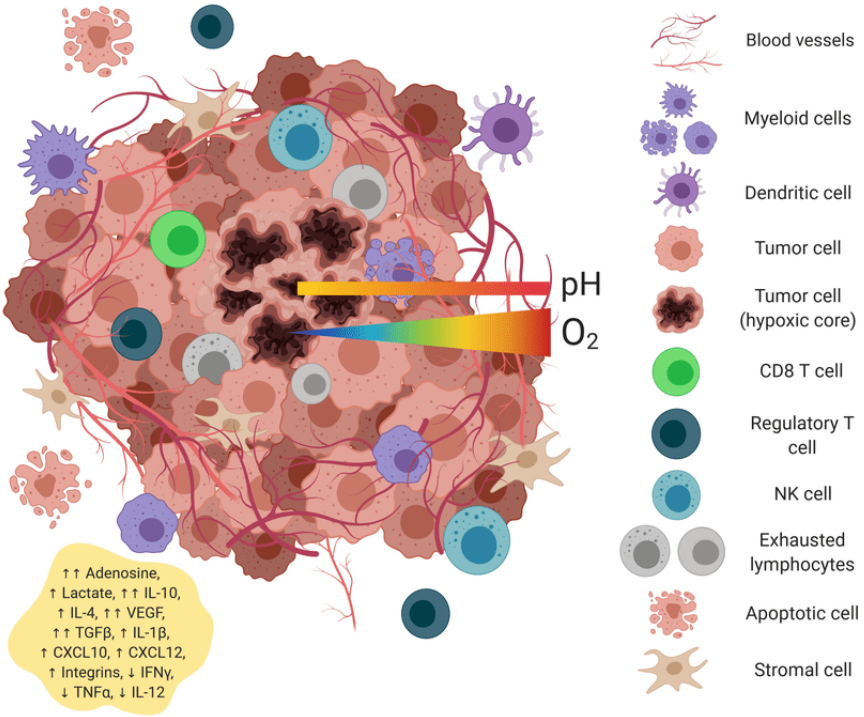

Figure 10: The TME is a complex environment of different cell types and soluble factors (e.g. chemokines and cytokines). Heterogenous tumor cells, stromal cells, and many different leukocyte populations form a network that is supported by dysregulated vasculature and collagen. Poor perfusion and tumor cells with high glycolytic activity create areas with low oxygen levels, acidic pH, and poor accessibility to nutrients. ©2019 Fernández, Luddy, Harmon and O'Farrelly (Licensed under CC BY 4.0)[346]

The TME is composed of different components surrounding the tumor cells and contributing to a unique tissue environment: endothelia cells, stromal cells such as fibroblasts, immune cells including myeloid and lymphoid cells, bone marrow-derived precursor cells, the extra cellular matrix, cytokines and other signalling molecules (Figure 10)[347, 348]. The TME plays a critical role in the development and progression of cancer as well as for anti-cancer drug efficacy [349, 350]. Furthermore, the TME can promote metastasis which is the leading cause for tumor related deaths [351, 352]. Tumor associated fibroblasts and immune cells like Mφs, or myeloid-derived suppressor cells (MDSC) are just one example of factors that support the tumor development and dissemination at various stages [353, 354]. Many studies have tried to dissect the different pro-tumorigenic and anti-tumorigenic components of the TME, but it has proven to be complex and greatly dependent on the tumor origin and molecular subtype. In the following chapters we will focus on how endothelial cells, chemokines, selectins and specific immune cell subsets can shape the TME of CRC and influence the clinical outcome of patients.

Endothelial cells

Endothelial cells are essential for the blood supply of the tumor tissue and therefore tumor progression but they are also the entry point for immune cells capable to facilitate tumor regression and elimination [355]. In many tumors, endothelial cells have an altered morphology and show distinct cellular and molecular phenotypes compared to normal endothelium. They display disproportionate branching, irregular diameters, chaotic flow patterns and increased permeability or leakiness [356]. Furthermore, tumor endothelium is surrounded by morphologically abnormal pericytes [357]. The transition from endothelial to mesenchymal cells has been shown to promote cancer development, metastasis, and drug resistance [358, 359]. Cancer-associated fibroblast can originate from mesenchymal cells and secrete various cytokines or growth factors like the suppressive cytokine TGF-β and the angiogenesis promoting vascular endothelial growth factor [360, 361]. In contrast, endothelial cells can upregulate the cytokines

CXCL9, CXCL10 and CXCL11 as well as adhesion molecules to increase tumor infiltration of beneficial T cells [362].

In this thesis we isolated endothelial cells from mouse intestinal tissues and worked with endothelial cell lines to improve the understanding of how they might interact with Treg and therefore influence transendothelial migration of beneficial T cells into tumors.

Selectins and integrin ligands

We investigated the expression of E-selectin and P-selectin on murine endothelial cells in paper I. In fact, there is very little research on selectins in colorectal cancer especially for endothelial cells. Many studies have evaluated soluble selectins from blood as a biomarker for CRC progression and metastasis [363]. Furthermore, there is evidence that selectins expressed on tumor cells promote metastases [364].

There is data on the expression of integrin ligands VCAM-1 and ICAM-1 which have been studied in paper II but again focusing on the expression in tumor cells or bulk tumor samples. One study by Mlecnik et al. found that both ICAM-1 and VCAM-1 gene expression in CRC were positively correlated with the density of T cell populations in certain tumor regions and also associated with prolonged disease-free survival [365]. On the other hand, ICAM-1 and VCAM-1 are both associated with active inflammatory bowel disease where they are upregulated in colonic tissue [366]. It has also been demonstrated that the infiltration of tumor-associated macrophages (TAM) was linked to VCAM-1 expression in tumor cells, which was mediated by cancer-associated fibroblasts [367]. Additionally, VCAM-1 expression in cancer cells promotes the invasiveness and metastasis of CRC [368].

The available data of selectin and integrin ligand expression in endothelial cells of tumors is scarce and highlights the need for new studies like this thesis to investigate the effects of these molecules on T cell migration into tumors.

Chemokines

Chemokines have been demonstrated to promote tumor growth, angiogenesis and metastasis but there is also evidence that they promote anti-tumor immunity [369, 370]. CXCL9, CXCL10 and CX3CL1 expression have been linked to a higher density of $CD8^+$ T cells in CRC which also correlated with increased disease-free survival. Tumors with high CXCL9 and CXCL10 expression were mostly infiltrated by $CD45RO^+$ memory T cells [365, 371]. Furthermore, tumors with high CX3CL1 expression were infiltrated by T cells with a distinct TCR repertoire compared to tumors with low CX3CL1 expression which was correlated to a better prognosis [372]. Our previous study in a mouse model of CRC has also shown that CXCL9 and CXCL10 expression increased in Treg depleted tumors followed by an accumulation of $CXCR3^+$ conventional T cells [314]. A study by Wendel et al. indicated that NK cells are relying on the interaction of CXCR3 and CXCL10 to infiltrate tumors in mice [255]. Most studies have investigated chemokine expression on tumor cells or in bulk tumor samples but very few have examined the functions on endothelial cells in tumor tissue.

The chemokine receptor CXCR3 plays a dual role in CRC by promoting tumor growth and metastasis when expressed on tumor cells or exhibiting anti-tumor effects when expressed on tumor infiltrating T_{EFF} cells [247]. CXCR3 is important for the generation of Th1 cells that secrete IFN-γ and exert anti tumor immunity [373]. A study from Musha et al. demonstrated an active recruitment of $CXCR3^+$ T cells into the invasive margin of colorectal tumors which was accompanied by Mφs and tumor cells expressing CXCL10 [374]. The CXCR3 ligands CXCL9 and CXCL10 are highly expressed in lymph nodes which might explain the migration and population with $CXCR3^+$ tumor cells [375]. Moreover, patients with tumors expressing CXCR3 did also show significantly shorter survival rates. It is important to point out that the CXCR3 variants which exist in humans seem to have different effects on tumors. T_{EFF} cells and Treg expressing CXCR3-A are attracted to the tumor which can lead to both anti-tumorigenic effects by the first and pro tumorigenic effects by the latte. Tumor cells expressing CXCR3-A show increased proliferation and

dissemination while they lower their expression of CXCR3-B with similar effects [376].

We explored the expression of chemokines in mouse and human tissue as well as specifically in endothelial cells in paper I and II of this thesis. These studies were important to understand the role of chemokines on the increased T cell infiltration into tumors when Treg were depleted in our tumor mouse model.

Myeloid cells

Myeloid cells are not the focus of this thesis, but we will give a short overview about their general functions and impact on tumor progression. They can be very abundant in the tumor stroma and influence the patient outcome. Myeloid subsets can be divided into mononuclear and polymorphonuclear cells. Mononuclear cells include Mφs and DCs which develop mostly from blood monocytes and dendritic cell precursors, respectively [377, 378]. Mφs are important for tissue homeostasis, immune surveillance, and inflammation [379]. The general role of classical DCs is to sample antigens in tissues and present it in the local draining lymph node to generate antigen specific T cell immunity or tolerance [380]. Plasmacytoid DCs are a specialized subset of DCs that can produce large amounts of IFN-α and contribute in creating a pro-inflammatory environment [381]. Granulocytes include neutrophils, eosinophils and basophils that have distinct nuclei with two or three divided lobes as well as mast cells that have unilobed nuclei. They are recruited to inflammatory sites to release inflammatory and toxic agents that protect against bacteria or parasitic infections; however, neutrophils are also efficacious phagocytes [382-384]. MDSC include immature myeloid progenitors and other monocytic and granulocytic cells that are functionally distinct from the conventional myeloid subsets and supress T cell activation *in vitro* [385].

Mφs are a heterogenous population in tumors and can have both pro-tumorigenic and anti-tumorigenic effects. They can secrete mediators like growth factors (e.g. epidermal growth factor) and cytokines (e.g. IL-6, TNF, IL-10, TGF-β) to shape the TME and support tumor cell survival

and proliferation [386-388] as well as other factors that modify tissue architecture and favour tumor cell migration, invasion and metastasis [389, 390]. They are typically divided into classical M1 and alternative M2 Mφs based on their functional profile *in vitro* though the phenotypes in tumors are much more fluent [391]. Nevertheless, M1 Mφs are thought to be involved in the direct killing of tumor cells, the production of angiostatic factors and the stimulation of anti-tumor T cell functions. On the other hand, M2 Mφs are believed to support tumor cell growth and invasion, enhance tumor angiogenesis and inhibit anti-tumor T cell functions [392]. In accordance with this model, a pan-cancer analysis by Gentles et al. associated the M2 phenotype with a less favourable prognosis than the M1 phenotype [393]. Furthermore, a meta study by Zhao et al. showed a better overall survival for patients with high infiltration of pan-Mφs but lower overall survival when Mφs with M2 phenotype were present [394].

The role of DCs in CRC is not fully understood and study results are contradictive because there is no consent about the markers for the identification of these cells. They are usually not very abundant in tumor tissue and difficult to study but they are considered important as they can present neoantigens on MHC-I to activate anti-tumor $CD8^+$ T cells and sustain anti-tumor immunity [395]. However, the presence of DCs in tumors has been linked to different patient outcomes. High intraepithelial infiltration of DCs has been correlated with the infiltration of $CD4^+$ and $CD8^+$ T cells which is considered beneficial for the outcome of patients but DCs in other areas of the tumor seem to decrease the disease-free survival [396, 397]. Another study by Nagorsen et al. found that the survival of patients was significantly better for tumors with high dendritic cell infiltration [398]. The knowledge of how DCs contribute to the development of CRC is limited and more detailed studies are needed.

Tumor infiltrating MDSC are considered to negatively affect the anti-tumor response by regulating the TME with pro-inflammatory mediators to promote a more suppressive environment and tumor growth [399]. MDSC can originate from monocytic or polymorphonuclear origin and are therefore a heterogenous population with diverse mechanisms to supress the adaptive and innate immunity [386]. MDSC, monocytes and

neutrophils infiltrating CRC are associated with advanced cancer stages and decreased survival [392, 400]. Furthermore, there is evidence that the presence of MDSC has a negative impact on the outcome of checkpoint blockade therapies like PD-1 or PD-L1 [401].

Natural killer cells

NK cells are subdivided into two main populations of $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ with frequencies of about 90 % and 10% in blood, respectively. The $CD56^{dim}CD16^{+}$ NK subset exhibits more cytotoxic potential than the $CD56^{bright}CD16^{dim/-}$ subset which is known to produce large amounts of cytokines like IFN-γ when activated [402]. However, most studies that examined NK cells in CRC used only CD56 and therefore we will talk about NK cells as $CD3^{-}CD56^{+}$ cells. It has been shown that NK cells are reduced in CRC tissue as well as the expression of the NK cell receptors CD61, NKG2D, NKp30 and NKp46 [403, 404]. The low expression of NK cell receptors was an indicator of disease progression such as histological grade, depth of invasion and lymph node metastasis [403]. In a study by Halama et al., reduced infiltration of NK cells was also observed despite normal MHC-I expression on tumor cells and high levels of chemokines whereas T cell numbers were positively correlated with high chemokine levels [405]. Patients with low numbers of NK cells in the tumor tissue show a worse disease outcome with significantly shorter survival rates [406, 407]. Furthermore, NK cells from peripheral blood of CRC patients showed a deregulated phenotype with poor capabilities of secreting cytokines and degranulation [404]. The frequency of NK cells from blood was a prognostic indicator where high percentages correlated with longer survival time [408].

NK cells were part of the clustering results from the mass cytometry data in paper IV. They were not a focus of this thesis, but we could show that their frequency among lymphocytes is decreased in CRC.

Regulatory T cells

The first evidence that Treg supress anti-tumor immunity was shown in a study with mice where Treg were depleted by a monoclonal anti-CD25 antibody which led to the regression of tumor growth [409]. Treg accumulate in most human tumors and can comprise up to 50% of $CD4^+$ T cells in the TME. They are generally associated with a poor prognosis in most cancers but $Foxp3^+$ T cells have also been correlated to a favourable prognosis in CRC [410]. One major problem is the evaluation of the patient outcome by immunohistochemistry (IHC) with Foxp3 because there are several T cell populations which can express Foxp3 after activation but not necessarily display immunosuppressive properties [127]. Interestingly, a study by Saito et al. showed that there are two distinct populations in CRC that show different functions: $Foxp3^{hi}$ effector Treg and $Foxp3^{lo}$ T cells. $Foxp3^{lo}$ T cells are considered to be activated non-Treg cells showing pro-inflammatory effects and their accumulation in CRC correlates with a better prognosis compared to tumors with higher numbers of suppressive $Foxp3^{hi}$ effector Treg [411]. Treg in the TME are generally having an activated and suppressive phenotype which differs from unaffected peripheral tissues shown by the upregulation of many inhibitory/checkpoint molecules like CTLA-4, T cell immunoreceptor with Ig and ITIM domains (TIGIT), Lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), OX-40, PD-1 and ICOS [412]. Moreover, apoptotic Treg in the TME are capable of releasing large amounts of immunosuppressive adenosine by converting ATP through the ecto-enzymes CD39 and CD73 [413]. One study by Kumagai et al. in non-small cell lung cancer, gastric cancer and malignant melanoma also showed a correlation of $PD\text{-}1^+$ Treg and the resistance to PD-1 immune therapy suggesting that Treg were activated by the treatment [414]. Several studies show the negative effects of Treg on the TME and T cell anti-tumor immunity, but depleting Treg systemically poses risks of immune-related adverse events like autoimmunity and related toxicities [415].

Treg and their role in the TME especially how they influence T cell migration has been the focus of this thesis. We have studied the effects of Treg depletion on endothelial cells and the migration of T cells into tumors

in paper I and II. Furthermore, we examined the effect of Treg depletion on T cell effector functions in paper III. The thesis uncovers new mechanisms of how Treg influence T cell migration into tumors and adds to existing evidence that Treg are detrimental for CRC patients.

Tumor-infiltrating lymphocytes

There are several lymphocyte subsets that can infiltrate a tumor, but tumor-infiltrating lymphocytes (TIL) are mostly referred to as Th1 effector cells and CTL which are implicated in killing tumor cells. However, there is a strong focus on Th1, Th17 and CTL as they have been associated to a particular clinal outcome. We will focus on these subsets and discuss other cell types in separate chapters. Patients with IL-17 producing Th17 cells have a poor prognosis while the presence of a Th1 type response or CTL has been linked to a prolonged disease-free survival [416]. Tumors with heterogeneous densities of $CD8^+$ T cells could be further differentiated into good or bad prognosis by including the density of IL-17 producing cells. Interestingly, the same study showed that a Th2 gene signatures had no impact on the patient outcome while a very recent study established a worse outcome [417]. In contrast, the survival benefit of a Th1 response in CRC is well established and linked to MSI tumors and the CMS1 type. It has been suggested that the frameshift mutations caused by the broken DNA repair machinery create a higher mutational burden which seem to explain the recognition of neoantigens by the adaptive immune system and a strong Th1 response in these tumors [418]. Furthermore, TIL from MSI tumors have increased expression of the checkpoint molecules PD-1, PD-L1, CTLA-4, LAG-3 and TIM-3 [419]. The density of TIL expressing the Th1 master regulator Tbet is also linked to a favourable outcome as well as to PD-1 expression in mainly MSI tumors but also a small fraction of MSS tumors [420]. One study by Mlecnik et al. further showed that MSI tumors were associated with immunoediting, a high density of Th1 and effector-memory cells, PD-1 expression, and mutation-specific CTL [421]. The Th1 response is also linked to an increased expression of CX3CL1 and the memory T cell response to an expression of CXCL9 and CXCL10 [365]. Additionally, it has been shown that Th17 cells inhibit the migration

of CTL by downregulation of CXCR3 via the expression of IL-17A [422]. Most MSS tumors show little T cell infiltration and are therefore considered to be immune-excluded or -deserted tumors (CMS2-CMS4). MSI tumors benefit the most from checkpoint blockade therapies because the existing anti-tumor immune response can be elicited, but MSS tumors might need neoantigen vaccines to improve the infiltration by Th1 and CTL before commencing immunotherapies [309].

There has been little data about the role of CD8αα in tumor tissue compared to the intensively studied CD8αβ subset. We examined both subsets in our mouse model in paper III and could show that they are differently affected by Treg.

Unconventional T cells

There are few studies in human CRC about the effects of unconventional T cells on the TME or the patient outcome. The phenotype of NKT cells in CRC has not been described probably due to their low abundance in tissues. In $APC^{Min/+}$ mice, NKT cells have been demonstrated to promote intestinal tumor development through the suppression of the Th1 response and accumulation of Treg [423]. In contrast, studies in other tumors have found a protective effect of iNKT cells by killing TAM in the TME and by converting MDSC with suppressive activity into APCs [424, 425]. In general, iNKT cells are considered to be beneficial for the anti tumor immunity while dNKT cells show a more suppressive or regulatory function in tumors [426].

MAIT cells accumulate in CRC tissue and show an exhausted phenotype [427, 428]. Exhausted MAIT cells are linked to a poor prognosis in hepatocellular carcinoma [429]. Furthermore, increased numbers of MAIT cells in CRC either assessed by their gene signature or IHC predict poor overall survival [430, 431].

The presence of γδ T cells in human tumors has been associated with a favourable outcome across 25 solid tumors in a meta-analysis of gene expression signatures [393]. However, most reports have found a negative

impact of γδ T cells which seems to depend on the TME. Wu et al. showed that Vδ1 cells in CRC were a major source for IL-17. They were polarized in an IL-23 dependent manner by inflammatory DCs which had been activated by microbial products caused by a loss of the barrier function [432]. Furthermore, a novel population of $CD39^+$γδ Treg with immunosuppressive effects has been identified [433]. Both γδT17 and $CD39^+$γδ Treg have been correlated to clinicopathological factors in patients with CRC. Consistent with these findings, Rong et al. found a high percentage of tumor infiltrating $Vδ1^+$ and low percentage of $Vδ2^+$ cells compared to unaffected tissue. $Vδ1^+$ cells showed strong inhibitory functions and were positively linked to the tumor stage [434].

In paper IV of this thesis, we investigated unconventional T cells and their expression of NK cell receptors and exhaustion markers which has not been comprehensively studied in colon tumor tissues. Furthermore, we show a detailed analysis of γδ T cells subsets and their location in tissues.

Checkpoint inhibitors and immunotherapy

Checkpoint molecules are inhibitory co-receptors on T cells that are important to restrict T cell functions in normal physiological settings, but this mechanism can by exploited by tumors to reduce anti-tumor immunity. Several checkpoint molecules like PD-1, CTLA-4, TIM-3, LAG-3, OX-40, 4-1BB and more have been identified and are under investigation to be used in immunotherapy [435]. Anti-PD-1 (and anti-PD-L1) antibody treatment has been the biggest success of immunotherapy in humans so far and therefore the discovery of PD-1 on T cells was acknowledged with a Nobel prize for medicine in 2018. Targeting the PD-1/PD-L1 axis has been most effective in melanoma but a subgroup of colorectal cancer patients is also responsive to the treatment [436].

The combination of Treg depletion and PD-1 therapy is so far not possible in patients because there is currently no drug which depletes Treg selectively. Reports on the depletion of Treg by CTLA-4 immunotherapy are conflicting because it seems to lead to depletion in a mouse model but not in human tumors [437, 438]. Therefore, in paper III we studied the

combinatory effect of these treatments in the mouse model described in the main methods. These studies can give indications about the benefit from such a combined therapy for MSS tumors when selective targeting of Treg will be possible in the future.

AIM

This thesis investigates by which means Treg can supress the T cell migration into intestinal tumors as well as the type of T_{EFF} cells that are affected. This is important for the evaluation of Treg depletion as a treatment option for CRC patients. Additionally, we performed a comprehensive analysis of unconventional T cells in CRC tissue which sets the foundation for future studies to harness their potential in adoptive cell therapy.

Specific Aims:

- Define the role of CXCR3 and its ligands in T cell migration into tumors
- Investigate the mechanisms Treg use to supress transendothelial migration of T cells into tumors
- Examine the effect of Treg depletion on different T cell populations in tumors
- Explore unconventional T cell populations and their phenotype in human CRC

MAIN METHODS

$APC^{Min/+}$/DEREG mice

Additionally to the $APC^{Min/+}$ mouse model, we employed the DEpletion of REGulatory T cells (DEREG) mouse model [439] to study the influence of Treg on endothelial cells and the migration of T cells. DEREG mice have been generated through bacterial artificial chromosome transgenesis and harbour a DTR-eGFP transgene under the control of the Foxp3 promoter. Foxp3 is the master regulator for Treg in mice. This enabled us to deplete Treg by administration of diphtheria toxin (DT) as mice have a high tolerance to DT (10^3-10^5 higher dose compared to humans). One of the drawbacks is that the incomplete transgene expression leaves about 2-5% Treg after depletion and DT injection must be repeated after a week. Furthermore, a treatment longer than two weeks leads to incomplete depletion because transgene negative $Foxp3^+$ Treg increase in number.

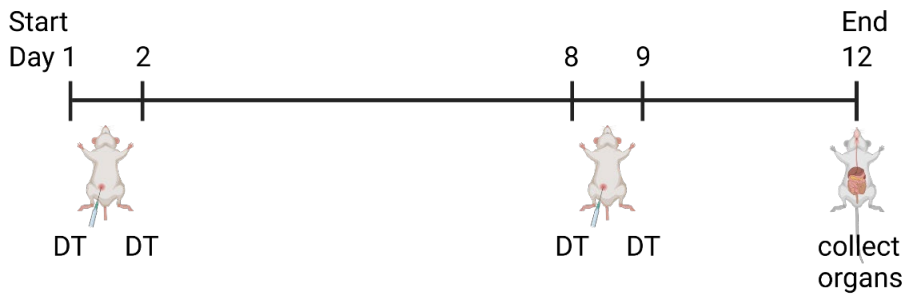

Figure 11: Schedule for the DT injection of mice. Created with BioRender.com.

$APC^{Min/+}$/DEREG mice were generated by breeding male $APC^{Min/+}$ mice (C57BL/6 background) with female DEREG mice. The $APC^{Min/+}$ genotype was determined by polymerase chain reaction of the APC gene in tail biopsies and the DEREG phenotype by flow cytometry analysing Foxp3-GFP positive cells in blood [440]. We injected DT intraperitoneal (i.p.) into $APC^{Min/+}$/DEREG mice for short-term depletion of Treg

(Figure 11). $APC^{Min/+}$ mice were injected with DT according to the same schedule and served as Treg competent control mice. $APC^{Min/+}$/DEREG and $APC^{Min/+}$ mice were used in paper I, II and III.

PD-1 immunotherapy

We also treated mice with i.p. injections of PD-1 or isotype control antibodies (kindly provided by Dr. Rene de Waal Malefyt, Merck Inc.) combined with DT in paper III (Figure 12).

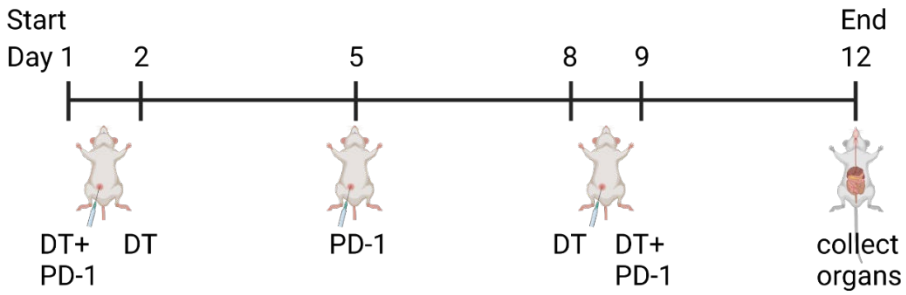

Figure 12: Schedule for the combination of PD-1/isotype and DT injections of mice. Created with BioRender.com.

Feeding of PDCC

In paper II we investigated the effect of the nSMase2 inhibitor phenyl (*R*)-(1-(3-(3,4-dimethoxyphenyl)-2,6-dimethylimidazo[1,2-*b*]pyridazin-8-yl)pyrrolidin-3-yl)carbamate (PDDC) *in vivo* by providing PDDC in the diet of the mice (Figure 13). This was an appropriate and safe way to keep sustained levels of PDCC in plasma and effectively inhibit nSMase2 [441]. Vehicle chow was used as a control for PDCC untreated mice as well as seven days before the start of the experiment to let the mice adapt to the new food.

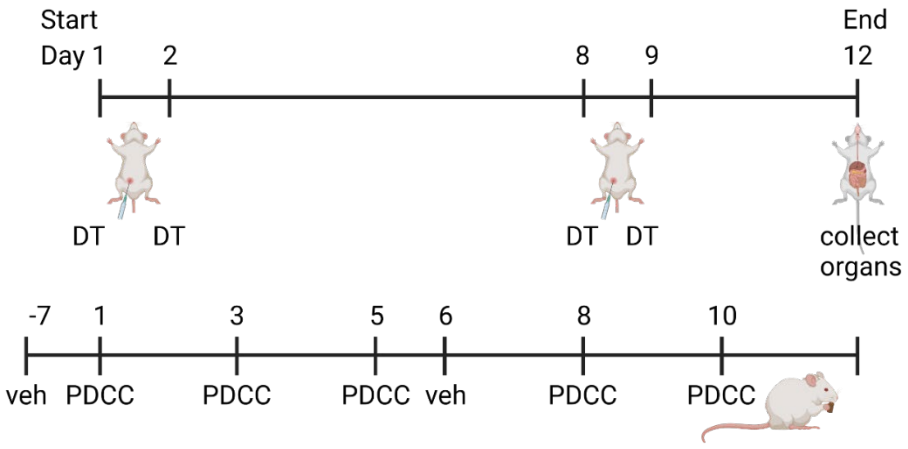

Figure 13: Schedule for the combination of DT injections and feeding of PDCC inhibitor/vehicle chow. Created with BioRender.com.

Collection of tissue and cell isolation

Murine samples

Mice were housed in a specific pathogen free environment and all experiments were approved by the regional animal ethics committee in Gothenburg. Small intestinal tumors and unaffected small intestinal tissue were collected and quickly processed to isolate immune cells and endothelial cells (Figure 14). First, epithelial cells were removed by Ethylenediaminetetraacetic acid (EDTA) containing buffer. This fraction also contained the IEL. Second, the tissue was enzymatically digested with either collagenase D to isolate lymphocytes or dispase, deoxyribonuclease and collagenase P to isolate endothelial cells. Cell suspensions were analysed with flow cytometry and sorted for some experiments.

Human samples

Colon tumor and unaffected colon tissues (>10 cm distance from tumor) were collected during curative surgery at Östra Sjukhuset. The studies were permitted by the Regional Research Ethics Committee of Västra Götaland and all volunteers gave informed consent before their participation. Patients which had undergone chemotherapy, radiotherapy or treatment

with immunomodulatory drugs were excluded from studies as well as patients with diagnosed autoimmune diseases. Patient samples were immediately brought to the laboratory to isolate immune cells and endothelial cells (Figure 14). Epithelial cells were removed by EDTA containing buffer and subsequently the tissue was enzymatically digested with a mixture of Liberase™ and deoxyribonuclease. Cells were incubated with fluorochrome labelled antibodies and analysed by flow cytometry or sorted.

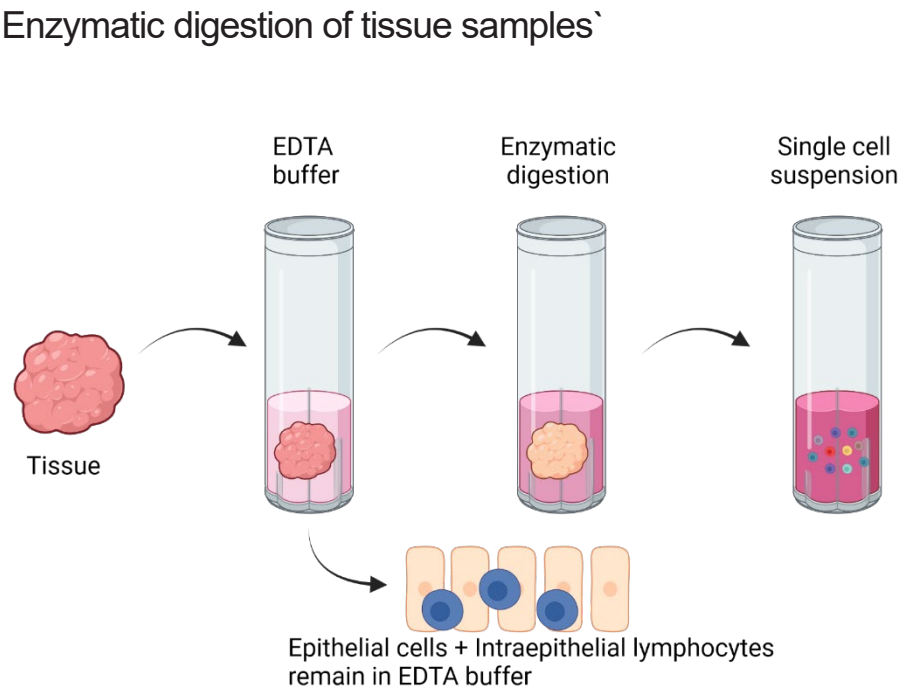

Figure 14: Workflow for enzymatic digestion of murine and human tissues to obtain a single cell suspension. Created with BioRender.com.

Enzymatic digestion is widely used to extract immune cells from tissue and usually gives a good cell yield. The main disadvantage is the lack of specificity of some enzymes leading to unwanted loss of lineage markers like CD8 (human samples) and especially chemokine receptors on the

surface of the cell. One always needs to be aware of this limitation and utilize proper controls e.g. use digestion enzymes with peripheral blood mononuclear cells and assess the loss of surface molecules. In some cases, surface molecules can be recovered by incubation at physiological conditions for some hours or overnight. Tissue disintegration without enzymes is possible but can lead to cell death and low yields due to the mechanical stress the cells are exposed to. We have been using small samples sizes of < 1g where enzymatic digestion is necessary to extract an appropriate number of cells. In case of better access to tissue, one could use a mechanical tissue disintegration approach with less or no enzymatic digestion and a further cleaning step by gradient centrifugation.

Flow cytometry

Flow cytometry provides high throughput multiparameter analysis of single cells in solution. Highly specific antibodies against molecules (mainly proteins) of interest are conjugated with a wide range of different fluorochromes. The cells of interest are processed into a single cell solution and labelled with these commercially available antibodies. The labelled sample is acquired on the flow cytometer (Figure 15). The flow cytometer takes up the sample through a pressurized system or peristaltic pumps and concentrates the cells into a stream of single cells which is surrounded by the so-called sheath fluid. The single cells pass an arrangement of usually 3-5 consecutive lasers where the fluorescent fluorochromes are excited with one specific wavelength. Every fluorochrome emits photons at a specific wavelength interval which is longer than the excitation wavelength. For every laser, this light is captured by optics perpendicular to the laser beam and is guided into an array of fluorescence filters ordered from lower to higher (or higher to lower) wavelength intervals. Each filter permits light, specific for the emission wavelength interval of a certain fluorochrome, through to a photon multiplier tube (PMT) or avalanche photodiode (APD). The light with higher wavelength gets reflected to the next filter. At the PMT or APD, the photons get converted into a digital signal which can then be visualized in the software. Due to the nature of

overlapping excitation and emission spectra of the fluorochromes, fluorescence spill over needs to be compensated which leads to a loss of signal resolution. Additionally, the cell size and granularity (complexity) are measured by forward scattered and side scattered light of the blue laser, respectively. The data is usually visualized as a dot plot with one parameter on each axis and cell populations are gated manually in the appropriate software. The recent development towards 30-40 parameters requires more sophisticated analysis tools like unsupervised clustering.

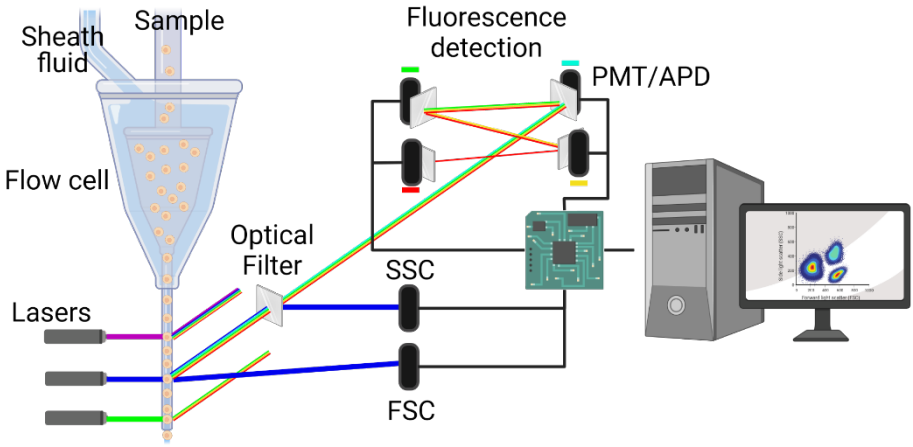

Figure 15: Schematic picture of the components of a flow cytometer (fluidics, optics and digital components). Created with BioRender.com.

Flow cytometry is the most important methods in the field of immunology and is heavily utilized to gain more insights into the complexity of different cell populations of the immune system. The high throughput of about 7000-10000 cells/second enables for fast data acquisition and detection of rare cell populations. The labelling of cells is rather easy and running a flow cytometer does not require special knowledge. Fluorescence spill-over compensation can be a hindrance if many cell markers are studied on the same cell, but good antibody panel design can minimize these effects. One other disadvantage is the autofluorescence nature of certain cell types e.g.

Mφs which pose a challenge in fluorescence-based assays. As the number of analysed parameters steadily increases with every new generation of flow cytometers, the analysis gets more complex and a big challenge that requires bioinformatics knowledge.

Mass cytometry

Mass cytometry delivers similar data as flow cytometry does, but heavy metal tagged antibodies are used for labelling instead of fluorochrome conjugated antibodies. The mass cytometer has a much lower throughput of about 300-500 events per second, but spectral overlap is neglectable. This makes it possible to detect 40-50 proteins on the same cell, increasing the amount of information per cell drastically. The number of labels is so far limited to about 50 by the purification processes for heavy metal isotopes while the machine has the capability to distinguish 135 different masses. The availability of many channels enables bar coding of samples which reduces batch to batch variability and reduces sample preparation times. Additionally, autofluorescence does not exist with this method which is a huge advantage for studying Mφs or other cells with higher complexity. On the other hand, cells are atomized before detection and cannot be distinguished by their granularity or size like in flow cytometry. The biggest challenge by far is the amount of data and to create an analysis pipeline which lets one explore the biological differences of the samples. Manual gating commonly used in flow cytometry is not an option for this amount of data and the introduction of spectral flow cytometry indicates that the competence for high dimensional data analysis is becoming inevitable.

Figure 16 shows a schematic view of the general principle of a mass cytometer. The cells are run into the machine via a pressured sample line that pushes the cell suspension into the nebulizer where a spray of droplets is formed. Each droplet usually harbours one cell. The droplets fly through a heated chamber to vaporize the liquid and the cells reach a plasma that atomizes/ionizes them. Each cell forms an ion cloud which is passing from atmospheric pressure through a vacuum interface into vacuum which is

needed for the ion optics. Subsequently, the ions are going through a quadrupole (high-pass ion filter) which removes all non-ions, photons, and low mass ions e.g. carbon or oxygen which are very abundant in all cells. Finally, ion clouds get pushed every 13 µs by a high voltage pulse into the Time-of-Flight chamber where ions are separated by their atomic mass. Light ions are flying at a higher speed than heavy ions reaching the detector first. Every mass has a detection band of about 20–25 ns. The detector signal is translated into a digital signal that can then be used to identify the cell events (gaussian distribution of signal) and register the expression of the protein markers for each event. Finally, the results can be shown in a dot plot like flow cytometry.

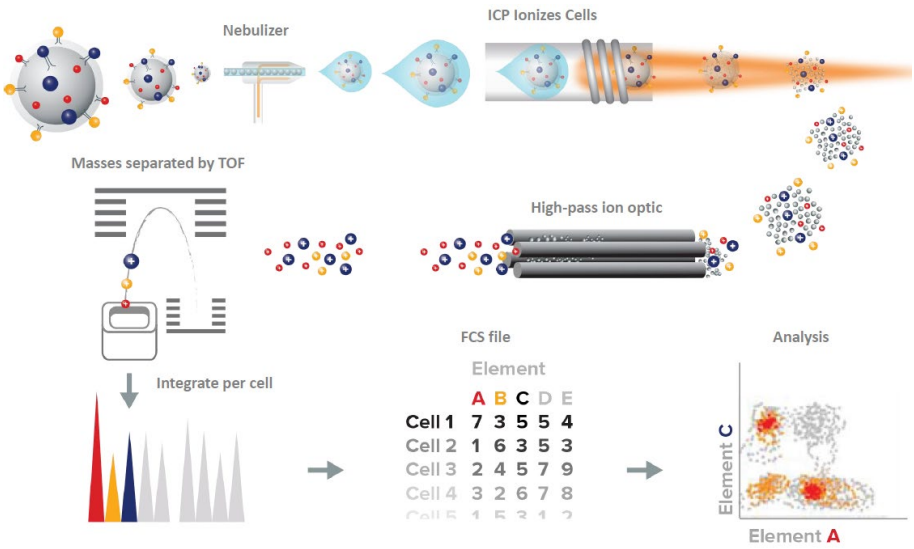

Figure 16: Schematic picture of the general principle of a mass cytometer. Courtesy of Fluidigm.

High-dimensional data analysis

High-dimensional data experiments are commonly used in an exploratory manner which is different from the traditional way of formulating a specific hypothesis and narrowing down on a specific target to investigate. This can quickly lead to new discoveries because of the unbiased nature of these methods. At the same time, one must be careful to not overlook artefacts or errors in the analysis as we have little insight in the decision tree of the algorithms. Additional experiments with other methods are always required to confirm new findings.

The parameters analysed per cell have been increasing rapidly through reduction of costs and better equipment. Researchers are generating large amounts of high-dimensional data from flow cytometry, mass cytometry or other methods like single cell RNAseq. This poses new challenges for the analysis and requires a different way of thinking. It is difficult to translate a panel with 40+ markers into results because serial gating traditionally employed in flow cytometry is not practical. The experiment design beforehand becomes more important as one might not be able to correct certain variations manually after the data has been acquired. It is important to have good quality samples to start with and that the equipment is in a good condition to avoid technical variance. As one mass cytometry application specialist has put it: "crap in, crap out". Additionally, it is crucial to avoid batch effects between samples because the employed algorithms are not informed and do take any data and process it in the same way. Barcoding of samples and normalization of data between samples can overcome some of the problems. Dimension reduction and unsupervised clustering are two very common algorithm classes that are used to visualize data in a 2D space and classify cells into different clusters, respectively (Figure 17). Further analysis is needed to extract quantitative results and show significant differences e.g. frequencies of cell clusters between a healthy and disease state.

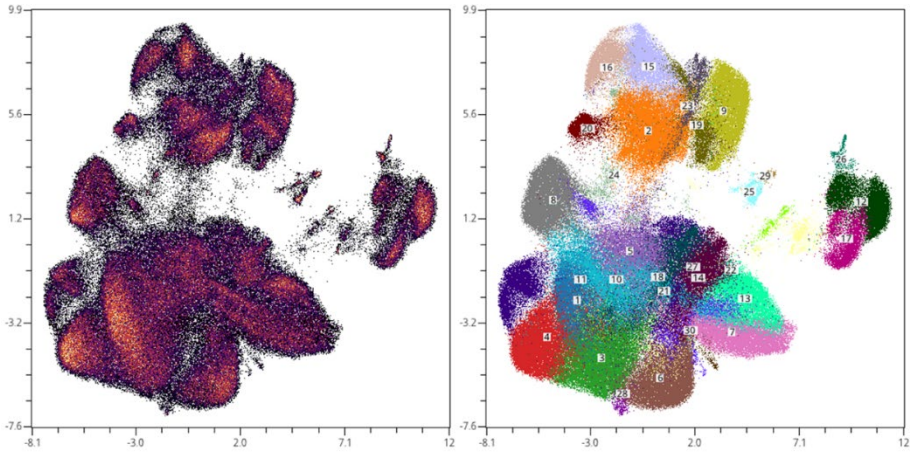

Figure 17: Dimensionality reduction of mass cytometry data with the UMAP algorithm (left, brighter colour = higher density) and overlayed unsupervised clustering with the Phenograph algorithm (right)

Immunofluorescence microscopy

In contrast to flow cytometry, IHC preserves the integrity of the tissue and thereby the location of cells at the time point of tissue extraction. We employed IHC in frozen murine tissue to visualize the location of different T cell subsets in tumors and unaffected tissue (Paper III) as well as to monitor the histology of the small intestine after treatment of mice with the PDCC inhibitor in Paper II (Figure 23). Furthermore, we stained formalin-fixed paraffin-embedded (FFPE) tissue from CRC patients to identify and quantify γδ T cells in paper IV. Figure 18 shows a typical example of the IHC workflow including quantification which was employed in this thesis. The importance of the location of T cells in tumors has been greatly recognised in the last decade and has led to new diagnostics tools like the Immunoscore [442]. IHC has been limited to the detection of 6-7 targets in the last years, but recent developments are extending this limit to about 30-40 now. As with mass cytometry, there is a transition from heavy limitations on the technical side to the difficulties of the analysis of this highly complex data. Advanced image analysis including artificial intelligence is developing this method into a high

throughput system like flow cytometry while preserving the spatial context of the cells. This is already applied to get a better understanding of the spatial relationship between different immune cells and cancer cells which might determine the type of therapy patients receive in the future.

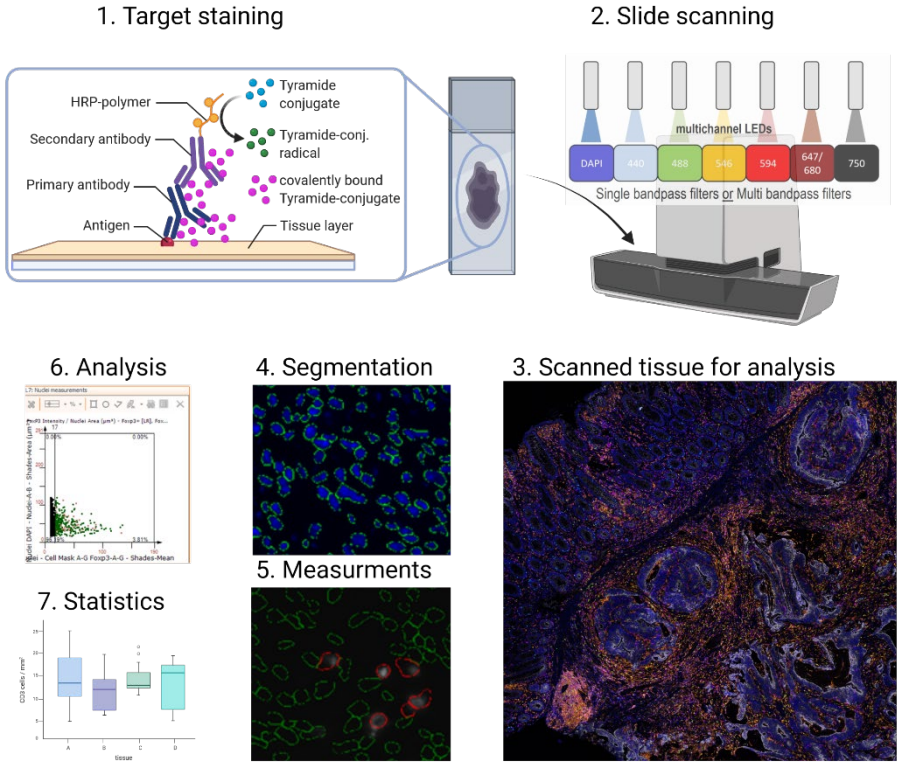

Figure 18: Workflow for IHC from tissue staining to results for a FFPE section. (1) Tyramide amplification method is applied for each antibody target (up to 6). (2) Whole tissue is scanned using a scanning microscope with a narrow band pass filter set. (3) Example of a tissue scan from a colon tumor with immune cell infiltrate. (4) Nuclei are segmented based on stained DNA (DAPI). (5) Measurements of cellular markers like surface expression, intracellular expression, or distances between cells. (6) Analysis of detected events. (7) Results are interpreted with statistical methods. Created with BioRender.com. Filter set image is a courtesy from Kromnigon, Sweden.

Biobanks are a big advantage for IHC on FFPE tissue. One can study cells in the tissue of many patients and correlate the results to survival data. On the other hand, proper tissue processing is crucial and has not always been done in a reproducible way in older archived tissue samples. Frozen tissues are easier to process but histological features are less preserved because of the freezing process. The staining process of frozen tissue is easier, faster, and usually more cost-effective. Frozen tissue is commonly used in murine studies while FFPE tissue is very accessible for human studies.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) is an easy and widely used method to quantify the RNA of a particular gene. It is a good and cheap method to confirm results from messenger RNA (mRNA) sequencing experiments. Proteins are constructed by translation of mRNAs into amino acids but because of the complex regulation processes, one must be careful to conclude gene expression solely from mRNA expression. First, complementary DNA (cDNA) is prepared from lysed cells or whole tissue lysates (bulk). Second, cDNA is amplified, but only if the target probe binds to the mRNA of interest. In each amplification cycle, fluorescent probes are incorporated into the newly formed double-stranded cDNA product and the signal can be read in real-time after each cycle, hence real-time PCR (Figure 19). Fluorescent signals are below the detection limit in the beginning, but the exponential amplification process will show a rising curve in fluorescence. The number of cycles to reach a certain threshold can be used for relative quantification as well as absolute quantification with a calibration curve. qPCR was used in paper I and II.

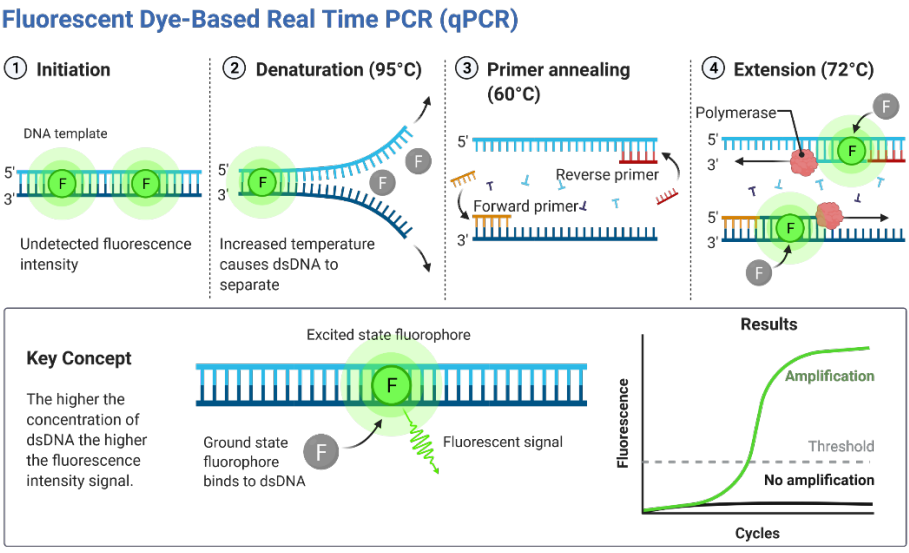

Figure 19: Schematic view of the reactions and the concept of the qPCR method. Adapted from "Fluorescent Dye-Based Real Time PCR (qPCR)" by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

Targeted and non-targeted RNA sequencing

We utilized non-targeted RNAseq in paper II for sequencing of RNA from sorted murine blood vessel endothelial cells of intestinal tumors from DT treated $APC^{Min/+}$ and $APC^{Min/+}$/DEREG, i.e. tumors with or without Treg. Endothelial cells were identified as $CD45^{-}EpCAM^{-}CD31^{+}Podoplanin^{-}$ cells and sorted on a BD FACSAria™ II Cell Sorter. RNA was isolated, amplified and sequenced with Illumina sequencing at Science for Life Laboratory, Stockholm, Sweden.

Non-targeted sequencing can be seen as a hypothesis generating approach where all RNA molecules are sequenced and aligned to an existing genome. In most cases, the interest is concentrated on mRNA which is translated to proteins in the cell. For this purpose, ribosomal RNA is usually removed in the amplification process as it is very abundant, and therefore sequencing would be less efficient. The advantage of untargeted

sequencing is the unbiased nature of this approach as one examines all possible transcripts, but this also means that the analysis is much more complex and usually requires help from bioinformaticians. The challenge is to identify genes of interest in perhaps hundreds of up- or downregulated genes which are not necessarily directly related to one's research question. One needs to make sense of the data and use existing literature and databases to aid in this process.

Targeted RNAseq on the other hand, identifies a specific set of mRNA molecules in the sample. This approach is fast and easy because all the targeted genes are usually well characterized, and their general functions are known. Hence, there are pre-designed data analysis pipelines where one can explore whole networks of genes without any knowledge of bioinformatics. One might be interested to compare inflammatory markers in between two treatment groups so it is not helpful to look at all mRNA, but rather at a specific set of genes that are well established for inflammatory processes. In paper II we employed this method (Nanostring) to look at a pre-designed immunology panel of 594 genes in treated (with the nSMase2 inhibitor 2,6-Dimethoxy-4-(5-Phenyl-4-Thiophen-2-yl-1H-Imidazol-2-yl)-Phenol (DPTIP)) and non-treated Human umbilical vein endothelial cells (HUVEC) cells.

Both non-targeted and targeted RNAseq are quantitative in the way that the expression of a certain gene transcript is measured relative to all transcripts in the sample. Absolute quantification can be performed with spike-ins (mixed in samples of RNA at known concentrations) but it is not widely used. Transcript levels are usually used as a substitute for protein abundance but due to post transcriptional modifications, they are most often not equivalent.

Primary endothelial cell lines and Treg co-cultures

Both HUVEC and human microvascular endothelial cells were utilised in paper II in the inhibition experiments and subsequent qPCR/Nanostring analysis, or flow cytometry analysis was applied. Furthermore, HUVEC

cells were utilized for co-culture experiments with Treg and Treg conditioned media (Figure 20). Endothelial cell lines are an easy to use *in-vitro* system to test the effect of specific compounds like DPTIP or cytokines which is difficult to do *in-vivo* due to the complexity of the environment and a more difficult read out of the effect. Though, this is also a disadvantage of these cultures because cells do not grow in their natural environment and do not fully mimic an endothelial blood vessel. This is especially true for solid tumors where the TME can be unique and affect cells in a different manner than in unaffected tissue. Co-cultures use the same principle but use cells that either mediate changes through cell to cell contact or secretion of mediator molecules.

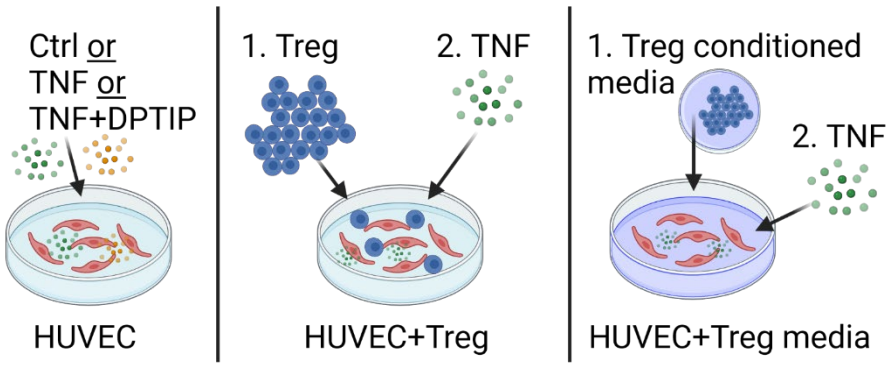

Figure 20: Cell cultures of endothelial cells (HUVEC). Addition of TNF and/or the inhibitor DPTIP (left) to the culture. Co-culture of Treg with HUVEC cells and subsequent TNF stimulation (middle). Culture of HUVEC with Treg condition media and subsequent stimulation with TNF (right). Created with BioRender.com.

Statistics

Statistical significance was evaluated using the Mann–Whitney test for unpaired and Wilcoxon signed-rank test for paired analysis. p values of < 0.05 were considered significant. Horizontal lines/bars in the figures show the median. Statistical analyses were performed in GraphPad PRISM software (GraphPad Software).

RESULTS AND DISCUSSION

Paper I - Regulatory T cells control endothelial chemokine production and migration of T cells into intestinal tumors of $APC^{Min/+}$ mice

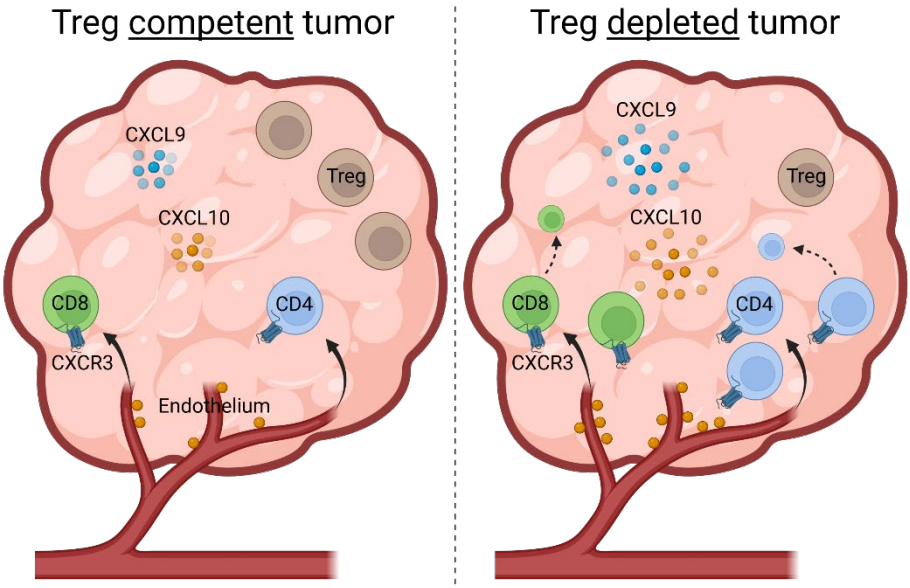

Figure 21: Graphical abstract of paper I. Tumors from Treg competent $APC^{Min/+}$ compared to Treg depleted $APC^{Min/+}$/DEREG mice. CXCL9 and CXCL10 expression in tumors is increased after Treg depletion as well as CXCL10 expression by endothelial cells. Migration into tumors is dependent on CXCR3 expression on T cells. Treg depletion increases the migration of $CD4^+$ and $CD8^+$ T cells into tumors and their proliferation. Created with BioRender.com.

Paper I follows up on a previous finding in our group that showed an increased accumulation of $CXCR3^+$ T cells in tumors after Treg depletion in $APC^{Min/+}$ mice [314]. Furthermore, Treg depletion increased expression of the chemokines CXCL9 and CXCL10 measured in bulk RNA from tumors which suggested a role of the CXCR3 receptor in the migration of

T cells. Previously, our group also showed that Treg from blood of colon cancer patients, but not healthy individuals, inhibit transendothelial migration of T cells *in vitro* [443]. Therefore, we were particularly interested if the expression of chemokines on endothelial cells as well as adhesion molecules affects migration of T cells into tumors. We specifically studied the role of CXCR3 in the migration of T cells and if that could contribute to the recruitment of T cells in addition to their proliferation in tumors.

Treg depletion increases migration of T cells into tumors

First, we assessed if the accumulation of T cells in Treg depleted tumors stems from increased migration or only their local proliferation. We labelled lymphocytes from MLN with Carboxyfluorescein succinimidyl ester (CFSE) and adoptively transferred them by injection into the tail vein of Treg depleted $APC^{Min/+}$/DEREG mice or Treg competent $APC^{Min/+}$ ctrl mice to determine the migration of these cells into different organs. We found a significant increase of $CFSE^+$ lymphocytes in tumors of Treg depleted mice but Treg depletion did not alter the migration into unaffected intestinal tissue, MLN, or spleens (Paper I, Fig. 1a). A more detailed view at the lymphocyte populations showed that $CFSE^+$ CD4 and CD8 T cells were both increased in tumors when looking at the number of cells per gram tissue (Paper I, Fig. 1c) but the frequencies of both subsets remained the same (Paper I, Fig. 1b). This could be explained by an increased infiltration of unlabelled endogenous CD4 and CD8 T cells into the tumors at the same time. Additionally, the absence of Treg does increase the proliferation of T cells measured by Ki-67 (data not shown) which also contributes to the increase of endogenous CD4 and CD8 T cells compared to $CFSE^+$ labelled cells. The effect of the Treg depletion on the migration and proliferation of $CD4^+$ non-Treg cells must be even larger then indicated because $CD4^+$ Treg accumulate in tumors, and their depletion leads to a decrease of total $CD4^+$ T cells. We also showed through staining of CD25 that $CFSE^+$ Treg recruitment into Treg depleted tumors decreased (Paper I, Fig. 1d). Treg depletion seems to alter the TME to inhibit further migration and/or proliferation of Treg which might also happen in a self-sustaining manner where Treg create a favourable TME

to recruit more Treg. Three studies in mice, two with subcutaneous MO4 melanoma and one with subcutaneous MC38 colon adenocarcinoma, have shown that Treg depletion leads to $CD8^+$ T cell infiltration and tumor regression [444-446]. However, we did not see any regression in our $APC^{Min/+}$/DEREG tumors in the short time interval of depletion and the TME in our model is most likely inherently different from a subcutaneous melanoma model. Nevertheless, we show that Treg depletion increases the infiltration and proliferation of $CD4^+$ and $CD8^+$ T cells in intestinal tumors which could be a valuable approach when combined with tumor vaccines and/or antibody immunotherapies.

CXCR3 is crucial for T cell migration into tumors

As mentioned earlier, the CXCR3-mediated chemotaxis might play a role in the recruitment of T cells into intestinal tumors in $APC^{Min/+}$ mice. We used the adoptive cell transfer system again but this time by labelling splenocytes with CFSE or CellTrace™ Far Red dye. Additionally, one fraction of cells was incubated with the CXCR3 agonist AMG487 [447] and equal amounts of non-treated and treated cells were injected into mice. We isolated all lymphocytes two days after transfer and compared the ratio of untreated/treated cells which migrated into specific tissues. The blocking of CXCR3 showed a significant reduction of lymphocytes migrating into unaffected intestinal tissue and intestinal tumors while the migration into MLN and the spleen was just slightly reduced (Paper I, Fig. 3a). Lymphoid tissues use different mechanisms of recruiting lymphocytes through HEV e.g. CCR7-CCL19/21 or α4β7/MAdCAM-1 and therefore CXCR3 blocking does not impact the migration to these tissues. In contrast, T_{EFF} cells migrating to peripheral tissues like the small intestine depend on the migration through CXCR3 [448] demonstrated by a 1,5-fold (median) reduction of lymphocyte migration into unaffected tissue and 3-fold into tumors. Furthermore, the hampered migration of specifically $CD4^+$ T cells was even more pronounced in tumors compared to the other organs and all lymphocytes (Paper I, Fig. 3b). It has been shown that the expression of CXCR3 ligands in the TME are associated with a higher T cell infiltration and prolonged disease-free survival [365] whereas a lack of

CXCL9 and CXCL10 are linked to a worse prognosis [449, 450]. Interestingly, when we combined CXCR3 blocking and Treg depletion there was no cumulative increase in migration of lymphocytes into tumors indicating that CXCR3 is the preferred mechanism of T cell migration into tumors.

Treg depletion does not alter selectins or their ligands *in vivo*

Chemokines attract T cells for transendothelial migration by activating integrins on T cells that bind to their ligands on endothelial cells to facilitate firm adhesion. However, selectins on endothelial cells are crucial for the initial tethering and rolling of T cells. Therefore, we investigated the role of E-selectin and P-selectin in the migration of T cells into intestinal tumors of $APC^{Min/+}$ mice. E-Selectin and P-Selectin expression was measured by flow cytometry on $CD31^+$ endothelial cells whereas the ligands PSGL-1 and cutaneous lymphocyte antigen (CLA) were detected on $CD4^+$ and $CD8^+$ T cells. We found that the expression of E-Selectin was decreased in tumor tissue compared to unaffected tissue but P- selectin was increased (Paper I, Fig. 2a). Both selectins are most often increased in an inflammatory site and P-selectin is rapidly upregulated whereas E-selectin is expressed at a later stage as it requires de novo synthesis [231]. The TME might inhibit the synthesis and/or the transport of E-selectin to the cell surface of endothelial cells and therefore decrease migration of T_{EFF} cells into the tumor. PSGL-1 was expressed on virtually all $CD4^+$ and $CD8^+$ T cells from unaffected and tumor tissue except for $CD4^+$ T cells where PSGL-1 was slightly increased in tumors compared to unaffected tissue (Paper I, Fig. 2c). PSGL-1 has been described to negatively regulate T cell responses in viral infections and cancer which might explain the increased frequencies of $CD4^+PSGL\text{-}1^+$ T cells in tumors [451]. Interestingly, neither the expression of E-selectin and P-selectin nor the expression of CLA and PSGL-1 were affected by Treg depletion regardless of the tissue type. This means that other cell adhesion molecule interactions are more likely to be influenced by Treg depletion but have not been identified.

Endothelial cells increase CXCL10 expression after Treg depletion *in vivo*

We showed that CXCR3-mediated T cell infiltration into tumors is important and that there were higher levels of CXCL10 mRNA found in tumor tissue compared to unaffected tissue in $APC^{Min/+}$ mice. CXCL10 can be expressed in various cell types like epithelial cells, endothelial cells, stromal cells, neutrophils or monocytes and therefore influence the TME [259]. We were particularly interested in the expression of CXCL10 on endothelial cells as they play an important role in the migration of cells. CXCL9 and CXCL10 were difficult to detect with antibodies at the time we carried out the study and therefore we used the Primeflow® mRNA assay to detect CXCL10 mRNA in endothelial cell *ex vivo* without stimulation. We found few cells (< 5% median) that expressed CXCL10 among lymphatic endothelial cells, DCs and epithelial cells (Paper I, Fig. 4). In contrast, about 8-13% of Mφs and blood endothelial cells expressed CXCL10 whereas T cells showed a large variation between samples (1-29%). Furthermore, Mφ frequencies and absolute numbers per grams of tissue were increased in tumors compared to unaffected tissue (Paper I, Fig. 5 and Supp. Fig. 4). It has been shown that CXCL10 expressing Mφs greatly contribute to anti-tumor immunity and are required for successful checkpoint blockade therapy in different solid tumors [452, 453]. Interestingly, there was no difference regarding tissue type (unaffected and tumor) or Treg depletion for any cell population except endothelial cells where the frequency of $CXCL10^{+}$ cells increased slightly after Treg depletion specifically in tumors. This small increase might not seem to be enough to explain the CXCR3 dependence for T cell recruitment we have seen. However, endothelial cells are in direct contact with cells from the bloodstream whereas Mφs secrete CXCL10 which then needs to be transported from the apical site to the surface of endothelial cells. Additionally, there is the possibility that only a small fraction of endothelial cells in a specific location of the tumor (e.g. the invasive margin) upregulate CXCL10 and therefore the effect seen here in all endothelial cells combined after Treg depletion is rather small.

Finally, we assessed the expression of CXCL9 and CXCL10 on endothelial cells in human unaffected intestinal and tumor tissue. Both chemokines

were expressed by endothelial cells but in varying frequencies as patient tumors can be heterogenous. There was a trend towards lower frequencies of $CXCL9^+$ and $CXCL10^+$ expressing cells in tumors but our cohort was too small to draw any conclusions.

Paper II - Regulatory T cells reduce endothelial nSMase2 expression which prevents T-cell migration into tumors

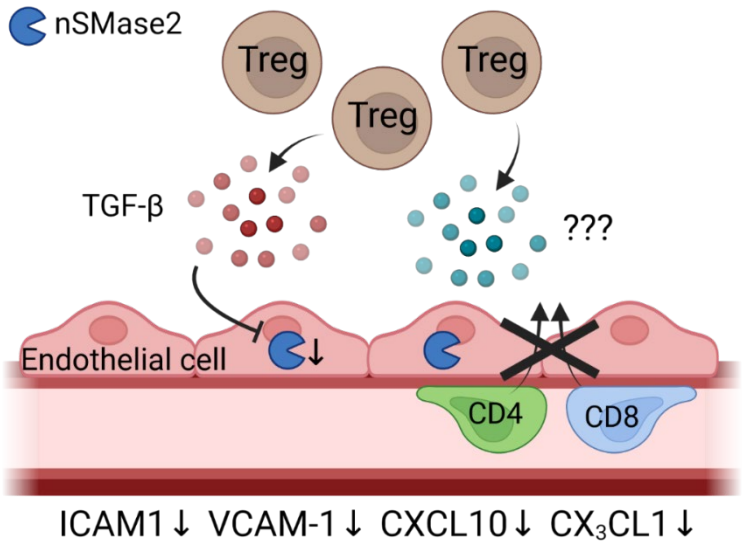

Figure 22: Graphical abstract of paper II. Treg decrease nSMase2 expression of endothelial cells through secretion of TGF-β *in vitro* but other unknown soluble factors secreted by Treg also contribute to this effect. Additionally, endothelial cells decrease the expression of ICAM-1, VCAM-1, CXCL10 and CX3CL1 when co-cultured with Treg *in vitro*. Inhibition of nSMase2 in our mouse model reduces T cell infiltration into tumors. Created with BioRender.com.

We have shown in Paper I that Treg affect the chemokine production of endothelial cells and thereby inhibit T cell migration into tumors of $APC^{Min/+}$ mice *in vivo*. However, the mechanisms of how Treg influence the chemokine production of endothelial cells in our model were still to be elucidated. We decided to use an unbiased approach by sequencing the mRNA of sorted endothelial cells to find a potential target. This was then followed by *in vitro* experiments with endothelial cell lines and *in vivo* experiments in our mouse model.

nSMase2 expressed by endothelial cells is potentially modulated by Treg

We sorted blood vessel endothelial cells from tumors of Treg depleted $APC^{Min/+}$/DEREG mice and Treg competent $APC^{Min/+}$ mice for mRNAseq. Several hundred genes were identified as differentially down- or upregulated but the gene Smpd3 (coding for the enzyme nSMase2) caught our attention as it is involved in TNF signalling [454] and can regulate the expression of selectins and cell adhesion molecules [455]. Additionally, it has been implicated in tumor progression and cell migration [456]. nSMase2 mRNA was increased over two-fold in tumor endothelial cells when Treg were depleted and it was in the top 15 upregulated genes with the lowest false discovery rate adjusted p-values. We backed the mRNAseq data by detecting increased levels of nSMase2 mRNA with qPCR in bulk tumor tissue of Treg depleted mice (Paper II, Fig. 2A). Furthermore, protein expression of nSMase2 was decreased in tumors compared to unaffected tissue in Treg competent $APC^{Min/+}$ mice (Paper II, Fig. 1A), but in human tissue we observed no significant difference (Paper II, Fig. 1B). However, some patients showed a strong reduction of nSMase2 in the tumor which might be correlated to the MSI status, CD8+ T cell and/or Treg infiltration which needs to be investigated in the future. We also performed *in vitro* experiments with HUVEC that showed increased nSMase2 expression after TNF stimulation (Paper II, Fig. 1C) which was attenuated when HUVEC were co-cultured with Treg (Paper II, Fig. 2B). Thus, the effect of Treg on nSMase2 expression could be regulated by similar mechanisms in our mouse model and human cells.

Treg inhibit the promoting effects of nSMase2 on the expression of chemokines and cell adhesion molecules

We were originally investigating the mechanisms behind the increasing chemokine production by endothelial cells in Treg depleted tumors in our $APC^{Min/+}$ mouse model which led us to the discovery of nSMase2. Now we used the nSMase2 inhibitor DPTIP [457] on TNF stimulated HUVEC to investigate the effects of nSMase2 on the expression of chemokines and cell adhesion molecules by targeted mRNA analysis (Nanostring). The analysis showed that the expression of VCAM-1, CXCL10, CXCL11, CX3CL1 were inhibited by DPTIP but not for ICAM-1 and CXCL9 (Paper II, Fig. 3B). We could then confirm the inhibitory effects by qPCR (Paper II, Fig. 3C) and at the protein level by using flow cytometry (Paper II, Fig. 3D). Furthermore, Treg had the similar inhibitory effects on VCAM-1, CXCL10 and CX3CL1 expressed by TNF stimulated HUVEC but surprisingly, ICAM-1 was also downregulated which was not seen with direct inhibition of nSMase2 through DPTIP (Paper II, Fig. 4A). These effects could also be replicated by culturing HUVEC with Treg conditioned media (Paper II, Fig. 4B). Importantly, these findings strengthen our results from the $APC^{Min/+}$ model that Treg inhibit molecules important for T cell migration and highlight the role of nSMase2. Most of the inhibitory effects of Treg on HUVEC were similar to the inhibition by DPTIP, except the reduction of ICAM-1 which seems to be regulated by another mechanism and does not involve nSMase2. Interestingly, the inhibitory effects by Treg were mediate by soluble factors *in vitro* which are partly unknown but adenosine and TGF-β have both been demonstrated to inhibit transendothelial migration *in vitro* [458, 459]. The half-life of adenosine in fluids like blood or plasma is just a few seconds and most likely does not contribute to the inhibitory effects in our culture system [460]. However, we could reverse a part of the inhibition observed by Treg when we blocked TGF-β signalling by adding the latency-associated peptide into the media which binds active TGF-β to form a latent complex (Paper II, Fig. 4C). This means there are probably other unknown soluble factors secreted by Treg that act on endothelial cells. Future studies should also investigate IL-35 since it has been shown that it inhibits the upregulation of VCAM-1 [461]. Blocking of IL-10 had no effect on the inhibitory effects of Treg soluble factors (Paper II, Supp. Fig. S5). A more

sophisticated approach to find new soluble factors could be the analysis of secreted proteins with chromatography-mass spectrometry.

In vivo expression of adhesion molecules and migration of T cells into tumors

Next, we looked at the expression of nSMase2 dependent chemokines and adhesion molecules in mice and humans. In the $APC^{Min/+}$ mice we found a lower frequency of VCAM-1 and CX3CL1 expressing endothelial cells in tumors compared to unaffected tissue (Paper II, Supp. Fig. S5). This correlates with lower nSMase2 expression, but Treg depletion had only minor effects on these molecules. However, the Treg depletion could possibly have only transient effects that are not detected after 12 days hence shorter time periods should be investigated in the future. Sphingosine-1-phosphate (S1P) can induce VCAM-1 expression and is generated from ceramide which in turn is produced from sphingomyelin in the cell membrane through nSMase2 activity [462, 463]. S1P can also induce an autocrine loop of the interferon regulatory factor 1 which in order can induce CXCL10 expression [464]. We have previously shown in mice that T cell migration into tumors is dependent on the CXCR3-CXCL10 axis and here we showed evidence that nSMase2 could influence the CXCL10 expression *in vitro*. Therefore, we were interested if the nSMase2 inhibitor PDDC could reverse the effects of Treg depletion which led to increased infiltration of T cells into tumors of $APC^{Min/+}$DEREG mice. PDCC treated mice showed a significant decrease in $CD4^+$ T cells in tumors compared to Treg depleted mice but frequencies were still somewhat higher than in Treg competent mice (Paper II, Fig. 5A). This suggests the existence of additional mechanisms that Treg use to inhibit transendothelial migration *in vitro*. Nevertheless, nSMase2 inhibition does show effects on T cell migration and targeting this pathway could be a viable treatment option to increase T cell infiltration into tumors. There was no change of the proliferation marker Ki-67 in $APC^{Min/+}$ mice only treated with PDCC. Thus, the inhibition of T cell proliferation by Treg is mediated through other mechanisms than suppression of nSMase2.

Furthermore, PDCC treatment did not lead to macroscopical changes in the small intestine based on IHC experiments (Figure 23).

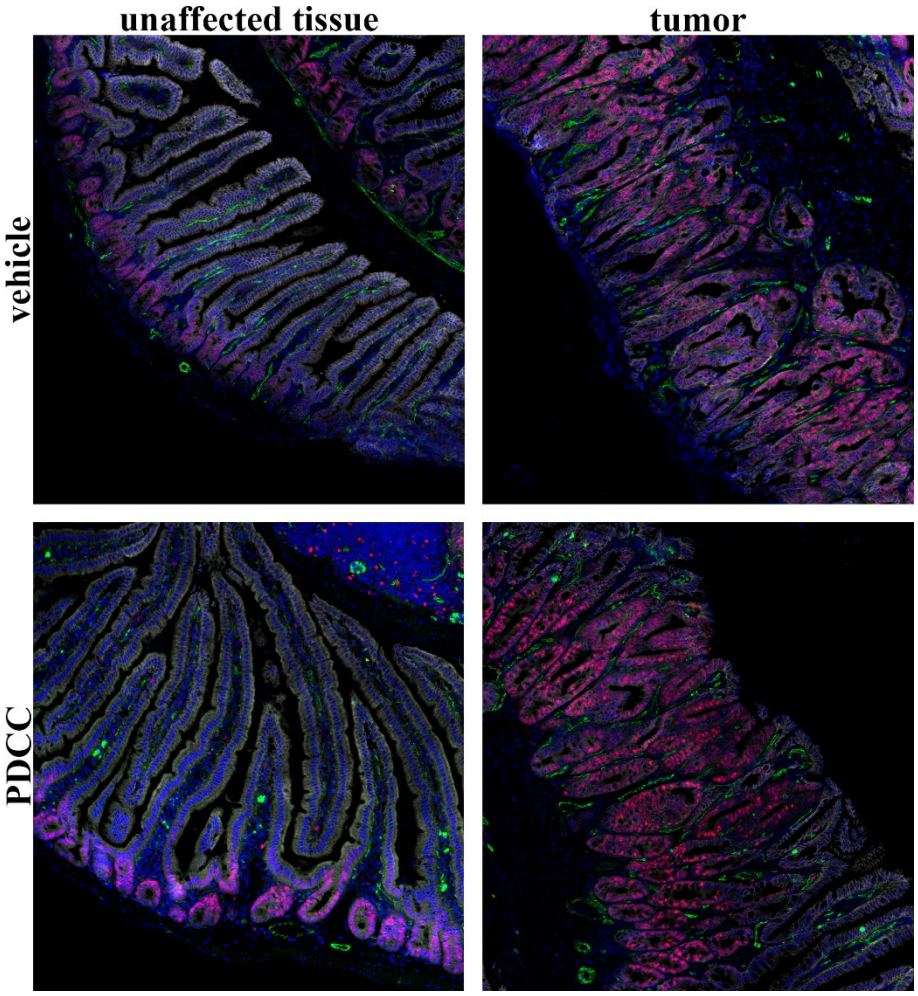

Figure 23: Representative fluorescence microscopy images showing the morphology of the small intestine of unaffected and tumor tissue from $APC^{Min/+}$ mice treated with PDCC or untreated (vehicle chow). Blue – nuclei, white – Epithelial cells, green – endothelial blood vessels, red – proliferating cells.

Paper III – Regulatory T cells specifically suppress conventional CD8αβ T cells in intestinal tumors of $APC^{Min/+}$ mice

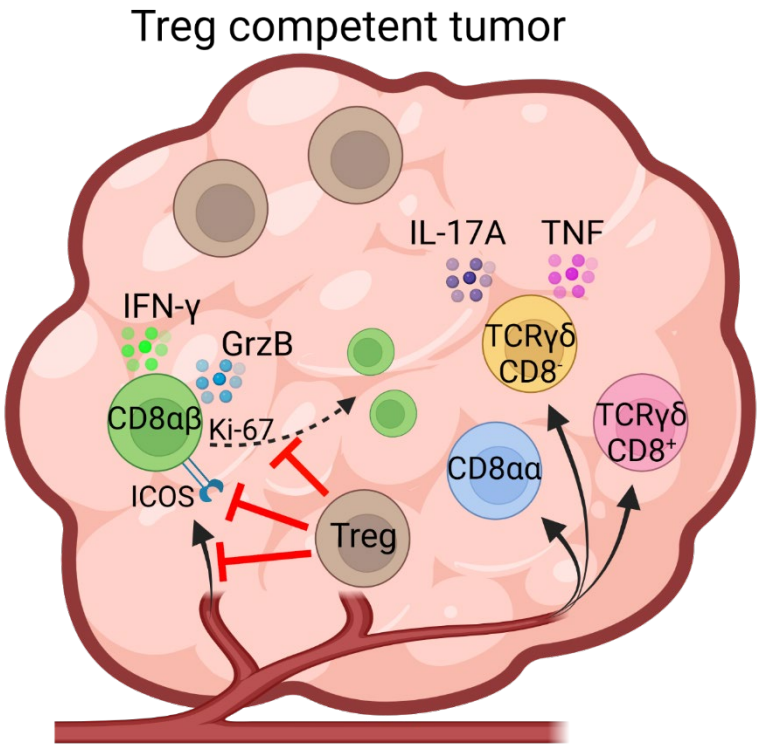

Figure 24: Graphical abstract of paper III. Treg specifically supress the secretion of Th1 associated cytokines and cytotoxic molecules in CD8αβ T cells as well as their activation (ICOS) and proliferation (Ki-67). In contrast, CD8αα and γδ T cells are not affected by Treg in the same manner and γδ T cells secrete IL-17A and TNF which might counteract anti-tumor immune responses. Created with BioRender.com.

We established before that Treg depletion leads to increased T cell migration into tumors of $APC^{Min/+}$ mice and that these effects are partly regulated by nSMase2 activity. In previous research we have also shown that $CD4^+$ and $CD8^+$ T cells are increased in Treg depleted tumors though

the phenotype of these cells was not studied in detail [314]. In this study, we focused on the phenotypic analysis of TCRβ$^+$CD8$^+$ T cells as they have been associated with anti-tumor immunity in CRC as well as γδ T cells that show both anti- and pro-tumorigenic effects. We also explored the effects of PD-1 treatment in combination with Treg depletion *in vivo*.

Treg depletion increases the numbers of CD8αβ T cells in tumors of APC$^{Min/+}$ mice

First, we compared the frequencies and absolute numbers of different T cells subsets (CD8αα, CD8αβ, TCRγδCD8$^-$ and TCRγδCD8$^+$; see Paper III, Fig. 1a) between tumors and unaffected tissue of APC$^{Min/+}$ mice. We found that there is a reduction of CD8αβ and CD8αα T cells but not γδ T cells in tumors (Paper III, Fig. 1b+c). This suggests that the tumors in our mouse model primarily represent the MSS type of human CRC in this regard because they also show low infiltration of CD8$^+$ T cells [421]. It has been shown that Treg accumulate in CRC, supress anti-tumor T cell responses and are associated with a worse patient outcome [411]. Consequently, we depleted Treg in our mouse model which led to increased frequencies and total numbers CD8αβ T cells in tumors but not for the other subsets (Paper III, Fig. 2a+b). Interestingly, few studies have looked at the possibility that increased numbers of CD8αα T cells could also contribute to overall CD8$^+$ infiltration which are known to possess suppressive capabilities [465, 466]. Tumor infiltrating CD8$^+$ T cells are associated with a beneficial prognosis, but we did not see a reduction of the tumor burden after Treg depletion. However, Treg depletion also increased the frequencies of proliferating and activated CD8αβ T cells determined by Ki-67 and ICOS, respectively (Paper III, Fig. 2c and 2d). ICOS is essential for T cell activation and has also been shown to indicate a T cell mediated anti-tumor response in a subcutaneous tumor mouse model [467, 468]. CD8αα, TCRγδCD8$^-$ and TCRγδCD8$^+$ T cells showed a less pronounced increase of proliferating cells and no difference in ICOS expression but about 40 % of TCRγδCD8$^-$ cells were ICOS$^+$ in both unaffected and tumor tissue. In summary, Treg depletion seems to have

the largest effect on CD8αβ T cells regarding frequencies/numbers, proliferation, and activation.

CD8αβ T cells in tumors increase Granzyme B and IFN-γ expression after Treg depletion

Next, we were interested in the cytotoxic capacity of CD8αβ T cells and their ability to produce cytokines that could increase anti-tumor immunity. We assessed the production of the cytotoxic molecule granzyme B (GrzB) and the ability of cells to degranulate as a sign for cytotoxic potential by detecting CD107a after Phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation (Paper III, Fig. 3). Furthermore, we analysed the expression of the cytokines IFN-γ, IL-2, TNF and IL-17A which have shown importance in tumor immunity (Paper III, Fig. 4). Again, Treg depletion affected only the CD8αβ T cell population which showed increased frequencies of $GrzB^+$ and IFN-γ^+ cells in tumors but degranulation was slightly reduced. CD8αβ T cell from unaffected tissue showed an increased ability to degranulate compared to the tumor which suggests that the cytotoxicity is supressed in tumors (Paper III, Supp. Fig. 7). Higher expression of GrzB has been associated with a lower risk of tumor reoccurrence in human CRC and both IFN-γ and GrzB are part of the Th1 response which is linked to a better clinical outcome [416]. However, the degranulation process is essential for GrzB to unfold its cytotoxic effects. CD107a is difficult to detect as it shows transient expression on the cell surface and is internalized immediately. PMA/ionomycin is a very common way to polyclonally stimulate T cells *ex vivo* to assess cytokine secretion but TCR specific stimulation with CD3/CD28 might lead to different results of CD107a expression. Interestingly, only TCRγδ$CD8^-$ showed high frequencies of IL-$17A^+$ cells in tumors and were about 2-fold (median) increased compared to unaffected tissue (Paper III, Fig. 4b and Supp. Fig. 9) but Treg depletion did not alter these frequencies. Conventional $CD4^+$ T cells did also express IL-17A but to lesser extent and there was also no increase in the tumor compared to unaffected tissue (data not shown). IL-17A is thought to promote tumor development and most studies have shown indirect

suppressive effects on anti-tumor immunity [432]. Furthermore, IL-17A seems to downregulate CXCR3 expression on $CD8^+$ T cells systemically in advanced stage CRC patients [422]. Tumors which show a Th17 gene signature are also associated with the worst disease-free survival [416].

CD8αβ T cells increase expression of checkpoint molecules after Treg depletion but treatment with PD-1 antibody shows no effect

Treg depletion did not lead to tumor regression. Interestingly, we found an increased tumor load after Treg depletion (Paper III, Supp. Fig. 5c) which we have not seen in previous studies with the same depletion scheme. This can be explained by a skewed female to male ratio (higher ratio in the Treg competent group) in the two groups in our experiments because surprisingly, females showed a lower tumor load than males (Figure 25).

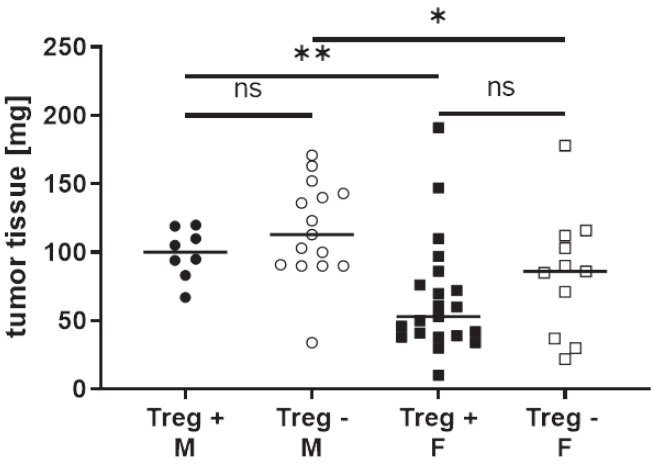

Figure 25: Tumor weight of Treg competent $APC^{Min/+}$mice (Treg+) or Treg depleted $APC^{Min/+}$/DEREG mice (Treg-) divided by sex. Mice used *in vivo* experiments of paper III (n=8, 15, 21, 11 from left to right). * $p<0.05$, ** $p<0.01$

The absence of tumor regression could be explained by tumor infiltrating T cells that show a dysfunctional exhausted phenotype. Exhausted T cells express inhibitory checkpoint molecules that are commonly targeted in immunotherapies to reverse exhaustion and increase tumor cell killing capabilities. Therefore, we assessed the frequencies of PD-1^+, TIGIT$^+$ and TIM-3^+ cells among the aforementioned T cell populations in unaffected and tumor tissue of $APC^{Min/+}$ mice (Paper III, Fig. 5a). All three checkpoint molecules were expressed at very low levels in unaffected tissue regardless of the T cell population or Treg depletion (Paper III, Fig. 5c). The frequencies of PD-1^+ and TIM-3^+ CD8αβ T cells were very low in tumors (median 3,1 % and 2,2 %, respectively) as well but significantly increased after Treg depletion (median 13 % and 12,9 %) (Paper III, Fig. 5b). However, based on the high increase in ICOS expression on CD8αβ T cells after Treg depletion, we could have expected a higher frequency of PD-1 expressing CD8αβ T cells, but the increase somewhat correlates with the increase in GrzB$^+$ and IFN-γ^+ cells. Interestingly, about 40% of TCRγδCD8$^-$ cells expressed PD-1 and/or TIM-3 specifically in tumors and Treg depletion had no effect on this expression. According to De Vries et al., the PD-1 expression on TCRγδCD8$^-$ cells could also mean that this population possesses an activated memory phenotype with anti-tumor activity [469]. However, we did not see a change in proliferation (Ki-67) or activation (ICOS) in γδ T cells when we additionally treated the mice with an anti-PD-1 antibody. The anti PD-1 treatment had also no effect on CD8αβ T cells in this regard and we did not see any tumor regression. Studies with patients show that PD-1 therapy is most effective in MSI tumors with an active immune response and T cell infiltration while most MSS tumors do not respond [470]. MSI tumors express more neoantigens because of frameshift mutations and furthermore, infiltrating immune cells express checkpoint molecules at high levels [418, 471]. We consider the $APC^{Min/+}$ mice mouse model to be more representative for MSS type tumors in patients which means that the results are somewhat expected and show that Treg depletion alone is not enough to create a sustained anti-tumor immune response. In the same mouse model, Wang et al. showed that the combined treatment of PD-1 with α-GalCer led to significant reduction of polyp numbers in the small and large intestine [472]. This demonstrates that combined treatments can have great effects

and are worth investigating. Treatment of MSS tumors might benefit from cancer vaccines to create a stronger anti-tumor response with tumor-specific T cell infiltration and shortly afterwards targeting of Treg and/or checkpoint molecules to potentiate the effect [473].

Paper IV – Unconventional T cells in colon cancer – phenotypic characterization

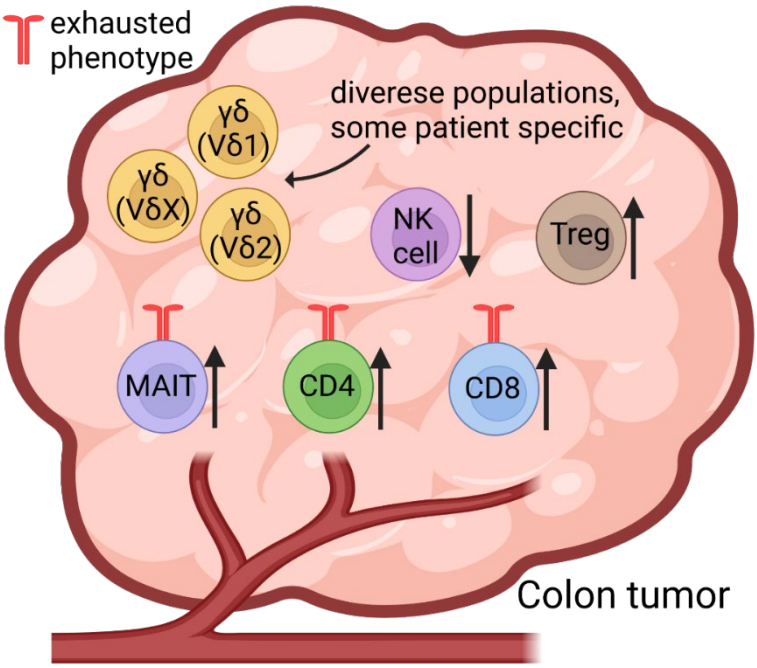

Figure 26: Graphical abstract of paper IV. We detected increased frequencies of exhausted $CD4^+$ and $CD8^+$ conventional T cells as well as an accumulation of Treg in colon tumors compared to unaffected tissue. In contrast, NK cells were greatly reduced in tumor tissues. Additionally, exhausted MAIT cells were enriched in tumors but expressed few activating or inhibitory NK cell receptors. γδ T cells showed diverse subpopulations while some of these were patient specific and might correlate with expansion of specific γδ clones. Created with BioRender.com.

Our findings in the $APC^{Min/+}$ mice that TCRγδ$CD8^-$ cells had a specific phenotype in tumors led to our interest in a better characterization of γδ T cells and other unconventional T cells in CRC. We used mass cytometry with a large antibody panel focused on unconventional T cells, NK cell receptors, checkpoint molecules and activation markers that have been shown to play a role in tumor immunity. The mass cytometry data built the basis for a more detailed analysis of certain cell types by flow cytometry and IHC.

Mass cytometry analysis confirmed increased frequencies of Treg and exhausted conventional T cell subsets in CRC

We preformed unsupervised clustering of $CD3^+$ OR $CD56^+$ cells from unaffected and CRC tissue of 8 patients. For this analysis, we used equal cell numbers from each tissue and applied the dimension reduction algorithm UMAP combined with the clustering algorithm Phenograph (Paper IV, Fig. 1a). The change in frequencies of the computed clusters were shown as a volcano plot with the p-values of the paired analysis and the fold change of the clusters between unaffected and tumor tissue (Paper IV, Fig. 1b). Furthermore, the median expression of immune cell markers for the different clusters was visualized in a heatmap (Paper IV, Fig. 1c). We could confirm results from previous studies that Treg frequencies (cluster 2 and 14) are increased in tumor tissue compared to unaffected tissue [474, 475]. Furthermore, there was an increase in $CD8^+$ and $CD4^+$ T cells with an exhausted phenotype in tumors (cluster 6, 11, 13) which has been reported as well [476, 477]. Both the $CD8^+$ and $CD4^+$ T cell populations expressed CD39 and CD103 which are markers considered to be expressed by tumor-specific T cells [478, 479] which could explain the lower frequencies in unaffected tissue (Paper IV, Supp. Fig. S4). Tumor-specific $CD39^+CD103^+$ T cells have been correlated to a better outcome for patients of ovarian, and head and neck cancer [478, 479] hence, it would be interesting to look for associations with the clinical and survival data in our study in the future.

Unconventional T cells could be detected in lower numbers compared to conventional T cells as would be expected. We did no detect reasonable numbers of iNKT cells with the Vα24-Jα18 antibody by manual gating in pilot experiments and therefore excluded this marker from the analysis in this study. iNKT T cells are also rare in blood and therefore they are difficult to study which raises the question of how important they are in tumor immunity of CRC if they are almost absent in unaffected and tumor tissue [480]. On the other hand, in mice they are considered to have anti-tumorigenic potential, but the NKT cell compartments in mice differ from humans which might explain the limited success of clinical trials targeting iNKT cells [481]. Additionally, we could confirm increased MAIT cell frequencies (by manual gating) in tumors compared to unaffected tissue [150, 482] whereas γδ T cell clusters (19, 21, 22) remained stable in frequencies. Both populations were analysed in detail in a separate analysis. NK cell frequencies (cluster 18 and 25) were greatly decreased in tumors compared to unaffected tissue.

Exhausted MAIT cells tend to be increased in tumors compared to unaffected tissue and express few NK cell receptors

Next, we used the same mass cytometry data set and applied a similar clustering approach for manually gated MAIT cells ($CD3^+CD161^{hi}$ $V\alpha7.2^+$ and 5-OP-RU $tetramer^+$) from four patients that showed sufficient numbers for analysis using unequal cell numbers from tissues (Paper IV, Fig. 2a). The interesting clusters among the 9 computed ones were a putative exhausted population with a proliferating $CD103^+$ $CD39^{hi}$ $PD\text{-}1^+$ phenotype (cluster 3) and a $CD4^+$ population with a similar exhausted $CD39^{hi}$ $PD\text{-}1^+$ phenotype (Paper IV, Fig. 2c)[428, 479]. The first was slightly increased in tumors compared to unaffected tissue while the latter was decreased but none of the changes were statistically significant probably due to the limited number of samples. This is in line with one of our previous studies that show exhaustion of MAIT cells in CRC and loss of polyfunctionality regarding cytokine secretion [428].

There are different activating and inhibitory NK cell receptors on T cells that can potentially be targeted within immunotherapy to improve anti-tumor immunity. The activating receptor NK2GD for example can recognize cancer cells that signal cellular stress through the expression of ligands like MICA or MICB and is being tested on CAR T cells in clinical trials [483]. Additionally, MAIT cells from the liver have been shown to use a NKG2D dependent but TCR/MR1-independent cytotoxicity [484]. MAIT cells are reported to express NK cell receptors, but most studies have been done in blood from healthy donors [485, 486] whereas expression in tumor infiltrating MAIT cells has not been studied in detail. In our mass cytometry data, we detected low frequencies of MAIT cells expressing activating (NKG2D, NKp30, CD16, CD352) or inhibitory NK cell receptors (CD158e1, CD158b, CD159a) except for CD161 which is a hallmark for MAIT cells (Paper IV, Fig. 2c). NKG2D is commonly found on conventional $CD8^+$ T cells [487] which usually possess the CD8αβ receptor whereas MAIT cells are mostly $CD8\alpha\alpha^+$ with innate-like properties and therefore most likely differ in expression of NK cell receptors. We extended our analysis by using flow cytometry and investigated the additional NK cell receptors DNAM1 (CD226), 2BA (CD244) and NKp80 (Paper IV, Fig. 2d). We could show that at least some patient showed substantial expression of CD94, CD158, NKG2D, NKp80 and 2B4 in tumor tissue which could possibly be associated with clinical parameters like MSI status. However, especially the inhibiting receptor CD158 was expressed on MAIT cells from the tumors in many patients which suggests a diminished NK cell receptor mediated cytotoxicity. This and the exhaustion of tumor infiltrating MAIT cells could partly explain why they do not exert anti-tumor functions that lead to tumor regression.

γδ T cells comprise patient specific subpopulations but cell numbers are reduced in the tumor epithelium compared to the mucosal epithelium

γδ T cells are one of the most abundant unconventional T cell populations in mucosal tissues. They have been associated with a better patient outcome [393] but IL-17 producing γδ T cells are also reported to promote

angiogenesis and accumulation of MDSC [432, 488]. Therefore, we investigated subpopulations of γδ T cells from the same mass cytometry data set as used before. γδ T cells were manually gated based on TCRγδ, Vδ1 or Vδ2 before equal cell numbers of each tissue (6 patients) were combined and subjected to clustering analysis (Paper IV, Fig. 3a). The 20 different clusters showed very diverse surface marker expression and none of them showed a significantly different frequency between tumor and unaffected tissue. Surprisingly, we found patient specific subpopulations in both tissue types. Every one of the six patients had at least one γδ T cell subpopulation with a substantial frequency which was unique to this patient or only shared by one other patient (Paper IV, Fig. 3c). We got suspicious about sample batch effects but a more careful look at the data did not reveal any problems and for most markers we also normalized the data in between samples. Interestingly, one patient (O463) with MSI-high status had two unique populations in both the unaffected and tumor tissue. It has been shown that the diversity of T cell receptor sequences in unconventional T cells differs between CRC tissue and adjacent mucosa which might be also the case for γδ T cells, and maybe a reason why we observed subpopulations with different phenotypes [489]. Flow cytometry analysis using the Vδ1, Vδ2 and Vγ9 confirmed a great variation of Vδ1 and Vδ2 populations between patients (Paper IV, Fig. 3c). There was also a substantial variation between patients in the $V\delta1^+$ and $V\delta2^+V\gamma9^-$ adaptive-like γδ T cells which were abundant in unaffected and tumor tissue (Paper IV, Fig. 4b and c). We showed that Vδ2 cells are abundant in unaffected and tumor tissue and not only in blood as commonly thought, but they showed less expression of tissue resident markers like CD103, CD38 and CD39 in comparison to Vδ1 cells. This suggests that Vδ1 cells might be better for adoptive transfer than Vδ2 cells that showed little success in clinical trials [490]. However, Vδ1 cells are difficult to isolate and expand [491]. IHC analysis of γδ T cell in tumor tissue and corresponding unaffected tissue revealed that γδ T cells are significantly reduced in the tumor epithelium compared to unaffected epithelium but numbers in the stroma are similar (Paper IV, Fig. 5b). This provides evidence that γδ T cells are unable to exert cytotoxic activity towards tumor cells because both are spatially separated.

CONCLUSION AND FUTURE PERSPECTIVES

In the first part of the thesis, we used the $APC^{Min/+}$ mouse model for intestinal tumors which carry a mutation in the APC gene that is commonly mutated in human MSS tumors. Patient with these tumors benefit the least from the current immunotherapies and new treatments are needed for this major patient group. Treg are thought to be an important factor in supressing anti-tumor repsonses in the TME and specific depletion of Treg could improve anti-tumor immunity and therefore patient outcome. We showed that Treg can inhibit the migration of $CXCR3^+$ T cells into tumors, possibly by acting on endothelial cells to decrease transendothelial migration. Endothelial cells upregulated their expression of CXCL10 when Treg were depleted which supports new research that shows the importance of chemokines in the migration of T cells into tumors [492]. Importantly, we also found that Treg reduce the expression of the enzyme nSMase2 in endothelial cells which is involved in the ceramide-S1P signalling pathway. nSMase2 regulates the expression of several chemokines and cell adhesion molecules important for transendothelial migration. The suppression of nSMase2 expression was partly mediated through TGF-β but other unknown soluble factors are also involved. Future studies should identify and characterize these soluble mediators as they could be targeted by therapies. Furthermore, it may be of interest to further investigate the role of the ceramide signalling pathway in cell migration and if there are possibilities to modulate it.

We also demonstrated that Treg specifically supress CD8αβ T cells in tumors by reducing their proliferation, activation and ability to suppress Th1 effector molecules, while CD8αα and γδ T cells were unaffected by depletion. This highlights the potential of Treg depletion in patients as tumor-infiltrating cytotoxic $CD8^+$ T cells are crucial for tumor regression and linked to a better patient outcome. Though, we could not show tumor regression after Treg depletion in our mouse model and the combination with PD-1 immunotherapy had no additional effects on tumor load or the Th1 response. Interestingly, there was a sex difference in tumor load and

there are indications that this could be related to sex hormones but more detailed studies are needed [493].

Treg depletion does most likely have beneficial effects in patients with an ongoing anti-tumor response like in CMS1/MSI tumors. Other tumor types might benefit from combined therapies that elicit the anti-tumor immune response and recognition before Treg are depleted. However, the specific depletion of Treg in tumors of patients is not yet possible but new research shows promising approaches to solve this issue by targeting TIGIT, ICOS or dual-targeting of CTLA-4 and CD47 [494-496].

Unconventional T cells can potentially exert anti-tumor functions through non-MHC restricted molecules without antigen exposure which make them a good candidate for adoptive cell transfer. However, this requires first a better understanding of the cell phenotypes and functions in tumors. We confirmed that MAIT cells are accumulated in tumors but show markers of exhaustion and little expression of activating NK cell receptors that could engage in TCR-independent cytotoxicity. Furthermore, γδ T cell subpopulations demonstrated a great diversity and were in some cases patient-specific which could be an indication for an altered diversity of TCR receptors. This emphasizes the need for more detailed studies of tumor-infiltrating γδ T cells, including TCR sequencing.

New model system like tumor scaffolds or organoid cultures from primary tumors could help to study the mechanisms of Treg suppression but mouse models remain important especially in CRC research. It has been shown that the gut microbiota impacts tumor development as well as effectiveness and toxicity of immunotherapies [497-499]. Inbred mouse strains in the laboratory do not fully represent this reality. Furthermore, there are species differences with regards to the development of unconventional T cells [500], which make studies of human tumors warranted. In upcoming studies, it would be desirable to use laboratory mice which are born to wild mice and better resemble the natural microbiota and human immune responses [501].

Treg depletion and the adoptive transfer of unconventional T cells are promising therapy options for the future, but our understanding of the

mechanisms and targets are just not sufficient yet. This thesis sought to contribute to the ongoing basic research that set the foundation for the development of new therapies for CRC patients

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Snoop Dogg

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