Recruitment of regulatory and conventional T cells to colon adenocarcinomas

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Abstract

Colorectal cancer is one of the most common malignant diseases, with an annual incidence of over one million cases worldwide. Although survival depends strongly on tumor stage at diagnosis, lymphocyte infiltration has been clearly correlated to a favourable prognosis in several studies. The aim of this thesis was to determine the mechanisms for lymphocyte infiltration in colon adenocarcinomas, with emphasis on the effect of regulatory T (Treg) cells on the recruitment of conventional T cells.

First, we characterized the lymphocyte infiltrate in human colon adenocarcinomas compared to surrounding unaffected tissue. In tumors, we detected substantial accumulation of $CD4^+$ $FOXP3^+CTLA4^+CCR4^+$ $CD39^+$ Tregs with potential to suppress anti-tumor immunity. Also, the frequencies of activated intratumoral, Th1 like T cells, important for anti-tumor immune responses, were decreased. The accumulation of $CCR4^+$ Tregs may be due to increased production of the ligand CCL22 in the tumor. Furthermore, MAdCAM-1 expression, an adhesion molecule used by lymphocytes to migrate to the gut, was decreased in tumor tissue, potentially contributing to shaping the repertoire of tumor infiltrating lymphocytes. Since directed lymphocyte migration is controlled by chemokines and chemokine receptors, we decided to investigate alternative mechanisms for lymphocyte recruitment to tumors by examining the mRNA levels of chemokine decoy receptors D6, DARC and CCX-CKR. By using real time RT-PCR, we detected strongly decreased levels of the chemokine decoy receptor D6, with affinity for inflammatory chemokines, in human colon tumors compared to unaffected mucosa, whereas there was no change in expression of DARC and CCX-CKR.

Further, we observed that Treg isolated from colon cancer patients inhibited transendothelial migration of conventional T cells *in vitro*, while Tregs from healthy control subjects had no such effect. Also, we detected elevated levels of the adenosine-generating enzyme CD39 on circulating Tregs from cancer patients. Adenosine suppress lymphocyte functions and indeed, exogenous adenosine resulted in inhibition of conventional T cell migration in our system, while blocking of adenosine receptors restored the migration of T cells from cancer patients.

To directly assess the function of Tregs in colon cancer, we crossed $APC^{Min/+}$ mice, a model of intestinal cancer, with DEREG mice that allow selective depletion of Tregs. The tumor tissue in these mice presents a similar distribution of T cells as human colon tumors, since there is decreased infiltration of activated T cells and accumulation of Tregs in intestinal tumor tissues. When depleting Tregs for 10 days, we detected improved $CD4^+$ and $CD8^+$ lymphocyte infiltration to tumors, indicating that accumulation of Treg impairs migration of conventional T cells into tumors.

Taken together, results from this thesis show differential lymphocyte composition in colon tumors compared to surrounding unaffected mucosa, possibly induced by accumulated Tregs and modulated chemokine decoy receptor and homing molecule expression in the local environment.

Original papers

This thesis is based on the following papers, referred to in the text by their assigned Roman numeral (I-IV):

I. Svensson H, Olofsson V, Lundin S, Yakkala C, Björck S, Börjesson L, Gustavsson B, Quiding-Järbrink M.
Accumulation of CCR4^{+}CTLA-4^{+}FOXP3^{+}CD25^{hi} regulatory T cells in colon adenocarcinomas correlate to reduced activation of conventional T cells. *PLoS One. 2012;7 (2):e30695.*

II. Langenes V*, Svensson H*, Börjesson L, Gustavsson B, Bemark M, Sjöling Å, Quiding-Järbrink M. **First author*
Mucosal expression of the chemokine decoy receptor D6 is decreased in colon adenocarcinomas. *Submitted*

III. Sundström P, Stenstad H, Langenes V, Theander L, Gordon Ndah T, Fredin K, Börjesson L, Gustavsson B, Quiding-Järbrink M
Regulatory T cells from colon cancer patients express CD39 and inhibit transendothelial effector T cell migration by an adenosin-dependent mechanism. *In manuscript*

IV. Langenes V, Fasth P, von Mentzer A, Ragahavan S, Quiding-Järbrink M
Depletion of regulatory T cells promotes infiltration of conventional T cells in gastrointestinal tumors in APC$^{Min/+}$ mice. *In manuscript*

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Abbreviations

APC	adenomatous polyposis coli
ATP	adenosine tri phosphate
CAM-1	cell adhesion molecule-1
cAMP	cyclic adenosine mono phosphate
CCX-CKR	chemocentryx chemokine receptor
CDR	chemokine decoy receptor
CRC	colorectal cancer
CTLA-4	cytotoxic T lymphocyte associated antigen 4
DARC	duffy antigen receptor for chemokines
DC	dendritic cell
DEREG	depletion of Treg
DPX	1,3-dipropyl-8-p-sulphophenyl
DT	diphtheria toxin
FAP	familial adenomatous polyposis
GALT	gut associated lymphoid tissue
HEV	high endothelial venule
HUVEC	human umbilical vein endothelial cell
IBS	inflammatory bowel syndrome
ICAM-1	inducible cell adhesion molecule 1
IEL	intraepithelial lymphocyte
ILF	isolated lymphoid follicle
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
iTreg	inducible Treg
LP	lamina propria
LPL	lamina propria lymphocyte
MAdCAM-1	mucosal adressin cell adhesionmolecule 1
MDSCs	myeloid derived suppressor cell
MHC	major histocompatibility complex
MLN	menesnteric lymph node
NSAID	non steroidal aniinflammatory drug
nTreg	natural Treg
PBMC	peripheral blood mononuclear cell
PNAd	peripheral lymph node adressin
PP	peyer's patch
PSGL-1	P-selectin glycoprotein ligand 1
RA	retinoic acid
ROS	reactive oxygen species
SI	small intestine
TAA	tumor associated antigen
TAM	tumor associated macrophage
TCR	T cell receptor
TLR	toll like receptor
Treg	regulatory T cell
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
vWF	von Willerbrand factor
WT	wild type

Introduction

Introduction to the immune system

The highly intricate processes constituting the immune system have evolved to defend the organism against pathogens including bacteria, viruses and parasites. We are constantly exposed to microbes and a major site for interactions between host and bacteria are mucosal sites, especially the gastrointestinal system, which is colonized with high loads of commensal bacteria. This requires efficient eradication of invading pathogens as well as regulatory control to avoid excessive immune responses that might lead to autoimmunity or pathologic inflammation due to repeated interaction with otherwise harmless commensal bacteria.

Acute inflammation is immediately activated in response to infection or tissue damage and mediated by innate immune cells including monocytes, macrophages and mast cells, infiltrating peripheral tissues. Microbial molecules activate monocytes and macrophages that respond by initiating inflammation, in part by secreting inflammatory cytokines to further activate and recruit neutrophils, mast cells and NK-cells. Inflammation is normally self-limiting and efficient in eradicating many pathogens, however, innate immunity has a limited diversity and sometimes fails to completely eliminate the inflammatory stimuli. Dendritic cells also infiltrate peripheral tissues and ingest antigens before migrating to lymph nodes to activate adaptive immunity, characterized by antigen-specificity, broad diversity and generation of memory. Adaptive immunity is mediated by T- and B-lymphocytes that specifically recognize microbes as well as non-microbial substances via their T-cell receptor and B-cell receptor, respectively. Activated lymphocytes are recruited to effector sites like the gut lamina propria to protect the host through several mechanisms referred to as effector functions. B-cells mediate humoral immunity by the production of antibodies that neutralises microbes and toxins as well as targets pathogens for phagocytosis. $CD8^+$ T-cells have a key role in cell-mediated immunity with their cytolytic capacity and efficient killing of host cells that are infected by pathogens. Another subset of T-cells, termed $CD4^+$ T helper cells, mainly function in secreting cytokines to enhance the function of other immune cells. Also, one subset of T helper cells, termed regulatory T cells, suppresses immune responses and is crucial in

controlling immune responses and for inducing tolerance to commensal bacteria and food antigens.

Although evolving from host-cells, tumor cells express antigens that are recognised as foreign by the adaptive immune system and tumor specific cytotoxic $CD8^+$ T cells as well as activated humoral immunity are observed in cancer patients. The factors determining the balance between tumor growth and activated anti-tumor immunity, are still poorly understood and are the focus of this thesis. Determining the processes by which tumors avoid elimination by the immune system will provide molecular pathways to exploit in the attempts to enhance tumor immunity in cancer patients.

Lymphocyte homing to the gut

Immature lymphocytes differentiate into mature, naïve T- and B-cells in the thymus and bone marrow respectively, to generate cells with the ability to recognise specific antigens through surface bound receptors that in T-cells are termed T cell receptors (TCR). When the maturation process in the thymus is complete, T-cells are released from the primary lymphoid tissue into the circulation to re-circulate between blood and tissue until it encounters its specific antigen. Naïve T-cells are imprinted to continuously recirculate through secondary lymphoid tissues, which they enter through high endothelial venules (HEV), until their TCR recognises an antigen [1].

Induction of adaptive immunity in response to intestinal antigens takes place in gut-associated lymphoid tissue (GALT) consisting of the mesenteric lymph nodes (MLN), small intestinal Peyer's patches (PP) as well as isolated lymphoid follicles (ILF) in the colon [2]. Antigens captured by antigen presenting cells (APC) are concentrated in these tissues via the afferent lymphatic vessels and presented via MHC class II to naïve T-cells (Th0) with the appropriate (TCR) [3]. Recognition of the cognate antigen by naïve T-cells in the context of appropriate co-stimulation results in proliferation and differentiation into effector T cells [1]. Entry of naïve lymphocytes to GALT requires the interactions between lymphocyte L-selectin and its carbohydrate ligands PNAd, expressed on HEV [4]. The entry process is further supported by the integrin $a4\beta7$, expressed by lymphocytes that bind to the endothelial mucosal adressin cellular adhesion molecule-1 (MAdCAM-1) [5]. Effector T cells activated in the GALT

preferentially home to intestinal tissue due to imprinting at the inductive site, resulting in up-regulation of integrins and chemokine receptors specific for the intestine [1]. Homing to the small intestinal mucosa requires lymphocyte expression of the integrin $\alpha4\beta7$ to facilitate binding to MAdCAM-1 expressed by endothelial cells throughout the gut, and CCR9 to respond to the ligand CCL25 expressed on small intestinal post capillary venules [6-8]. A subset of $CD8^+$ T cells referred to as intraepithelial lymphocytes (IEL) express $\alpha E\beta7$ (CD103) which makes them localize to the epithelium [9]. The majority of LP regulatory T cells (Tregs) in mice also express $\alpha E\beta7$, indicating a role for this integrin in Treg recruitment [10]. Homing of lymphocytes to the colon also depends on $\alpha4\beta7$/MAdCAM-1 interactions, whereas most T-cells in the colon lack CCR9 expression as CCL25 is not produced by the colon epithelium to recruit CCR9 expressing cells. Instead, CCL28 is present in the colon epithelium to recruit CCR10 expressing lymphocytes, however the complete mechanism for lymphocyte homing to the colon is still poorly defined. CCL28 is also produced in the small intestine where it is believed to attract IgA lymphoblasts [11]. There is evidence that dendritic cells (DC) in the MLN or PP are educated to induce the gut-specific homing receptor $\alpha4\beta7$ and CCR9 in T cells upon activation [12]. The mechanism behind the imprinting of homing-specific qualities in T cells is not completely understood, but the production of retinoic acid (RA) by lamina propria DCs seemingly plays an important role as RA alone induce $\alpha4\beta7$ and MAdCAM-1 in lymphocytes stimulated with anti-CD3 and anti-CD28 *in vitro* [13,14].

Transendothelial migration

The extravasation of lymphocytes is a multi-step process mediated by the adhesion molecules expressed by lymphocytes and their ligands on the vascular endothelium. Several distinct molecular steps are involved in the entry of lymphocytes into lymphoid tissue via HEVs and peripheral effector sites such as the intestinal lamina propria; rolling, activation, firm adhesion and transmigration [15]. The initiation of lymphocyte rolling on the endothelium is mediated primarily by selectins, including L-selectin expressed by naïve lymphocytes and P-selectin as well as E-selectin expressed by the activated endothelium. P- and E-selectin on the endothelium is recognized by glycosylated P-selectin glycoptrotien ligand-1 (PSGL-1) present on all lymphoid cells whereas L-selectin binds to a group of carbohydrate structures collectively termed PNAd, exclusively expressed in HEV, present in secondary lymphoid organs. L-selectin

is expressed on most lymphocytes except on effector memory cells [16]. In Payer's patches, MAdCAM-1 also mediate rolling of lymphocytes through interactions with L-selectin, which has not been observed in other lymphoid organs [5]. The primary adhesion molecules, mediating rolling of lymphocytes on the endothelium, are constitutively active and hence need no previous activation to bind their ligands. Rolling on the endothelium significantly reduce the speed of travelling lymphocytes, enabling their chemokine receptors to interact with chemokines immobilised on the endothelium. Signalling through chemokine receptors is crucial for the activation of integrins on the lymphocyte surface into a high affinity state, necessary for subsequent firm adhesion [1]. T cell homing to lymph nodes is facilitated through the constitutive expression of CCL19 and CCL21, present on the surface of HEVs to recruit CCR7 positive cells, including most T lymphocytes. B cells on the other hand, express CXCR4 and CXCR5 and are recruited to lymph nodes via interactions with CXCL12 and CXCL13 respectively [17,18]. Firm adhesion is mediated via binding of $\alpha L\beta 2$ (LFA-1), $\alpha 4\beta 1$ (VLA-4) and, especially important for intestinal homing, $\alpha 4\beta 7$ to their respective ligand intercellular CAM-1 (ICAM-1), vascular CAM-1 (VCAM-1) and MAdCAM-1 [15]. Thus, MAdCAM-1 can initiate rolling of lymphocytes on the small intestinal PP HEV through L-selectin, whereas its participation in firm adhesion requires activation of the $\alpha 4\beta 7$ integrin. This cascade of interactions will ultimately lead to the arrest and crossing of lymphocytes through the endothelium.

Inflammation caused by infectious agents, autoimmunity or malignant transformation causes multiple alterations in the mucosal vasculature. This results in the accumulation of lymphocytes within the lymph nodes to enhance the probability of antigen encounter by specific T- lymphocytes as well as promotion of effector cell extravasation into the lamina propria. Up-regulation of P-selectin is detected within seconds after inflammatory exposure whereas increased ICAM-1 expression typically starts within hours [11]. E-selectin, ICAM-1 and MAdCAM-1 expression is also induced by inflammatory cytokines such as LPS, IL-1 and TNF-α [19,20]. Both experimental models of chronic colitits and inflamed intestinal tissue from patients suffering from IBD display elevated MAdCAM-1 expression and elevated influx of $\alpha 4\beta 7^{+}$ T cells [21,22]. The importance of this infiltration route into inflamed intestinal mucosa is demonstrated in animal models where blocking of $\alpha 4$ or $\beta 7$ both reduce intestinal inflammation [23,24].

The molecular events determining lymphocyte traffic are similar in homeostatic and inflammatory conditions, however, the phenotype of lamina propria lymphocytes reveal differential expression of chemokine receptors described in the *chemokines and chemokine receptors* section.

Lamina propria lymphocytes and intestinal immunity

The mucosal surface of the intestine is a critical barrier between the host and external environment, making it a potential entry site for microbes. As the intestine is densely colonized with harmless commensal bacteria, intestinal immune responses must be tightly regulated to avoid excessive, tissue-damaging inflammation. The intestinal mucosa consists of a single layer of epithelial cells and the lamina propria (LP) harbouring lymphocytes and other immune cells (Fig 1 and 3A). Some or the epithelial cells are termed goblet cells and produce mucus that protects the epithelium from immediate contact with commensal bacteria. Another defence mechanism is provided by secretion of antimicrobial peptides, among them defensins and cathelicidins, produced by paneth cells, another type of specialized epithelial cells, localized to the crypts. Also, secretory dimeric IgA is present in the intestinal lumen to block entry of microbes and associated toxins to the intestine [25]. Invading bacteria that cross this first line of defence will encounter non-circulating intraepithelial lymphocytes (IEL) that are scattered around the epithelial lining. Although IEL is a heterogenous population, the majority express CD8 and contains a higher proportion of γδ T cell receptor expressing cells compared to conventional T cells of the LP where the majority express αβ TCR. IEL display less diversity in their antigen receptor than conventional T cells and are believed to recognise antigens commonly encountered in the gut where some IEL can respond to antigens without prior priming. The function of human IEL is not well described but seemingly IEL are important for the integrity and healing of the epithelium, as well as induction of immune responses against enteric pathogens [26]. B-lymphocytes found in the LP are mostly IgA secreting plasma cells probably generated in the PP, and are scattered throughout the LP although rarely in the villi [2]. The majority of secreted IgA holds a dimeric structure and are secreted into the intestinal lumen, via epithelial cells, to bind and neutralize bacteria, viruses and toxins [27,28]. The majority of LP lymphocytes are MHC II restricted $CD4^+$ that are evenly distributed in the LP compartment. LP MHC class I restricted $CD8^+\alpha\beta^+$ T cells preferentially migrate to the

epithelium, however some 40% of LP T cells are $CD8^+$ [2]. LP $CD8^+$ T cells harbours cytolytic activity and compared to other tissues, the LP $CD8^+$ T cells mediate particularly long lasting immunity and display enhanced survival in response to systemic viral infection [29-31]. In fact, cells with an effector memory phenotype ($CD62L^-CCR7^-CD45RO^+\alpha4\beta7^+$) predominates among LP T lymphocytes, suggesting that a major proportion of the LPL are antigen-experienced [32].

Macrophages and dendritic cells, which are professional antigen presenting cells and critical for T cell activation, are abundant in the LP and have the ability to induce both protective immunity against invading pathogens and immune tolerance to harmless commensal bacteria and food antigens [33]. Besides being a major source of TNF-α, LP macrophages, in contrast to macrophages in other tissues, also produce significant amounts of the anti-inflammatory cytokine IL-10 [34]. Functionally, macrophages in the LP are characterized by their unique combination of high phagocytic capacity as well as ability to hinder excessive inflammatory responses and induce oral tolerance [33,35].

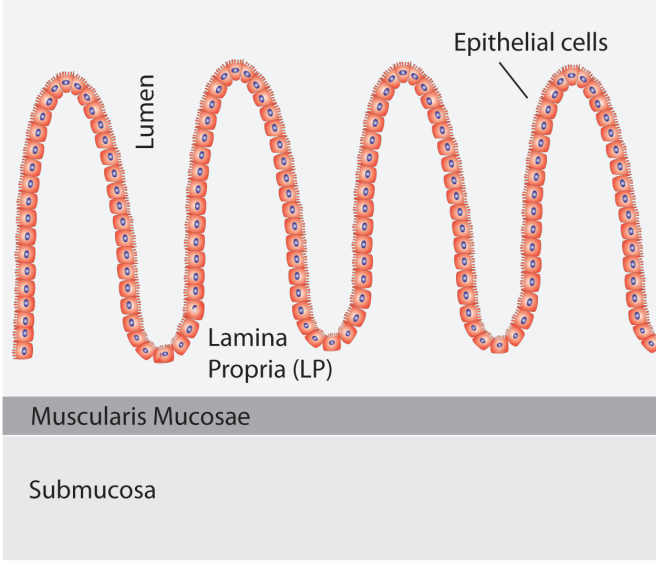


Fig 1 The colon mucosa. A single layer of epithelial cells protects the host from the outside environment and from coming in direct contact with commensal bacteria, pathogens and food antigens in the lumen. Beneath the epithelium is the lamina propria (LP) that harbours a wide range of immune cells, but also secondary lymphoid tissue. The lamina propria is supported by the muscularis mucosae, which also separates the LP from the submucosa, consisting of dense connective tissue and some adipose tissue.

Lamina propria T helper subsets

$CD4^+$ T helper cells orchestrate immune responses and are of particular importance in regulating local intestinal immunity. All $CD4^+$ subsets can be found in the intestinal LP including Th1 controlling cell-mediated cytotoxicity against intracellular bacteria, virus and tumor cells, Th2 inducing protection against helminth infection and allergy, Th17 producing inflammatory cytokines in response to extracellular bacteria and regulatory T cells (Treg) guiding immune homeostasis and regulating immune responses [29]. The $CD4^+$ helper phenotype and function are determined by cytokines released from antigen presenting DCs and surrounding cells during activation. However, $CD4^+$ are highly plastic and although a certain phenotype was induced by DC activation, a change in the surrounding cytokine profile may induce a shift in both phenotype and function in T helper cells [36]. Further, antigen concentration and co-stimulatory signals also influence differentiation of $CD4^+$ T cells [37]. As already indicated, the lamina propria is a highly complex immunologic site where control of immune responses is crucial. LP DCs seem to be of significant importance in inducing the immunosuppressive nature of the LP, through the release of RA during T cell activation [14]. Although several subsets of DC are localized in the LP, they can be classified into two major populations based on their ability to activate T-cells. In mice, $CD11b^+$ DCs induce Th1 and Th17 polarization whereas $CD103^+CX3CR1^-$ DCs are involved in the induction of Tregs [34]. $CD103^+CX3CR1^-$ DCs has also been described as classical DCs that transport LP antigens to the mesenteric lymph node (mLN) to induce adaptive immune responses [35]. Most LP $CD103^+$ DCs in mice and humans express high levels of RALDH2 (retinal dehydrogenase) and hence have the capacity to produce RA to control intestinal Th differentiation. RA has been suggested to inhibit inflammatory Th17 induction and promote Treg differentiation *in vitro* [38]. Further, rats deficient in the RA precursor vitamin A display decreased Th2 responses [39] whereas supplementation of vitamin A in mice inhibit Th1 but promote Th2 differentiation, indicating that RA protects the intestine against exaggerated cell mediated immunity [40,41]. The major Th subsets infiltrating the LP are described below (Fig 2).

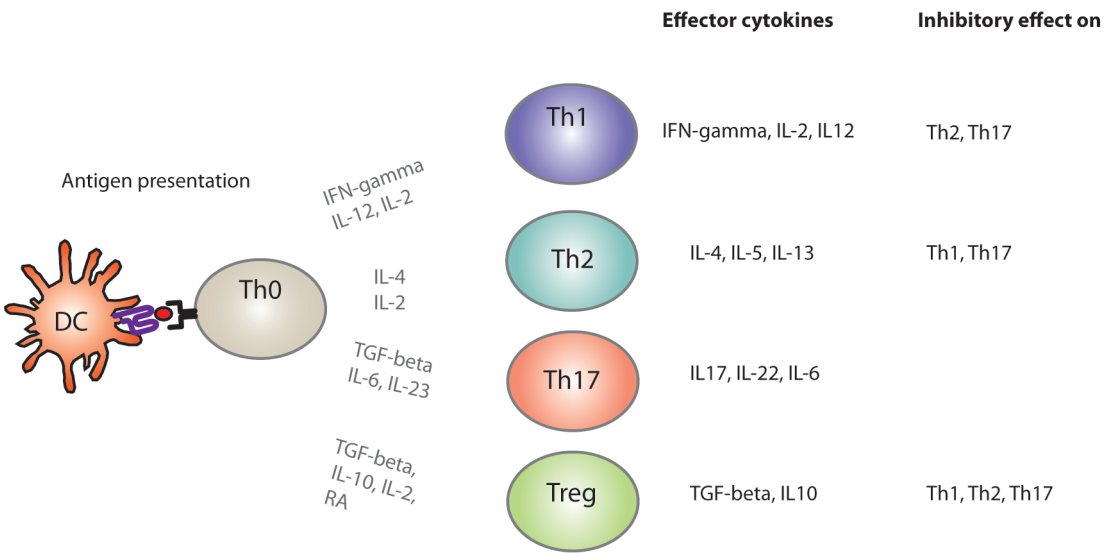


Fig 2 Depending on the cytokine profile, dendritic cell (DC) activation of of naïve $CD4^+$ T cells (T0) results in the differentiation into distinct subsets of T helper cells (Th1, Th2, Th17, Treg). Each T helper subset has their unique production of effector cytokines and subsequent impact on immune responses. In addition, the cytokines produced by certain T helper subsets commonly inhibit the differentiation of other T helper cell subsets.

Th1 cells are necessary in mounting cellular immune responses against intracellular pathogens and tumor cells, and are induced by IL-12, TNF-α and IFN-γ secreted by antigen presenting DCs, macrophages and NK-cells in inflammatory lesions. Th1 cells further contribute to the production of IL-2, IL-12 and IFN-γ, resulting in enhanced activation by macrophages and cytotoxic NK-cells. IL-2 and IL12 also induce proliferation of $CD8^+$ T cells, the population mediating antigen specific cytotoxicity [42]. Th1 cells in the LP are crucial in efficient generation of protective $CD8^+\alpha\beta^+$ effector memory responses [29]. However, many antigens that pass the mucosal barrier are harmless and excessive Th1 responses are in part avoided via the presence of RA, IL-10 and TGF-β in the intestinal LP. Uncontrolled activation of Th1 may result in intestinal inflammatory disease as shown in an experimental model of colitis where over-expression of STAT-4, a transcription factor driving Th1 gene expression, results in colitis due to excessive IFN-γ production by $CD4^+$ T cells [43]. Moreover, administration of IL-12 to mice with chemically induced colitis resulted in more severe inflammation whereas antibody blocking of IL-12 completely abrogated inflammation [44]. Human IBD is characterized by excessive levels of inflammatory cytokines including IL-12 and IFN-γ [45]. Further, *in vitro* stimulation of lamina propria $CD4^+$ isolated from IBD

patients produce significantly more IFN-γ but less of the Th2 cytokine IL-4, compared to control $CD4^+$ cells [46].

Th2 responses are fundamental for the induction of antibody production from B-cells in response to extracellular pathogens but seemingly also to tumor cells as cancer patients display tumor antigen specific antibodies (described later). IL-4 is essential for the development of Th2 cells and its presence during DC priming induce the ability of T cells to produce Th2 signature cytokines including IL-4, IL-5 and IL-13 [47]. Although the participation of Th2 cells in the induction of colitis is not fully elucidated, it has been reported that LP T cells from ulcerative colitis lesions produce elevated levels of IL-5 upon *in vitro* stimulation compared to controls. In contrast, LP lymphocytes from Crohn's disease lesions did not, indicating differences between intestinal compartments [46].

In the LP, the differentiation of inducible Treg **(iTreg)** is promoted by TGF-β and IL-10, produced by various cell types including recruited natural **(nTreg)**, DCs, macrophages and epithelial cells [48,49]. Tregs are indispensible for maintaining gut homeostasis and to prevent the development of colitis in response to commensal bacteria, via their production of TGF-β and IL-10 [29,50]. In contrast to wt, transfer of $CD4^+CD25^+$ Treg from TGF-β1 deficient mice do not protect recipient mice from colitis [51]. However, IL-10 expression in adoptively transferred Treg is dispensable but not the presence of IL-10 in the LP, as blocking of IL-10 resulted in elevated Th1 cytokines and impaired wild type (wt) Treg function [52].

Proinflammatory **Th17** cells are, like iTreg, generated in response to TGF-β, but only in the presence of the inflammatory cytokine IL-6. Also, IL-23 is important in supporting the survival and effector functions of Th17 cells. [53]. Moreover, a recent publication shows that IL-1β is required for Th17 differentiation of naïve, human T cells in response to *Candida albicans* stimulation but not to *Staphylococcus aureus*, indicating differential requirements for T helper cell differentiation depending on the pathogen [54]. The inflammatory qualities of Th17 responses are demonstrated in the EAE (experimental autoimmune encephalomyelitis) model where T cell specific block of TGF-β results in poor differentiation of Th17 cells and resistance to EAE [55]. Further, IL-6 deficient mice

are also protected against EAE due to low numbers of Th17 cells and immune responses dominated by $Foxp3^+$ cells. Th17 effector cytokines include IL-17, IL-22 and IL-6 and in the intestinal lamina propria, Th17 cells protect the host against extracellular bacteria and fungi [56,57].

The cytokines governing Th induction usually have counter-regulating effects as well. IL-4 is not only an inducer of Th2 responses but simultaneously block differentiation of Th1 cells by suppressing the expression of the IL-12Rβ2 chain, resulting in unresponsiveness to IL-12 and inhibited IFN-γ production. Similarly, IFN-γ producing T cells inhibit the differentiation of Th2 cells and possibly of Tregs by inhibiting IL-10 expression [58,59]. Conversely, Treg inhibit Th1 differentiation by secreting IL-10, which reduces IL-12 production by APCs. Moreover, TGF-β is reported to suppress both Th1 and Th2 responses [60]. Proinflammatory Th17 and regulatory Treg induction are reciprocally connected and both induced by TGF-β. In the context of low TGF-β and simultaneous availability of IL-6 and IL-21, $CD4^+$ cells differentiate into Th17 while high TGF-β levels suppress the Th17 phenotype in favour of Tregs [61]. Also, both IL-4 and IFN-γ seem to have inhibitory impact on Th17 differentiation [62,63]. Notably, the induction of specialised properties and effector functions in $CD4^+$ cells are usually studied *in vitro* and therefore lack the influence of complex *in vivo* immune processes, which might affect the fate of $CD4^+$ T cells.

Chemokines and Chemokine receptors

The human chemokine system is composed of more than 50 chemokines and 18 chemokine receptors. Chemokines are small, chemotactic molecules produced by most cell types and are devided into subgroups based on their structural as well as functional properties [64,65]. The major structural chemokine subgroups are C, CC, CXC and CX_3C which describes the conserved position of N-terminal cysteins [66]. Chemokines also harbour distinct functions with homeostatic chemokines being constitutively expressed and are involved in maintaining lymphocyte recirculation, important for antigen sampling and immune surveillance. Inflammatory chemokines are released upon inflammatory insult to guide the migration of effector cells to peripheral as well as lymphoid tissues [67]. Except for the most recently discovered chemokine decoy receptors, binding of chemokines is usually restricted within the structural subclass,

with CC chemokines having affinity for CC chemokine receptors and so forth. Chemokines may however bind several receptors and several chemokine receptors have affinity for more than one chemokine [64,65]. The interaction between chemokines and corresponding receptors induce cytoskeleton rearrangements and conformational changes in membrane integrins, due to activation of G proteins. All conventional chemokine receptors are G-protein coupled and induce migration of cells [65,68,69]. Chemokine receptor expression is commonly used to classify functional subsets of lymphocytes. Th1 polarization of $CD4^+$ T cells *in vitro* induce the expression of CXCR3 and CCR5 [70]. Interestingly their ligands are produced in various inflammatory states including colitis, where CXCL10 is dramatically up-regulated and infiltrating $CXCR3^+$ T cells produce high amounts of IFN-γ [71,72]. Th2 cells on the other hand express CCR4, CCR8 and CCR3 [67,73]. Notably, CCR4 memory T cells also express CXCR3 and CCR5, making it difficult to distinguish the effector subtypes solely based on their chemokine receptor expression [74].

Chemokine decoy receptors

Chemokine decoy receptors (CDR) possess some unique features as compared to conventional chemokine receptors as they promiscuously bind chemokines of different structural and functional classes. When interacting with ligands, the CDR internalise the complex without inducing cell migration. In contrast to other chemokine receptors the CDR lack the functional DRYLAIV motif, necessary for down-stream G-protein coupling and intracellular signalling [75]. The CDR family includes D6, Duffy antigen receptor for chemokines (DARC) and ChemoCentryx chemokine receptor (CCX-CKR) [76]. With varying affinity for over 15 pro-inflammatory CC chemokines, D6 is proposed to facilitate resolution and limit inflammation. This protective role is mediated via regulation of pro-inflamamtory CC chemokines, demonstrated in experimental models of psoriasis-like dermal inflammation, autoimmune diabetes, autoimmune encephalomyelitis and colitis [77-81]. Recently, D6 has also been shown to impair entry of APCs from effector sites to lymph nodes during inflammation. $D6^{-/-}$ mice display accumulation of CC-chemokines outside lymph nodes and retention of CCR7 expressing APCs in the peripheral tissue [82]. D6 expression is observed in several types of tissues including placenta, leukocytes, lung, skin and gut, partly on lymphatic endothelial cells [83,84]. Little is known about the regulation of D6 expression, but Weber *et al* have

Table I Chemokine receptors and their corresponding ligands (chemokines)

	Chemokine receptor	Chemokine
Inflammatory	CXCR3	CXCL9, CXCL10
	CXCR1,2	CXCL8
	CCR4	CCL22
Homeostatic	CCR9	CCL25
	CCR10	CCL28
	CCR7	CCL19, CCL21
Chemokine decoy receptors	D6	CCL22, CCL2, CCL3, CCL3L1, CCL4, CCL5, CCL7 CCL8, CCL11, CCL13, CCL17, CCL23, CCL24
	DARC	CCL2, CCL5, CCL7, CCL11, CCL13, CCL14, CCL17, CXCL5, CXCL6, CXCL8, CXCL11
	CCX-CKR	CCL19, CCL21, CCL25

demonstrated constitutive cycling of D6 to and from the cell surface, independent of surrounding chemokine concentrations [85]. Further, D6 expression has been connected to GATA1 expression in leukocytes, a transcriptional regulator of hemopoietic cell differentiation. Knocking down GATA1 in mouse DCs also block D6 expression *in vitro* [84]. The function of DARC, expressed on blood endothelium, is partly regulatory as the receptor is suggested to remove excess chemokines from the circulation [86]. In addition, DARC has the ability to transport chemokines from the basal to the apical side of endothelial cells to make them accessible to passing lymphocytes, which promotes adhesion and transmigration [87]. CCX-CKR is expressed in the thymus, intestinal lymph node and skin where it binds to and scavanges the homeostatic chemokines CCL19, CCL21 and CCL25 [88,89]. CCX-CKR is suggested to be involved in the suppression of Th17 immune responses as CCX-CKR$^{-/-}$ mice display earlier onset of EAE due to exaggerated Th17 immunity compared to wt controls [88]. Moreover, these mice display

a spontaneous Sjögren's syndrome like pathology, charecterised with lymphocytic lesions as well as defect lymphocyte distribution in the thymus, indicating a role for CCX-CKR in T cell regulation [90].

Colorectal cancer

With over a million new cases every year, colorectal cancer (CRC) is the third most common malignancy worldwide and the fourth biggest cause of cancer mortality [91]. Despite detection and treatment advantages over the recent decades, the 5-year survival is approximately 54% in Europe [92]. Risk factors for sporadic CRC, other than age and gender (colorectal cancer is more prevalent in males) [93] are intake of high fat/low dietary fibre diet, smoking, alcohol and inflammatory bowel disease (IBD) involving the colon [94]. The most frequent type of sporadic tumors that arise in the colon is adenocarcinoma, where the malignant transformation occurs in epithelial cells and commonly found in ascending colon [94]. In addition to surgery, which is the primary treatment for CRC, radiotherapy and chemotherapy are traditionally used to avoid relapse and to prolong survival of the patient [93,94].

Studying the contribution of lymphocytes in colorectal cancer requires an appropriate *in vivo* model. Approximately 20% of colorectal cancer cases are hereditary, where mutation in the *Adenomatous polyposis coli* (APC) tumor suppressor gene is a major cause of disease [93]. Also, the majority of sporadic colorectal cancer lack functional APC which is considered an early event in tumor formation, as small colorectal adenomas and late stage carcinomas display similar loads of APC mutations [95]. Inactivation of APC results in exaggerated Wnt-signalling and uncontrolled proliferation, which in turn leads to the formation of benign adenomas [96]. Individuals with hereditary familial adenomatous polyposis (FAP), who has a germ-line mutation in the APC gene, can develop hundreds of adenomas at the age of 15 and most unscreened patients have developed colorectal cancer at the age of 40 [97]. Carcinoma development requires additional mutations, typically in genes controlling proliferation, apoptosis and DNA integrity [98]. In this thesis we have used the $APC^{Min/+}$ model of intestinal cancer, which is heterozygous for APC and described in detail in the *key methodology* section.

Tumor immunity

Lymphocyte recruitment to tumors

Effector T cells need to infiltrate tumors to have anti-tumor effects and both murine and human data show that infiltration of $CD4^+$ and $CD8^+$ T cells is necessary for tumor immunity [99,100]. In a mouse model of pancreatic cancer, intratumoral $CD4^+$ T cells induced the production of CXCL9 and CXCL10 through IFN-γ that enhanced the recruitment of CXCR3 expressing $CD8^+$ T cells [101]. In contrast, combined blocking of CXCL9 and CXCL10 significantly inhibited the recruitment of tumor-specific effector cells. The same study also showed that a combined blocking of CCL2, CCL3 and CCL5 inhibited effector cell recruitment, suggesting that multiple chemokine receptors and chemokines are involved in recruitment of lymphocytes to tumor sites [101]. When Galon *et al* investigated the impact of T helper associated gene clusters in CRC, they found that Th1 associated genes were expressed in patients with better disease-free survival. By immunohistochemistry they confirmed their finding as high infiltration of $CD8^+$ cells in colorectal tumors strongly correlated with disease free survival [102]. Phenotypic examination of intratumoral effector T cells shows that $CD8^+CD45RO^+$ memory cells are associated with absence of early metastasis and increased survival [103].

Tregs are strongly accumulated in tumors and in the circulation of cancer patients [104]. Although a number of murine studies have demonstrated that Treg suppress anti-tumor immune responses and promote tumor progression [105], the role of Treg in human malignancies is still not obvious. Curiel *et al* demonstrated that infiltration of functional Tregs in ovarian cancer correlates to tumor progression and reduced survival [106]. Subsequently, similar findings have been reported for a number of solid carcinomas [107-111]. Circulating and tumor infiltrating Tregs isolated from cancer patients do not produce IFN-γ but produce significant amounts of IL-10 and TGF-β, suggesting that Tregs are functional and inhibit immune responses in malignant disease [112,113]. A critical question in tumor immunity is how Tregs and effector T cells are recruited to tumors. Human $CD4^+CD25^+$ T cells isolated from healthy donor blood express CCR4 and CCR8 and are chemotactically responsive to CCL22/CCL17 and CCL1 respectively [114]. Mature dendritic cells and macrophages, constituting a large proportion of the leukocyte

infiltrate in tumors [115], produce these inflammatory chemokines and potentially recruit Tregs to the tumor site [114]. Indeed, Tregs infiltrating ovarian and breast tumors have a high expression of CCR4 and blocking of CCR4 reduced *in vitro* migration of Tregs isolated from ovarian tumors [106,108]. $CCR4^+$ cells are attracted to CCL22 and CCL17, however blocking of CCL22 but not CCL17 inhibited Treg migration in the *in vitro* system, proposing a selective role for CCL22 in tumor immunity [106]. *In vitro* suppression assays, where $CD4^+CD25^+$ Treg are co-cultured with autologous effector cells, show that the increased fractions of circulating and intratumoral Treg isolated from CRC patients are functional [116,117]. These findings indicate that Tregs impair tumor immunity in colorectal cancer and have a negative impact on disease outcome. However, in contrary to what is reported for other solid tumors, tumor infiltrating Treg seem beneficial for disease outcome in CRC [118]. High density of tumor infiltrating $Foxp3^+$ lymphocytes is not only correlated with improved survival in patients with CRC [118,119], but also seem to improve prognosis in patients in treatment with chemotherapy or chemoimmunotherapy [120]. Reports are however inconclusive as a recent study show that the suppressive ability of circulating Tregs correlates to disease recurrence in CRC patients with liver metastasis [121].

Cancer associated inflammation

Already in the late 19^{th} century, Rudolf Virchow noted that cancer commonly originates from sites of chronic inflammation and that tumors harboured leukocytic infiltrates [115]. Indeed, subsequent research revealed that inflammatory cells and mediators are found in most solid tumors and that many cancers appear in tissues with a history of chronic infection or inflammatory disease [115,122]. Patients suffering from IBD are pre-disposed to colon cancer, and 20% of affected individuals develop malignant tumors within 30 years of disease onset [91]. Further, several epidemiological studies strongly links inflammation to cancer as they consistently show that regular intake of aspirin or other NSAID's (non steroidal anti-inflammatory drugs) reduces the risk as well as overall mortality of colorectal cancer [123-125]. Another well-described inflammation-associated cancer in humans is gastric cancer with a strongly elevated risk in *H. pylori*-positive individuals with chronic gastritis compared to *H. pylori*-negative individuals [126].

The requirements for transformation of cells into tumor cells are described by Hanahan and Weinberg and are referred to as the six hallmarks of cancer: sustained proliferative signalling, evasion of growth suppressors, resistance to cell death, induction of angiogenesis, replicative immortality and the ability to invade and metastasise [127]. Since the relationship between inflammation and cancer has grown increasingly strong, the six hallmarks of cancer were recently extended, including inflammation as a seventh hallmark [128,129]. The chronic inflammation associated with IBD or *H. pylori* infection results in the recruitment of immune cells with the ability to produce mediators that facilitates malignant transformation of local epithelial cells as well as support of the progression and survival of solid tumors. Macrophages and neutrophils that are found at sites of chronic inflammation release reactive oxygen species (ROS) that induce DNA damage and genomic instability, which are held as critical driving forces behind tumor initiation [130]. Macrophages are plastic and polarize into M-1 (classic inflammatory response and anti-tumor) or M-2 (pro-tumor) depending on the local environment [131]. In established tumors, tumor associated macrophages (TAM), that are polarized into M-2, are the major cell infiltrate and they support tumor growth by secretion of the Treg recruiting chemokines CCL17 and CCL22, induction of Tregs, secretion of immunosuppressive IL-10, production of VEGF and TNF-α to induce angiogenesis and induction of tissue remodelling to facilitate invasion and metastasis [132]. The maturation of immature myeloid cells is partially blocked in patients with colorectal cancer resulting in accumulation of MDSCs (myeloid derived suppressor cells) in the circulation and inside tumors of the patients [133]. MDSCs are characterized by high expression of arginase-I and iNOS (inducible nitric oxide synthase) and the ability to suppress NK-cells as well as adaptive immunity [134,135]. Moreover, MDSCs has been shown to induce $Foxp3^+$ Tregs [136]. Tumors also recruit immature dendritic cells (DC) but block their otherwise subsequent maturation, leading to very few mature DCs in the tumor that can initiate adaptive immunity. Instead, immature DCs release proangiogenic cytokines that promote tumor survival [137,138]. Seemingly, the tumor microenvironment in CRC favour polarization of chronic inflammation rather that acute inflammation and subsequent activation of proper adaptive immunity.

Immunosurveillance in cancer

Cancer incidence is significantly higher in individuals with congenital or acquired immunodeficiency and immunosuppressed transplant recipients, compared to the average population [139,140]. Further evidence for immunosurveillance in cancer comes from Shankaran *et al* who elegantly demonstrated that chemically induced and spontaneous tumors develop earlier and more frequent in $Rag2^{-/-}$ mice that completely lack mature lymphocytes compared to wt controls [100]. Immune cells, preferentially macrophages and lymphocytes, infiltrate tumors in response to malignantly transformed cells with accompanying tissue destruction, hypoxia and release of cytosolic content due to the high rate of necrosis in tumor cells [141]. Hence, immunosurveillance of tumors is suggested to start with an inflammatory response with subsequent activation of adaptive immunity that eliminates malignant cells before they are detectable [142]. However, through mechanisms only starting to emerge, tumor immunity fails to eliminate all tumor cells in the case of cancer disease and moreover, tumor growth is even supported by certain immune cells, described above. These interactions between the immune system and tumor cells is a process termed "The three E's of immunoediting"- Elimination, Equilibrium and Escape- proposed by Schreiber *et al* [142]. *Elimination* represents successful immunologic elimination of tumor cells. The *equilibrium* phase describes immunoediting of tumor cells; a process where tumor cells evolve under the selective pressure of immune reponses, resulting in the survival of less immunogenic tumor cell populations. In the *escape* phase, tumor cells that have acquired the ability to circumvent recognition by the immune system progress into a detectable tumor mass [142].

Anti-tumor immune responses in colorectal cancer

Tumors express immunogenic tumor-associated antigens (TAA) that are recognised by the immune system [143] and CRC patients evidently mount humoral and cellular tumor specific immune responses [144,145]. Besides NK-cells, dendritic cells and macrophages, one crucial factor in the destruction of tumor cells is the activity of tumor-specific $CD8^+$ lymphocytes, that recognise tumor cells via antigen presentation by MHC class I molecules [146,147]. Control of tumor cells by $CD8^+$ cells require IFNγ [100] and is mediated via perforin, granzyme B, TRAIL or FasL dependent mechanisms, all which induce apoptosis in target cells [148]. IFNγ is secreted by NK-cells and T cells present in

the tumor, and animal models where IFNγ is deleted or tumors are made IFNγ-insensitive, display significantly more chemically induced and spontaneous tumors compared to wt counterparts [142]. IFNγ does not only activate cytotoxic immune responses but also have direct anti-tumor effects, including anti-proliferative signals and inhibition of angiogenesis as well as making tumor cells more immunogenic by inducing MHC class I expression [142]. Th2 immune responses are proven inefficient in protecting the host from malignancy, whereas infiltration of Th1 $CD4^+$ T helper cells secreting IFNγ, enhances tumor immune responses as they boost $CD8^+$ T cell activation and proliferation [42]. $CD4^+$ T helper cells are also necessary for generating $CD8^+$ memory cells, suggested to be superior to other $CD8^+$ T cells in the control of tumors [149,150]. Clinical data reveals that high densities of $CD3^+CD45RO^+$ memory T cells in colorectal tumors correlates with improved overall survival of the patient. The importance of Th1 responses in colorectal tumors is supported in the same report, as patients that expressed high levels of intratumoral Th1-related genes (IFNγ, CD3, CD8, granzyme B) in their tumors had a better prognosis than individuals with low expression [151]. The role of Th-17 cells in tumor immunity is unclear as they harbour both tumorigenic and anti-tumor functions. This dual role of Th17 cells in malignancy seem to depend on the tumor microenvironment [152]. However, there is evidence that the infiltration of Th17 cells correlates with poor prognosis in CRC patients [102,153]. Murine studies further indicate pro-tumorigenic qualities in Th17 cells as shown by their ability to mediate tumor progression in spontaneous and bacteria induced colon cancer [154].

Tumor immune escape

According to the model put forward by Dunn *et al*, immunoediting is responsible for shaping non-immunogenic tumor cells that can avoid recognition from the immune system through a number of mechanisms [142]. Tumor specific CD8+ T cells recognise tumor cells through the presentation of tumor antigens via MHC class I molecules. Commonly, tumor cells lose MHC class I expression, or the ability to process antigens for presentation on MHC I, and thereby circumvent detection by CD8+ T cells. MHC class I signals “self” to the immune system and cells that lack expression are still recognised as foreign by NK-cells, that normally respond with cytolytic attack [155]. However, tumors also alter the MHC I expression and thereby NK-cells also fail to detect them [156]. Even

within the same tumor, TAA expression is heterogenous, Tumors are known to alter the expression of TAA and low levels or suboptimal combinations of antigens could avoid detection by the immune system [157]. Moreover, tumor cells can resist T-cell mediated killing by defects in death receptor signalling normally induced by Fas ligand (FasL) and TRAIL, expressed on T-cells. By down-regulating Fas and TRAIL receptor 1 and 2 or components in their signalling pathways, tumors become resistant to cell mediated immunity [148]. Tumor cells produce numerous soluble mediators with immunomodulatory properties, most notably TGF-β and IL-10 [158]. TGF-β abrogates T cell effector functions as well as proliferation and differentiation. IL-10 inhibits the priming of $CD8^+$ T lymphocytes by dendritic cells in vitro and levels of IL-10 in the serum of cancer patients have a negative prognostic impact [159]. Another property of IL-10 and TGF-β is that they promote the induction of regulatory T cells.

Regulatory T cells

In 1995, Sakaguchi *et al* discovered that maintenance of self-tolerance and control of autoimmunity were dependent on a subpopulation of $CD4^+$ T cells co-expressing CD25, the α-chain of the IL-2 receptor [160]. This discovery identified regulatory T cells (Treg) that until then was only known as a certain population of $CD4^+$ T cells that seemed to suppress immune responses rather than boosting them [161]. When transferring $CD4^+$ T cells, depleted from $CD25^+$ cells, into nude mice lacking functional T and B cells, they developed multiple autoimmune disease that was prevented by restoring T cell composition or by co-transfer with $CD4^+CD25^+$ lymphocytes [160]. A molecular definition became available with the discovery that $CD4^+CD25^+$ Treg express the transcription factor Foxp3 (forkhead box p3), which is now recognised as the master regulator of Treg development and function [162]. Mutations in the Foxp3 gene results in multiorgan autoimmunity as seen in in the Scurfy mouse or in the human equivalent IPEX disease (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) [163].

The generation of Tregs in the thymus is indispensible for immune homeostasis and self-tolerance. Thymus derived Tregs (nTreg) develop from $Foxp3^-$ precursors, expressing T cell receptors with intermediate affinity for self-antigens [164]. This process is IL-2 dependent and generates cells that evidently maintain peripheral immune homeostasis

[165]. It is now clear that a subset of $CD4^+Foxp3^+$ Tregs develops outside the thymus in peripheral tissues such as the gut. These inducible Tregs (iTregs) appear in the gut in response to food antigens, in chronic inflammation and in malignant tissues. Conditions known to favour iTreg development are chronic low dose exposure to antigens in combination with suboptimal co-stimulation in the presence of TGF-β and IL-2. In the gut, DCs are potent inducers of iTregs as they produce high amounts of TGF-β and retinoic acid that further enhance the induction of iTregs. iTregs are important in mucosal tolerance and in suppressing immune responses to neo-antigens in tumors and transplants [166,165]. However, it is not clear if Tregs of different origins have distinct functions or if they act synergistically. Great effort has been made to find absolute and specific markers to separate Tregs from conventional T cells ($CD4^+CD25^{-/low}$ and $CD8^+CD25^-$). To date, no such molecules are discovered and multiple markers are used to identify Treg. Commonly, $CD4^+Foxp3^+$ $CD25^+$ cells are regarded as regulatory T cells in mice, however, human lymphocytes display a more heterogenous CD25 expression and Treg are identified as $CD4^+Foxp3^+CD25^{hi}$ [167,162]. Treg can also be identified by their constitutive expression of CTLA-4 (cytotoxic T lymphocyte associated antigen-4) and low expression of the IL-7 receptor CD127 [168,169]. In addition, the identity of Treg is commonly verified by their high production of IL-10 and TGF-β and absence of IL-2 and IL- 4 production [170,171].

Suppressive functions of regulatory T cells

Several mechanisms have been suggested for $CD4^+Foxp3^+$ Treg mediated immune suppression. Tregs are known for their hypoproduction of IL-2 and for inhibiting T cell proliferation *in vitro* by limiting the access of IL-2 through the high expression of **CD25** [172]. *In vitro* experiments show that when adding exogenous IL-2 to co-cultures of Tregs and autologous conventional T cells, Treg suppression of the latter is abrogated [173]. This is further evidenced by *in vivo* studies in mice where deletion of IL-2 or the IL-2 receptor gene, disrupt immune regulation by Tregs and result in autoimmunity, which can be reversed by adoptive transfer of wt $CD4^+CD25^+$ T cells [174,175].

CTLA-4 is indispensible for Treg function, as evidenced by Wing *et al* who found that mice with Treg specific deletion of CTLA-4 failed to control immune responses and developed autoimmune disease [176]. Activation of T cells and antigen presenting cells

depend on co-stimulatory signals mediated via CD28, expressed by T cells, that induces immune activation through binding of CD80/86, present on the surface of antigen APCs. CTLA-4, which is highly expressed in Tregs, out-compete CD28 due to stronger affinity for CD80/86, leading to reduced activation of target cells [177]. In addition, Tregs seem to enhance this effect by CTLA-4 mediated down-regulation of CD80/86 expression in target APCs *in vitro,* making the effect even more significant [178]. However, the suppressive function of human Treg *in vitro* has also been reported to be independent of CTLA-4 [179].

Damaged or activated cells release ATP, which promotes immune responses in part by inducing production of IL-2 [180]. ATP can be hydrolysed into **adenosine** through the action of the ectoenzymes **CD39** and **CD73**, preferentially expressed by Tregs in mice [181]. Hence, ATP concentrations are reduced and instead adenosine, that has a suppressive effect on T cell cytokine production and proliferation, becomes available. Human Tregs can be divided into a $CD39^+$ and a $CD39^-$ fraction and expression of CD73 is low or absent [182]. However, endothelial cells among others express CD73 and presence of Tregs can potentially facilitate the conversion of ATP into adenosine in human tissues [183].

TGF-β and **IL-10** are signature immune suppressive cytokines and although they are produced by Tregs, their role in Treg suppressive function is somewhat unclear. We have previously shown that human Tregs exert regulatory functions *in vitro* independent of IL-10 and TGF-β, similarly to what others report on human Tregs [179,184]. In contrary, *in vivo* murine expriments reveal that TGF-β is required for Treg control of immune homeostasis and inflammation specifically in the intestine. Interestingly, IL-10 is only essential for Treg suppressive activity in the intestine of mice as $IL\text{-}10^{-/-}$ mice develop severe intestinal inflammation but are free from multiple autoimmune disease [185]. The discrepancy of results obtained from human *in vitro* studies and murine *in vivo* models suggest that experimental setup affects Treg function, and that differences between species might exist. Moreover, it is not clear whether several subtypes of Tregs exist or if their function is merely context dependent.

Aim of the thesis

The general aim of the thesis was to determine the mechanisms for conventional and regulatory T cell infiltration in colon adenocarcinoma, compared to surrounding unaffected mucosa, and possible effects of $CD4^+Foxp3^+$ T cells on conventional T cell migration.

The specific aims were to:

- Determine the phenotype of intratumoral lymphocytes and the mechanisms for their recruitment to human colon tumors.
- Define the expression of chemokine decoy receptors in human colon tumors.
- Determine the role of circulating $CD4^+CD25^{hi}$ T cells on *in vitro* migration of conventional T cells in colorectal cancer patients.
- Examine the effect of $Foxp3^+$ T cells on homing of conventional T cells to intestinal tumors in a mouse model of intestinal cancer.

Key methodology

Volunteers

Thirty-one (Paper I) and forty-one patients (Paper II), with some overlap, were included in these studies after informed consent and approval from the local Regional Board of Ethics in Medical Research in west Sweden (Paper I, Table I, Paper II, Table I). Blood was collected from cancer patients before surgery or one year post tumor resection and from age- and sex matched healthy donors. All patients had histologically verified adenocarcinoma and none of the participants had a history of autoimmune disease. Patients who received chemotherapy or radiation therapy within the last three years before surgery were excluded from the study. During or immediately after partial colectomy, strips of tissue were collected together with unaffected mucosa, minimum 5 cm away from the tumor, for downstream applications prior to LPL isolation (Fig). In this study, all parameters analysed in tumor mucosa (also referred to as tumor) are compared to unaffected mucosa from the same patient.

The $APC^{Min/+}$ mouse model of intestinal cancer and DEREG mice

The APC multiple intestinal neoplasia mouse ($APC^{Min/+}$) is heterozygous for a nonsense mutation in the APC gene, which is a homologue of the human APC [186]. Similarly to humans, impaired function of APC in C57BL/6 mice manifests in the formation of 30-100 adenomas along the small intestine. The colon usually display a low number of adenomas (1-4) that instead grow considerably larger compared to those in the small intestine [187]. The $APC^{Min/+}$ mouse adenomas rarely progress into invasive adenocarcinoma and have not been reported to metastasise [187]. $APC^{Min/+}$ males, kindly provided by Professor Sven Pettersson, Karolinska Institute, were bred to C57BL/6 females at the Department of Experimental Biomedicine, University of Gothenburg. To enable specific depletion of $Foxp3^+$ T cells we crossed $APC^{Min/+}$ males with female "depletion of Treg" (DEREG) mice, kindly provided by Professor Tim Sparwasser, Hannover University. DEREG mice express green fluorescent protein (GFP) and a high affinity Diphteria toxin (DT) receptor under the control of the Foxp3 promoter [188]. DEREG Tregs can be tracked by their GFP expression but also selectively depleted by administration of DT. Genotyping was performed in 4 weeks old mice by flow cytometry (detecting GFP expressing Treg) or PCR (heterozygosity of APC

allele). For flow cytometry and co-culture experiments, mice were sacrificed at week 10, 14, 18 and 22. The Government Animal Ethics Committee, Göteborg, approved all animal handling.

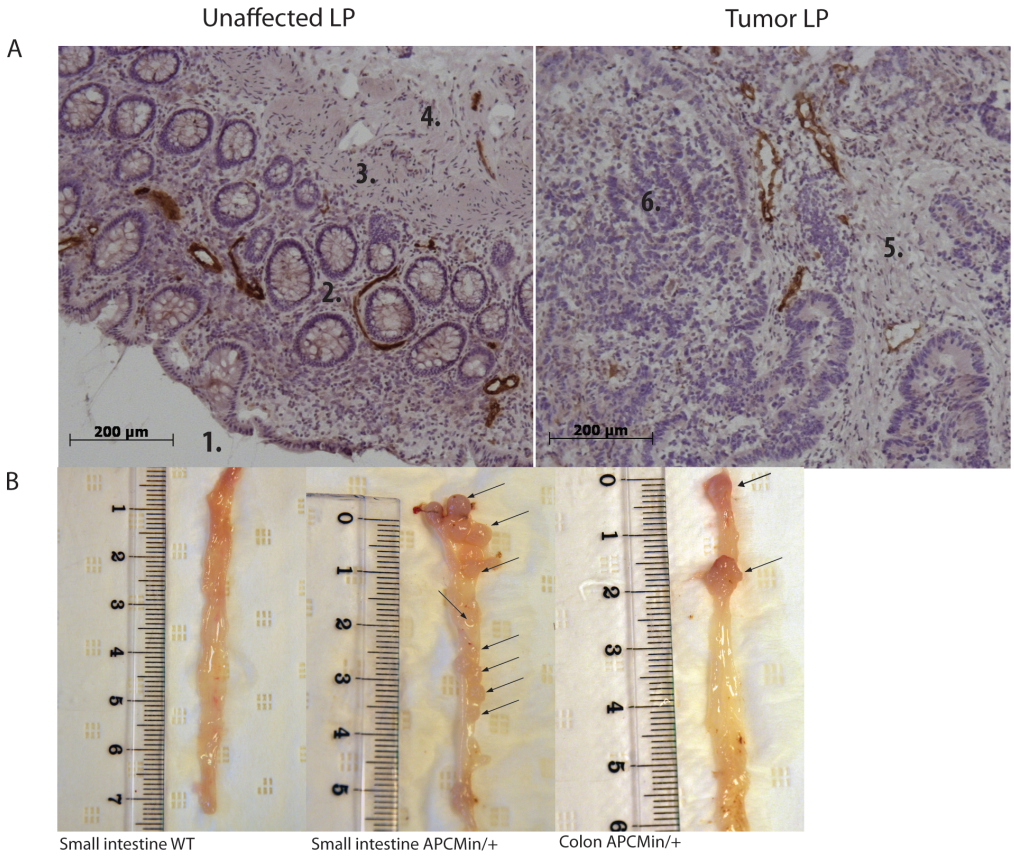


Fig 3 A. Staining of MAdCAM-1 in tumor and unaffected colon mucosa from the same patient, counterstained with hematoxylin. The unaffected colon mucosa display normal architecture with the epithelial lining (1) separating the intestinal lumen from the underlying lamina propria (2) which is infiltrated with lymphocytes. Below the lamina propria is the muscularis mucosae (3) and submucosa (4). Colon tumors commonly display patches of epithelial cells (6) interspersed with stromal cells (5). Lymphocytes rarely infiltrate the tumor epithelium but tend to cluster in the stroma. **B.** Proximal small intestine, and colon (whole length) longitudinally opened from WT (left), and $APC^{Min/+}$ mice (middle and right). Tumors are indicated by arrows.

In vivo depletion of Tregs

Administration of 0.02 mg/kg bodyweight on four consecutive days or day 1,2, 7 and 8, efficiently depleted Tregs in DEREG mice. In parallel, control animals were identically

DT treated. To confirm Treg depletion by flow cytometry analysis on whole blood, blood was collected at day 5. At day ten, mice were sacrificed and, spleen, MLN and intestines were collected for FACS analysis. Intestines were also collected for tissue immunofluorescence and LPL isolation.

Isolation of human PBMC and lamina propria lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation on Ficoll-Paque (Pharmacia). Dynabeads (Dynal Biotech AS) were used for depletion of $CD4^+$ and $CD8^+$ T cells and $CD14^+$ monocytes according to manufacturer's protocol (Paper III). Human colon LP was cut into pieces before incubation in HBSS (Sigma), containing 1mM EDTA/DTT, to remove epithelial cells and IEL. Subsequent isolation of LPL was performed with collagenase III/DNAse digestion (Sigma) at a concentration of 100U/ml and 0.1U/ml respectively, for 2 h or until tissue was completely resolved, at 37°C. This isolation procedure results in optimal yield of LPL, with minimum contaminating IEL and without altering surface expression of the markers analysed in this thesis [189].

Isolation and co-culture of mouse lymphocytes

After removal of PP, the small and large intestine were cut into small pieces for isolation of LPL. First, tissue was incubated in HBSS containing 2 mM EDTA (Gibco), 10% FCS and 1,5% HEPES (Gibco) at 37°C, to remove epithelial cells and IEL. The remaining small intestine was digested with collagenase D (Roche) whereas the large intestine was digested with collagenase VIII (Sigma). Cell debris was removed by filtration through a nylon mesh and lymphocytes were counted using trypan blue.

For co-culture experiments, single cell suspensions were prepared from spleen and subsequently filtered through nylon mesh before red blood cell lysis in 0.07 M NH_4Cl_2, pH 7.3. The autoMACS cell separating system was used for isolation of CD4+ T cells according to manufacturer's protocol (Minltenyi). Subsequently the CD4+ fraction was further separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations using anti-CD25 (PE) antibodies detected with anti-PE microbeads (Miltenyi). Remaining $CD4^-CD8^-$ cells were irradiated at 1000 rad and used as APCs for down stream *in vitro* assays. $5x10^4$ responder $CD4^+CD25^-$ cells were incubated with $2x10^5$ APC, 0.5μg/ml soluble anti-CD3

and $CD4^+CD25^+$ Tregs at various Treg:T responder ratios (1:1, 1:2, 1:4, 1:8) in 96 well plates for 72 h. In parallel whole speen cell fraction cells were incubated alone with anti-CD3 and APC. Thymidine (1μCi) was added during the last 6-8 hours of incubation. Plates were harvested in the Tomtec/Wallac system and proliferation through thymidine incorporation was measured by Trilux 1450 Micro beta counter system.

Murine *Helicobacter* infection

The *Helicobacter pylori* Sydney strain 1 (SS1) [190], adapted to infect mice was grown on Columbia Iso-A agar plates for 3 days before cultured in liquid Brucella broth (BD) over night, at micro-aerophilic conditions. The infection dose was determined by optical density (OD) and adjusted to OD 1,5, corresponding to approximately 1 x 10^9 bacteria/ml. Before infection, viability was assessed microscopically and bacteria were also titrated onto Columbia Iso-A culture plates for CFU quantification. Lastly, bacteria were intra-gastrically administered to 8 week old C56/Bl6 pathogen-free females (Harlan, Netherlands) at a dose of 3 x 10^8 bacteria/ml, via a feeding-needle under anaesthesia. The *Helicobacter felis* strain CS1 (ATCC) was grown on Columbia-Iso plates for 40-48 hours before washed with 1 ml PBS/plate and suspension was adjusted to OD 1 before infecting C56/Bl6 pathogen-free females with 200 μl as described above. One third of the longitudinal stomach was collected at day 1, 3, 5 and 7 post-infection (ss1) or week 4, 6 or 8 post-infection (*H.felis*) for RNA extraction and down-stream real time PCR. Histological scoring and urease test verified *Helicobacter* infection. All infected animals were successfully infected whereas control animals were free from *Helicobacter*.

Flow cytometry

Human LPL from both tumor and unaffected mucosa were incubated with anti-CD4, CD8, CD19, CD56, CD69, CD45RA, CD45RO, CD103, CXCR3, CD127, CD25, CD39 (AbD serotec), CD73, CCR4, CCR5 and CD31 (BD Biosciences). Integrin α4β7 was detected by biotinylated monoclonal antibody ACT-1, kindly provided by Dr. Picarella, Millenium Inc, Cambridge. Mouse cells were incubated with anti- CD4, CD8, CD25 and CD69. For downstream intracellular staining of Foxp3 (clone PCH101 (human), clone FJK-16s (mouse) eBioscience) and CTLA-4 (BD Biosciences), human and mouse cells were fixated and permeabilized using the Foxp3 staining buffer kit, according to manufacturers protocol (eBioscience). Lastly, human cells were incubated in cellfix (BD

Bioscience) and aquired on FACS Calibur or LSR II before analysis using the Flow Jo software (Tristar). Lymphocytes were identified by their characteristic forward and side scatter and 7AAD$^+$ (Sigma) or Live/Dead$^+$ (Live/Dead Cell Stain Kit, Invitrogen, Molecular probeS) cells were considered dead, and excluded from analysis in Flow Jo.

Flow cytometric cell sorting

CD4$^+$CD25^{hi} Treg was separated from PBMC by flow cytometric sorting on a FACSAria (BD Biosciences). Cells were sorted using anti-CD4, CD8 and CD25. CD4$^+$CD8$^-$CD25^{hi} cells were sorted as Treg and remaining cells were collected as a Treg depleted fraction (Paper III, Fig 3A).

Immunohistochemistry and immunofluorescence

After excision, human and mouse tissue was immediately frozen in OCT and stored in -80°C until use. Human tumor and unaffected mucosa was analysed for the expression of Foxp3 (eBioscience), CD4, CD8 (DAKO), MAdCAM-1 (clone 10G3 kindly provided by Dr. Sirpa Jalkanen, University of Turku), PNAd (MECA-79), Lyve-1 (R&D systems), biotinylated vWF (DAKO) and DARC (Everest biotech). 8 μm tissue sections were fixed in ice-cold acetone before rehydrated in PBS following blocking of endogenous biotin (Molecular probe, Invitrogen). For detection with DAB, tissue peroxidases was blocked in glucose peroxidase buffer, before incubation with primary antibodies for 30-60 min. Double staining with DARC and vWF primary antibodies were detected with donkey anti-mouse DyLight 549 and streptavidin-Alexa Fluor® 488 for 30 min. When performing double stainig with Foxp3 and CD4 or CD8, Foxp3 staining was enhanced using a tyramide Alexa Fluor® 488 TSA kit (molecular probes, Invitrogen) and CD4 or CD8 was detected with goat anti-mouse IgG1 Alexa Fluor® 549 (Molecular probes, Invitrogen). Lastly, sections were mounted with DAPI containing ProLong® Gold (Molecular probes, Invitrogen). For immunohistochemical detection, sections were incubated in streptavidine-HRP (ABC, Elite, Vectastain) for 45 minutes before incubation with DAB (Histolab). Sections were then counterstained with hematoxylin and mounted with Mountex (Histolab). Suitable isotype controls were used in parallel with all the above-mentioned primary antibodies. Images were acquired using the Axiovision 4.7.2 (Carl Zeiss) software and quantification was made in Biopix 2.0.21.

RNA isolation and real time PCR

Strips of tissue were incubated in RNA later (Ambion) over night at 4°C and subsequently stored in -80°C until use. Before RNA isolation, using the RNeasy mini kit (Qiagen), frozen tissue was mechanically homogenised (Tissuelyser II, Qiagen). Obtained RNA was run on agarose gels to confirm integrity, DNAse treated to eliminate contaminating DNA and concentration was spectrophotometerically measured (Nanodrop ND-100). 500 ng RNA template was used to synthesise cDNA using the Omniscript kit (Qiagen) in a total volume of 20μl. Real time PCR analysis on human material was performed on 40 ng cDNA in a reaction mixture of 1 x Taqman Universal PCR Master Mix and 1 μl Taqman primer (Table II, Applied Biosystems). Mouse mRNA expression was analysed using 40 ng cDNA, 1 x Power SYBR green mastermix (Applied Biosystems) and oligonucleotide primers (Table II) to detect MAdCAM-1 and β-actin. Mouse primers were designed in Primer Express software 2.1 (Applied Biosystems) and ordered from Eurofins MWG (Germany). Difference in gene expression was calculated using the comparative CT (threshold cycle) method, also termed 2-ΔΔCt [191]. Data are presented as fold change, normalised to 18s or β-actin, which are stably expressed in both tumor and unaffected intestinal mucosa. Assays were run at standard thermal cycling conditions, described for the 7500 real time PCR system (Applied Biosystems).

Detection of cytokines and chemokines

Human proteins were extracted by incubation in 2% saponin, 100 μg/ml soybean trypsin inhibitor, 350 μg/ml pefabloc and 0.1% BSA (Sigma) over night at 4°C. Suspensions were centrifuged and supernatants were subsequently collected for detection of chemokines. CCL22 and CCL8 were detected using Duoset ELISA (R&D Systems) according to manufacturer's protocol. The chemokine concentrations were normalized to total protein as measured with the BCA protein assay (Thermo Fisher Scientific) following desalting on Zeba™ micro desalt spin columns (Thermo Fisher Scientific). CCL2 and CCL5 were detected with cytometric bead array (CBA) chemokine analysis (BD Biosciences), also according to manufacturer's protocol. Mouse cytokines were measured in supernatants from co-culture experiment using CBA Flex set (BD Biosciences) targeting soluble IL-10, IFNγ, IL-1β, IL-17A and IL-13. CBA and CBA Flex set

samples were acquired on LSR II and data was analysed using the FCAP software (BD Biosciences).

Table II Primers used for mRNA detection in Taqman (human) and Sybr Green (mouse) based assays.

Human

Target	Primer ID
D6	Hs00174299_m1
DARC	Hs01011079_s1
MAdCAM1	Hs00369968_m1
18 s	Hs99999901_s1
CCX-CKR	Hs00356608_g1

Mouse

Target	Primer sequence
MAdCAM1	Forward 5'AACTGGTGCTGACCCATAGAAAGGA3' Reverse 5'GGGCTCAGCAGAGGTCGTGTT3'
Beta-actin	Forward 5'CTGACAGGATGCAGAAGGAGATTACT3' Reverse 5'GCCACCGATCCACACAGAGT3'

Transendothelial migration (TEM) assay

TEM was analysed in an *in vitro* transwell chamber system [192,184]. HUVEC cells were subcultured from the first passage and subsequent passage 4-6 were used for TEM experiments were they were grown to confluence on fibronectin coated filters of the upper chamber in 6-well transwell plates before the start of experiments. Basolateral to the upper chamber, either 5 x 10^7 CFU *H. pylori* Hel312, 100 ng/ml TNF-α, IL-1β, IL-8, 1 and 0.1 μg/ml LPs or 30 μg/ml polyI:C was added 4 h prior to addition of 10^6 PBMC at the apical side of the confluent endothelium. At 37°C, PBMC (whole fraction, $CD4^+CD25^{hi}$ depleted or $CD4^+CD25^-$ cells reconstituted with $CD4^+CD25^{hi}$) were allowed to migrate through the endothelium for 16 h before collected, counted and identified by flow cytometry. To estimate the quantity of migrating cells, migrated cells were counted using True Count beads (BD Biosciences) during FACS analysis. In some experiments, PBMC were stained with 1 μM CSFE for 30 min at 37 °C prior to TEM, to enable detection of proliferating cells. To examine the role of adenosine in mediating migration in our system, 10 μM of the stable adenosine analogue 2-chloro-adenosine (Tocris) was added to the migrating PBMC from healthy donors at the start of the experiment. In other experiments we blocked adenosine receptors in migrating lymphocytes from cancer patients, by addition of 25 μM 1,3-dipropyl-8-(p-sulphophenyl) xanthine (Sigma), which is a general adenosine receptor antagonist, 30 min before the start of the experiment.

Statistical analysis

To determine statistical significance between groups in paper I, II and II, the non-parametric tests, Wilcoxon Signed rank test or Mann Whitney was applied. In Paper IV, 2-way ANOVA and Bonferroni post-test were applied for multiple comparisons and Student's t test for calculating statistical significance between two groups. P values of <0.05 were considered statistically significant and are indicated in graphs by asterix. All statistical analyses were performed in Prism GraphPad 5 or SPSS 14.0.

Results and discussion

Lymphocyte frequencies in tumor and unaffected colon mucosa

Tumor infiltrating lymphocytes strongly correlate to disease outcome in CRC and even seem to have a superior predictive power of prognosis than more conventional methods [151]. Recent studies reveal that type, density and location are crucial for clinical outcome and therefore we examined the lymphocyte infiltrate in colon tumors compared to that of unaffected lamina propria from the same patient [102]. When performing a general characterization of intratumoral lymphocytes in colon tumors we found no difference in $CD4^+$ or $CD8^+$ T frequencies between tumor and unaffected colon mucosa. The distribution of $CD45RO^+$ memory cells was also similar between the two tissues, and so was the fraction of $CD3^-CD56^+$ NK-cells among lymphocytes. $CD19^+$ B-cells were on the other hand significantly decreased in tumor compared to unaffected tissue ($p<0.001$) (Fig 4). The ratio CD8/Foxp3 seems to be a strong prognostic marker for patient outcome in addition to absolute frequencies, meaning that relative frequencies of $CD8^+$ T cells in relation to other lymphocyte subsets are highly relevant and perhaps more so than the frequency of $CD8^+$ T cells alone [193-195]. Therefore, although not low compared to unaffected tissue, the relative density of $CD8^+$ T cells might be too low for effective anti-tumor activity. Further, the fraction of $CD45RO^+$ T cells inside colorectal tumors are also correlated to survival in patients with CRC and even if frequencies here are similar to unaffected colon mucosa, proper tumor immune-responses might depend on the relative frequency of $CD45RO^+$. Again, this means that if the ratio between memory T cells and suppressive cells is low, there might be poor immune resonses in the tumors examined. Also, considering that tumors mount immune responses and that the tumor microenvironment harbours activated, inflammatory cells, it is remarkable that the lymphocyte infiltrate is similar to that of adjacent resting tissue [196]. In contrast to T cells, $CD19^+$ B cells are not considered crucial for tumor immunity [197]. Still, B cells are reported to contribute both to tumor progression, in part by inhibiting anti-tumor cytotoxic lymphocytes [198,199], and to correlate with improved survival in ovarian cancer, making the role of B-cells in cancer somewhat diffuse [200]. We have previously demonstrated decreased frequencies of $CD19^+$ B cells also in gastric cancer [201] and even if not further discussed in this thesis, studies to examine the role of B-cells in CRC are ongoing in our lab.

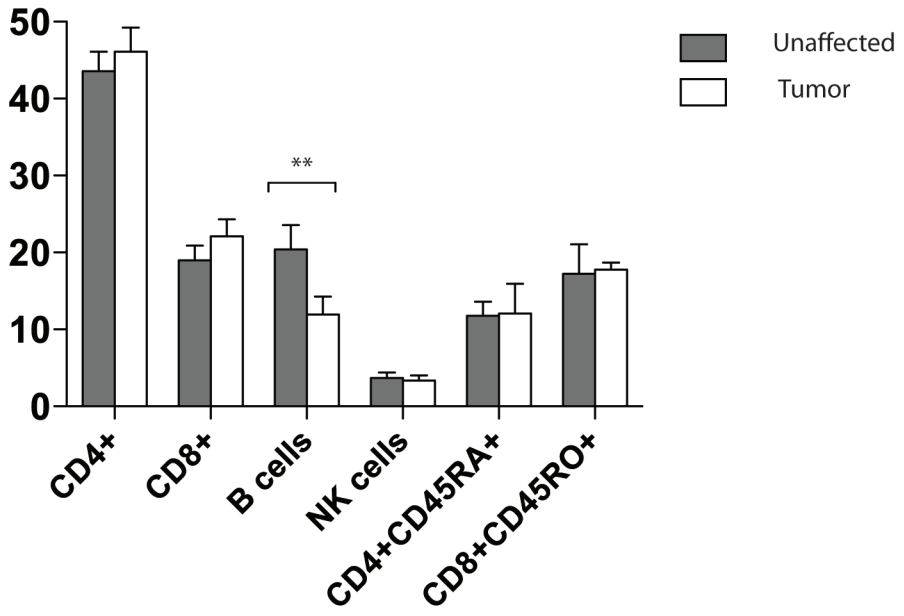


Fig 4 Lymphocyte subsets in tumor and unaffected mucosa. Frequencies of $CD4^+$ and $CD8^+$ T cells, $CD3^-CD56^+$ NK cells, $CD19^+$ B cells, and $CD45RA^+$ or $CD45RO^+$ $CD4^+$ and $CD8^+$ T cells among LPL from tumor and unaffected mucosa from patients with CRC were evaluated using flow cytometry. The bar graph represents the mean value ± SD of 10 patients for each cell subset and tissue source.
** $p<0.01$

Treg are accumulated in colon tumors and stably express Foxp3

The identification of human Treg is more demanding than in mice, not only because of heterogeneous expression of CD25 in human $CD4^+$ T cells (Fig 5) but also that Foxp3 is transiently expressed in activated conventional T cells [202,203]. Still, in tumor and unaffected mucosa we found that $CD4^+CD25^{hi}$ cells invariably expressed Foxp3 and had a low expression of CD127, making them putative Treg (Paper I, Fig 1B) [169]. We found that $CD4^+Foxp3^+CD25^{hi}$ Tregs were significantly accumulated in colon tumors ($p<0.001$) compared to unaffected mucosa (Paper I, Fig 1A), as we have previously reported for gastric tumors [184]. To verify that the $CD4^+Foxp3^+CD25^{hi}$ cells detected were not activated conventional cells we determined the methylation status of the Foxp3 promoter region by Ms-SNUPE (methylation sensitive single nucleotide primer extension analysis). Analysis revealed that the promoter region of $CD4^+Foxp3^+$ was almost completely demethylated (97-100% demethylation compared to 53-100% in

$CD4^+Foxp3^-$ cells) (Paper I, Fig 1D), suggesting that Foxp3 expression is stable and that the cells we detect are therefore Tregs [204].

Intratumoral Treg are $CCR4^+CTLA4^{hi}CD39^+$ and preferentially localized to the tumor stroma

When characterizing tumor-infiltrating Tregs we found clear differences compared to Tregs isolated from unaffected colon mucosa. The integrin $a_E\beta_7$ (CD103) anchors intraepithelial lymphocytes to E-cadherin on epithelial cells [205] and is further suggested to identify antigen experienced memory/effector Treg that develops in the periphery [206]. We noted that the fraction of $a_E\beta_7^+$ Tregs was larger in tumor compared to surrounding unaffected tissue ($p<0.05$), suggesting that these Tregs are induced locally in the tumor (Paper I, Fig 4A). In a murine model of colon cancer, where tumor cells were subcutaneously injected, the majority (~90%) of tumor-infiltrating Tregs were $a_E\beta_7^+$ [10]. This is in contrast to our results where Tregs only partially express $a_E\beta_7$, and might depend on the fact that the murine tumor was induced by injected cells or on differences between species. Some murine tumor-associated Tregs express Granzyme B that appears to be involved in their suppressive activity [207]. However, in this study no detectable levels of Granzyme B were found in human Tregs (data not shown). In contrast, we observed high expression levels of CTLA-4 on Tregs infiltrating colon tumors, similar to that of Tregs from unaffected tissue, and virtually all Tregs were $CTLA4^+$ (Paper I, Fig 3D/ data not shown). CTLA-4 in Tregs is indispensible for Treg mediated suppression of autoimmunity and anti-tumor responses [176] and high expression levels in tumor infiltrating Tregs indicate that active suppression of immune responses is on-going. To assess the potential of intratumoral Tregs to use adenosine for suppression of immune responses, we determined the expression of CD39 on $CD4^+CD25^{hi}$ cells. Virtually all tumor infiltrating Tregs expressed CD39 and the expression was significantly higher ($p<0.001$) compared to that of Tregs isolated from unaffected tissue (Paper III, Fig 5D). In contrast and opposite to what is reported for murine cells [181], CD73 was low on Treg isolated from either tissue (Paper III, Fig 5A). However, since endothelium express CD73, Tregs in colon tumors still have the potential to facilitate conversion of ATP into adenosine [208]. Fibroblasts could be an additional partner to Tregs in the conversion of ATP to adenosine as one report observed that fibroblasts in the breast tumor stroma was strongly CD73 positive [209]. A relevant

question is whether $CD39^+$ Tregs are recruited to tumors or if the ectonucleotidase expression is induced once the Tregs infiltrate the tumor. Comparing chemokine receptor expression between $CD39^+$ and $CD39^-$ circulating Tregs from cancer patients indicated that $CD39^+$ Tregs are selectively recruited to colon tumors as expression of CCR4 ($p<0.01$) and CCR6 ($p<0.01$) were upregulated compared to $CD39^-$ Tregs (data not shown). This is further supported by previous reports, showing that the ligands for both CCR4 and CCR6 (CCL22 and CCL20) are produced by colon tumors as well as our present data, observing increased intratumoral levels of CCL22 in colorectal cancer [210,211]. $CD39^+$ Tregs also expressed higher levels of CTLA-4 ($p<0.01$) compared to $CD39^-$ Tregs suggesting that $CD39^+$ Tregs have multiple suppressive effects (Paper III, Fig 6).

A number of studies suggest that Treg mediated suppression depends on cell-cell contact and hence Tregs need to be in close vincinity of target effector T cells to control immune responses. Immunofluorescent staining of colon tumors revealed that Tregs are accumulated in the stromal compartment of the tumor and that they rarely infiltrate the tumor mass, similar to what has been observed by others [212]. $CD4^+$ and low numbers of $CD8^+$ lymphocytes were also localized to the tumor stroma and sometimes in close contact to Tregs indicating that cell-cell dependent regulation is facilitated (Paper I, Fig 2).

Intratumoral conventional T cells display decreased frequencies of $CD69^+$ and $CD25^{int}$ cells and increased expression of CTLA-4

As T lymphocytes are central in tumor immunity we evaluated the degree of activation in the T cell populations of both tumor and unaffected colon mucosa. In contrast to $CD4^+CD25^{hi}$ putative Treg, activated conventional T lymphocytes display intermediate CD25 expression and were clearly and significantly reduced in colon tumors ($p<0.001$) compared to healthy tissue (Paper I, Fig 1C). A recent publication suggests that the majority of circulating human $CD4^+CD25^{int}$ T cells is memory T cells as they simultaneously expressed CD95 and CD45RO [213]. Memory T cells correlate with less metastasis in patients with CRC and are held as the most potent anti-tumor cell [150,149]. Therefore, having decreased frequencies of $CD4^+CD25^{int}$ lymphocytes

possibly means that both activated T cells and memory T cell activity may be present in low numbers, favouring tumor progression.

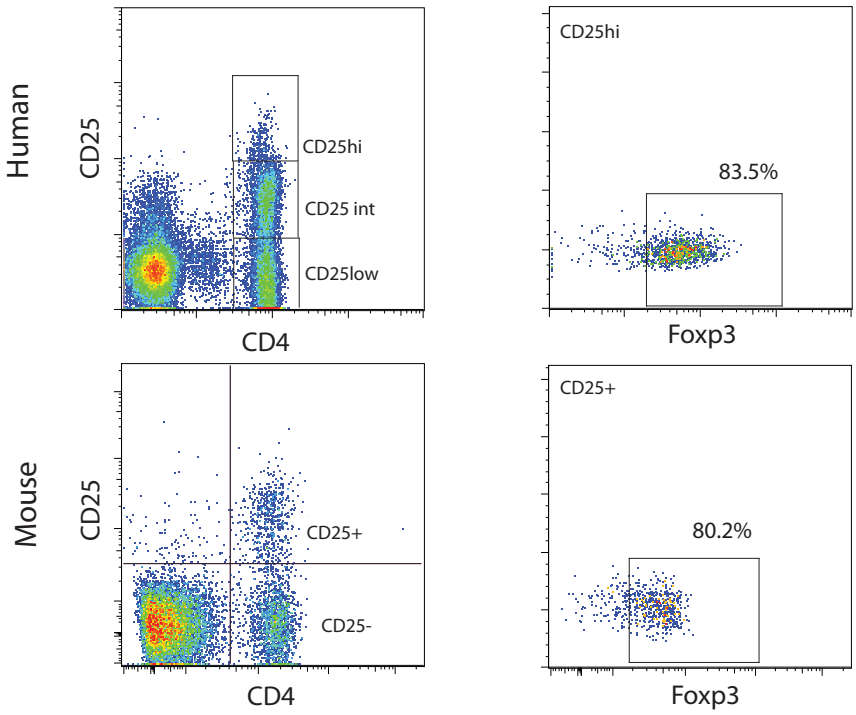


Fig 5 Upper panel: FACS plot showing characteristic $CD25^{low}$ (resting), $CD25^{int}$ (activated) and $CD25^{hi}$ (Treg) expression in $CD4^+$ T cells, among human PBMC (left). The right plot shows $Foxp3^+$ cells in the $CD4^+CD25^{hi}$ gate, which is the majority**. Lower panel:** Mouse lymphocytes (here isolated from the spleen) have a different distribution of CD25 compared to human lymphocytes, with $CD25^-$ (conventional T cells) or $CD25^+$ (Treg) $CD4^+$ T cells. The right plot shows $Foxp3^+$ cells in the $CD4^+CD25^+$ population.

In addition, the recently activated $CD69^+$ T cell fraction among $CD8^+$ and especially $CD4^+$ T cells was significantly decreased ($p<0.05$ and $p<0.01$ respectively) in tumor compared to unaffected mucosa (Paper I, Fig 3A). Although frequencies of T cells displaying activated phenotypes are decreased in colon tumors, we found that Granzyme B expressing $CD8^+$ T cells were more abundant among lymphocytes in tumors ($p<0.01$), showing the precense of putative cytotoxic effector cells at the location (Paper I, Fig 3B). However, the ratio of $CD8^+$Granzyme B^+ T cells to $CD4^+CD25^{hi}$ Treg was higher in the unaffected tissue than the tumor mucosa ($p<0.01$, Paper I, Fig 3E). Thus, although CTL are present, based on their Granzyme B expression [214], Tregs may still outnumber

these cells and create a favourable environment for malignant progression. One cause of insufficient cytolytic effects could be the increased CTLA-4$^+$ frequencies among intratumoral conventional T cells ($p<0.001$, Paper I, Fig 3C),suggesting them to be more susceptible to down-regulation of effector functions through ligation of their CTLA-4, compared to cells in unaffected colon mucosa [215]. Our data describes a tumor microenvironment where tumor immune responses and immune suppression co-exist and disease outcome probably depends on the balance between these intricate processes.

Simultaneous decrease of endothelial MAdCAM-1 and infiltration of $\alpha4\beta7^+$ conventional T cells in colon tumors

Compared to homing of lymphocytes to healthy colon mucosa, little is known about lymphocyte homing to colon tumors. To improve the knowledge about recruitment of lymphocytes to colon tumors, we analysed adhesion molecule expression in tumor infiltrating conventional CD4$^+$ and CD8$^+$ T cells as well as CD4$^+$Foxp3$^+$CD25^{hi} Tregs compared to lymphocytes isolated from adjacent unaffected colon mucosa. In addition, endothelium from both tumor and unaffected tissue was analysed for the expression of the homing molecule MAdCAM-1. Again, like we previously reported for gastric tumors [216], we observed a significant decrease in $\alpha_4\beta_7$ expressing CD4$^+$ T cells ($p<0.05$) in tumors compared to unaffected mucosa (Paper I, Fig 4A). Also, among Tregs the frequency of $\alpha_4\beta_7$ expressing cells was decreased in tumors from 4 out of 9 patients, and expression of $\alpha_4\beta_7$ was occasionally as low as 50% of that detected in Tregs from unaffected mucosa ($p<0.05$, Paper I, Fig 4A). MAdCAM-1, expressed by endothelial cells in the gut, mediates homing of $\alpha_4\beta_7$ expressing cells and decreased frequencies of $\alpha_4\beta_7^+$ lymphocytes in tumors could be explained by down-regulation of MAdCAM-1 on tumor blood vessels. Indeed, the mRNA levels of MAdCAM-1 were decreased in the majority of tumors ($p<0.05$), which was also confirmed by immunohistochemical detection of MAdCAM-1 (Paper I, Fig 5A,B). These data demonstrates differences in homing mechanisms between unaffected and malignant colon mucosa that in part can be explained by changes in the tumor-associated endothelium. Down-regulation of MAdCAM-1 might be one of several mechanism used by tumors to avoid infiltration of gut-homing effector T cells. Apparently, Tregs still accumulate in tumors and may not be as dependent on $\alpha_4\beta_7$/MAdCAM-1 interactions as Tregs infiltrating normal LP.

Besides integrins and selectins, lymphocyte homing is navigated by expression of chemokine receptors and available chemokines. An altered production of these chemokines in the tumor microenvironment could thus explain differential homing of lymphocytes to colon tumors compared to surrounding mucosa. The chemokine receptor CXCR3 is expressed by activated Th-1 lymphocytes and cytotoxic lymphocytes and $CXCR3^+$ lymphocytes are abundant in normal intestinal mucosa [217]. We found that $CXCR3^+$ conventional $CD4^+$ and $CD8^+$ T cell frequencies were clearly lower in tumors compared to unaffected tissue ($p<0.01$, Paper I, Fig 6A). *In vitro* studies imply that the host benefit from intratumoral $CXCR3^+CD4^+$ Th1 helper cells, with their ability to expand CD8+ T cells and induce IFN-γ production [218]. Interestingly, as IFN-γ is crucial for control of malignant growth, a shift in the $CXCR3^+$ fraction of lymphocytes might contribute to tumor progression through inadequate local production of IFN-γ. It has been shown in gastric cancer tissue supernatants that compared to non-cancerous gastric tissue, there is a shift in Th-responses [219]. Malignant gastric tissue produced the Th-2 related cytokine IL-4 but only modest levels of IFN-γ. One possible explanation for impaired recruitment of $CXCR3^+$ lymphocytes to tumors would be decreased production of the CXCR3 ligands CXCL10, CXCL9 and CXCL11. However, in contrary to what was expected, CXCR3 ligands were present in higher levels inside tumors compared to surrounding tissue from colon cancer patients, and could thus not explain the low CXCR3 frequency (data not shown). Interestingly, $CXCR3^+$ T cells have been reported to be evenly distributed in normal colon tissue whereas in colon tumors this population is mainly localized at the invasive margin of the tumor, indicating poor ability to infiltrate the tumor tissue [220]. The chemokine receptor CCR4 is preferentially expressed on Th-2 helper T cells and Tregs and cells expressing CCR4 migrate towards CCL22 and CCL17. We previously reported that gastric tumors harbour more $CCR4^+$ lymphocytes and CCR4 recruiting chemokines compared to surrounding tissue [221]. Here, we demonstrate that lymphocytes infiltrating colon tumors also display a Th2/Treg chemokine profile as CCR4 was significantly higher in $CD4^+$ T cells isolated from tumors compared to unaffected mucosa ($p<0.001$, Paper I, Fig 6B). A parallel finding was that the corresponding CCL22 but not CCL17 tissue concentration was three-fold upregulated in tumor tissue ($p<0.001$, Paper II, Table II) Further, we observed that intratumoral Tregs had significantly higher expression of CCR4 than Tregs

in surrounding tissue ($p<0.05$), indicating that CCL22 and CCR4 contribute to Treg recruitment ($p<0.001$, Paper I, Fig 6B). CCL22 is mainly derived from macrophages and dendritic cells [222] commonly infiltrating colon tumors, but can also be secreted from Th2 T cells [223,70].

Chemokine decoy receptor D6 is decreased in colon tumors

Chemokines are regulated by chemokine decoy receptors that each has an exceptional ability among chemokine receptors to recognise a wide range of chemokines. To find mechanisms for altered lymphocyte recruitment and accumulation of Tregs in colon tumors, we determined the mRNA expression levels of D6, DARC and CCX-CKR compared to unaffected mucosa. We found that D6 mRNA expression was significantly elevated in tumors compared to surrounding tissue ($p<0.001$) with a median decrease of 15-fold (Paper II, Fig 1A). In contrast, no differential expression was detected for DARC or CCX-CKR, which demonstrates that D6 expression is selective and not due to general down-regulation of chemokine decoy receptors (Paper II, Fig 1B,C). Our initial analysis included 13 patients, revealing decreased D6 levels ($p<0.05$, Fig 6) but similar DARC and CCX-CKR expression, we expanded the D6 study group to enable correlation analysis between D6 and tumor/patient parameters.

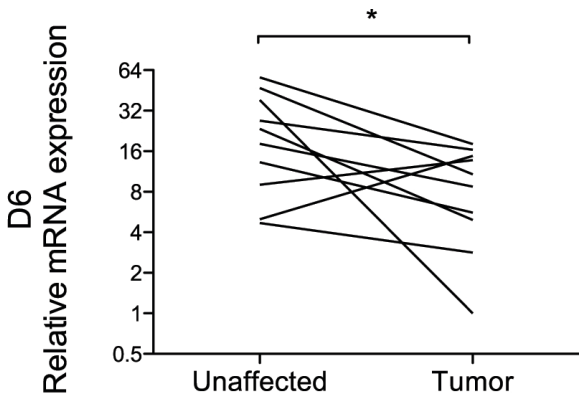


Fig 6 Initial experiments showing the expression of D6 mRNA in tumor and unaffected mucosa from the same individual (n=13). Data was obtaind with real time PCR and valueswere calculated using the$\Delta\Delta$Ct method as shown by fold change and relative to a calibrator samle.18s was used as endogenous control. The graph show paired values. *= $p<0.05$

Not long after the discovery of D6, the receptor was reported to suppress malignant transformation in mice treated with the skin irritant 12-O-tetradecanyol phorbol-13-acetat. $D6^{-/-}$ animals were highly susceptible to tumor formation of the skin following exposure compared to wild type controls, implying that absence of D6 results in an imbalance of chemokine levels that supports tumor development [224]. More recently, D6 expression was positively correlated to disease-free survival in breast cancer patients, further indicating that D6 contribute to the control of tumor progression [225]. Another study demonstrates that D6 expression is positively associated with absence of lymph node metastasis, tumor stage and relapse-free survival [226]. A recent publication revealed that hepatic tumor induction in D6-/- mice, results in accumulation of intrahepatic macrophages and decreased infiltration of neutrophils in liver tumors, compared to wt controls [227]. In contrast to the reports above, Vetrano *et al* reported increased D6 expression in human IBD-associated colon tumors compared to unaffected tissue [228]. However, in support of our observations, the same study also showed that D6 deficient mice are more susceptible to chemically induced colon cancerogenesis than wild type mice [228]. Still, the human data from Vetrano *et al* are contradictory to our results, which clearly show that D6 mRNA is substantially and significantly decreased in tumor mucosa. The discrepancy may depend on the fact that the patients included in the present study have no history of IBD.

D6 expression in murine *Helicobacter* infection

Helicobacter pylori chronically colonize the stomach mucosa and although causing gastritis in all infected individuals, most of them are asymptomatic. However, in 2 % of infected individuals, which is half the world's population, *H. pylori* causes gastric cancer [229]. Due to the strong relationship between *H. pylori* induced gastritis and and cancer, the bacterium is classified as a class I carcinogen for humans [230]. Since D6 have been shown to induce resolution and block the development of inflammation-induced skin tumors, we investigated the role of D6 in murine *Helicobacter* infection. We observed no difference in mRNA D6 expression in mice infected with the *Helicobacter pylori* SS1 strain at day 1, 3, 5, and 7 post-infection compared to wt controls (Fig 7) or week 2, 4, 6 and 8 as well as 6 months post-infection (data not shown). The *H. pylori* SS1 strain is

originally isolated from humans and has been adopted to also infect mice, who are not naturally colonized with *Helicobacter* [190]. Therefore, we reasoned that SS1 may not be optimal in the examination of inflammatory responses, as the murine infection only causes mild gastritis [231]. To increase the grade of inflammation in the stomach mucosa, we infected mice with *Helicobacter felis*, which cause more severe gastritis compared to *H. pylori* SS1 [231,232]. However, similar to SS1, we detected no alterations in D6 mRNA expression in *H. felis* infected mice compared to wt controls at week 4, 6 or 8 (Fig 7) or day 3, 5 and 7 post-infection (data not shown). Interestingly, and similar to our results, D6 mRNA is not altered in mice infected with *M. tuberculosis*, but still controls the infection, as D6 deficient mice display more severe inflammation and increased mortality in response to *M. tuberculosis* compared to wt controls [233]. Seemingly, the receptor needs not to be up-regulated to regulate inflammation, while its absence, results in excessive inflammation. Therefore we can perhaps not exclude a role for D6 in *Helicobacter* infection, although not further examined in this thesis.

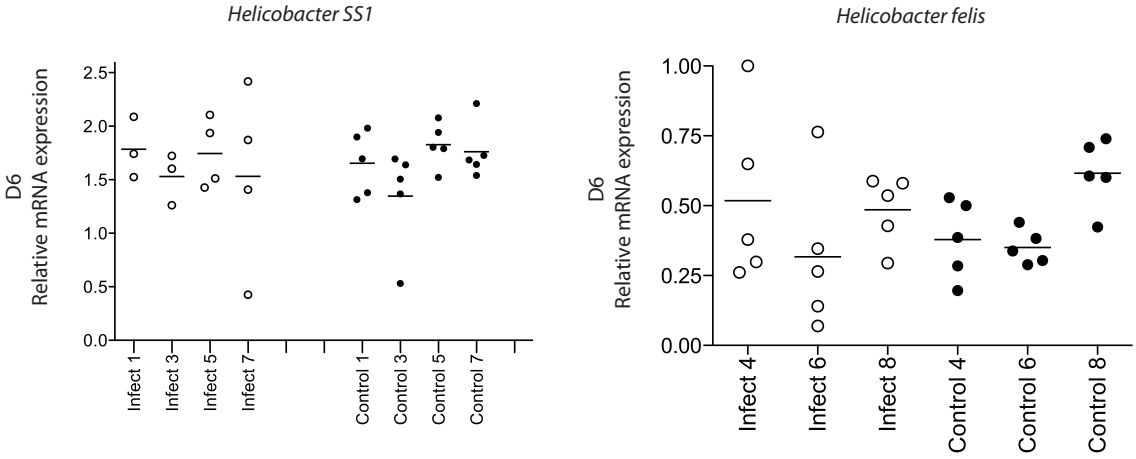


Fig 7 Expression of stomach D6 mRNA in C57/Bl6 mice infected with *Helicobacter pylori* SS1 (left) or *Helicobacter felis* (right) at selected time points. D6 mRNA was analysed on day 1, 3, 5, and 7 post-infection (*H. pylori*) or week 4, 6 and 8 post-infection (*H. felis*) Values are calculated using the ΔΔCt method and shown as fold change relative to a calibrator sample, using β-actin as endogenous control. Lines show mean values.

Lymph vessel density is elevated in tumor mucosa

Angiogenesis is a prominent feature in solid tumors, and colon tumors secrete angiogeneic mediators, including VEGF that promotes generation of both blood and lymph vessels. As D6 and DARC are mainly expressed by lymph endothelium and blood endothelium respectively in the gut [83], we examined the possibility that our results reflected changes in vessel density rather than regulation of chemokine decoy receptors. Decreased D6 expression in the tumor mucosa could not be explained by any decrease in lymph vessel density, as immunohistochemical staining of the lymph endothelial marker Lyve-1 [234] revealed higher lymph vessel density in tumor compared to unaffected mucosa ($p<0.05$, Paper II, Fig 2A). Thus, if lymph vessels are the principal source of D6 in the colon, the decreased D6 expression detected is even more pronounced on a per cell basis. An altered blood vessel density in colon tumors could conceal possible alterations of DARC expression and thereforee we determined the blood vessel density in tumors and surrounding mucosa. Double immunofluorescent staining with DARC and von Willebrand Factor (vWF) revealed that blood vessel density was elevated in some tumors compared to unaffected mucosa, although it was not significantly altered between the groups (Paper II, Fig 2B. Appedix Fig 1). Further, when related to vWF, DARC protein expression confirmed our mRNA data, as differential protein expression related to blood vessel density was not detected (Paper II, Fig 2C,D). Attempts to perform the corresponding staining procedure for D6 have been made, without reliable detection of D6 protein in colon tissue.

D6 expression correlates to tumor stage and location

The majority of tumors examined in this study featured decreased D6 mRNA expression, but not all and therefore we examined possible correlations between strongly decreased D6 in the tumor and tumor characteristics. To date, it is not clear whether there are age or gender specific differences in decoy chemokine receptor expression in humans. As D6 expression in unaffected colon mucosa varied considerably between individuals, we correlated D6 expression to gender and age of the patient without finding any relationship (data not shown). To compensate for the individual variation of D6 expression, we used the difference in D6 expression between unaffected and tumor mucosa when relating D6 expression to different tumor parameters. D6 expression was correlated with tumor stage and D6 expression was significantly lower in T3 and T4 staged tumors compared to T1 and T2 ($p<0.05$, Paper II, Fig 3A). The more advanced

the tumor, the less D6 mRNA was expressed. In addition to tumor stage, location of tumors had a significant impact on D6 expression, as levels were significantly lower in tumors from sigmoid colon compared to the cecum ($p<0.05$, Paper II, Fig. 3C). Further, although no significant correlation was observed, a trend towards less D6 expression was seen in medium differentiated tumors as compared to poorly differentiated and mucus-producing tumors. Even though D6 expression was clearly heterogeneously expressed in unaffected colon mucosa, it is important to emphasise that D6 expression in tumor associated colon was consistently lower independent of tumor or patient characteristics.

Treg/Th2 recruiting CCL22 is elevated in tumor mucosa

The contributing mechanisms of chemokine decoy receptors in tumor development and progression are largely unknown. D6 is proposed to scavenge CC chemokines to avoid excessive inflammation and hence increased availability of inflammatory CC chemokines is a possible consequence of decreased D6 expression [235]. We used protein extracts from tumor and unaffected colon mucosa to determine and compare the levels of selected D6 chemokines sequestered by D6. CCL22 that recruits CCR4 expressing lymphocytes is sequestered by D6 and was three-fold upregulated in colon tumors compared to unaffected mucosa ($p<0.001$, Paper II, Table II). CCL22 is secreted by tumor infiltrating macrophages [236] but has been shown to be secreted by intestinal epithelium and some cell lines of colon cancer in vitro but it is not known if it occurs *in vivo* [237]. However, simultaneous production of CCL22 and suppression of D6 expression by colon tumor cells would be a sophisticated strategy to recruit immunosuppressive Tregs. This indicates that decreased D6 expression within the tumor environment could be a mechanism used by tumors to recruit Tregs and Th2 lymphocytes. Also, CCL2 was elevated in some colon tumors although statistical significance was not achieved (Paper II, Table II). Why some, but not all CC chemokines sequestered by D6 are elevated in tissues with decreased D6 could depend on the varying affinity spectrum of D6 specific chemokines. CCL22 and CCL2 display particularly high affinity for the D6 receptor and thereforee they would probably be the chemokines most affected by D6 expression [81,238]. In mice with allergic inflammation of the lung, CCL22 regulation by D6 occurs only at certain concentration spans, which might be the case also in colon tissue [239]. The concentrations of CCL5 and CCL8 were

not different between tumor and unaffected tissue, which is partly consistent with data in murine models where D6 expression has no effect on CCL5 levels during pregnancy and in allergic inflammation of the lung [240,239]. Moreover, reduced D6 expression might result in poor induction of immune responses to tumor antigens, due to impaired migration of antigen presenting cells to lymph nodes, as recently reported [241].

Treg impair the migration of conventional T cells through activated endothelium

We have previously demonstrated that circulating Tregs from patients with gastric and colon adenocarcinoma inhibit migration of conventional T cells through *Helicobacter pylori* activated endothelium *in vitro* [184]. Hence, besides suppressing immune responses inside tumors, this observation offers another mechanism by which Tregs could reduce T cell infiltration into colon tumors. To exclude that the suppressive effect on T cell migration is *H. pylori* specific, we first induced TEM with LPS and PolyI:C [242,243] and could confirm that migration of $CD4^+$ and $CD8^+$ T cells, from healthy subjects, was strong and similar to that of *H.pylori* induced TEM (Paper III, Fig 1B,C).

LPS and PolyI:C was subsequently used to induce and compare TEM of gastric and colon cancer patient T cells, to that of T cells from healthy controls. LPS and PolyI:C induced only minor migration of $CD4^+$ T cells from cancer patients compared to $CD4^+$ cells from healthy volunteers, similar to that induced with *H. pylori* (Paper III, Fig 2A). $CD8^+$ T cell from cancer patients also had a reduced migration capacity with all stimuli examined compared to their counterparts from healthy volunteers (Paper III, Fig 2A) although not as pronounced as for the $CD4^+$ T cells. None of the migrating PBMC presented any cell division activity during experiments, as visualized by staining with CFSE prior to TEM, regardless of stimuli (data not shown). Together, these results demonstrate a reduced T cell TEM in cancer patients compared to in healthy volunteers, regardless of the stimulus used to activate the endothelial cells.

Having established an *in vitro* system for detecting T cell migration through endothelium activated with LPS and PolyI:C, we examined the influence of Treg from colon cancer patients on the migration of conventional T cells. When patient PBMC populations were depleted of $CD4^+CD25^{hi}$ cells, TEM of $CD4^+$ T cells subsequent to activation by LPS or Poly:IC was significantly increased compared to the whole PBMC fraction ($p<0.01$ and $p<0.05$ respectively. Migration levels of Treg depleted PBMC from patients were thereby similar to those of healthy controls. Addition of $CD25^{hi}$ Tregs to the $CD4^+CD25^{-/low}$ fraction resulted in a significant decrease of migration that was almost back to the level of whole PBMC fractions (Paper III, Fig 3C). The same effect was also observed for $CD8^+$ T cell migration through LPS activated endothelium ($p<0.05$, Paper III, Fig 3E). In

contrast, migration of conventional T cells isolated from healthy individuals was similar with and without depleted $CD25^{hi}$ Tregs, indicating that suppression of TEM by Tregs from cancer patients is not simply due to higher frequencies but also dependent on differences in Treg function compared to healthy controls (Paper III, Fig 3B,D).

Having observed increased frequencies of CD39 on intratumoral Tregs, we also examined the presence of the ectonucleotidases CD39 and CD73 on circulating $CD4^+CD25^{hi}$ Tregs. Indeed, $CD4^+CD25^{hi}$ Tregs from cancer patients expressed significantly more CD39 compared to corresponding cells from healthy individuals ($p<0.05$, Paper III, Fig 5A). Depending on experimental setup, Tregs have been shown to facilitate immune suppression through surface-bound or secreted TGF-β or CTLA-4, Granzyme B cytotoxicity, transfer of cAMP through gap junctions or production of IL-10 or adenosine [244-246,181,247]. It is not clear whether Tregs have the ability to use all of these effector mechanisms or if distinct subsets of Tregs with different suppression functions exist. We have previously reported that the Treg effect on TEM of conventional T cells in gastric cancer patients is independent of IL-10 and TGF-β, and hence suppression is mediated via other pathways. Adenosine is a potent immunomodulatory substance that acts on both T cells and dendritic cells to reduce T cell mediated immune responses [248-252]. During the last years, it has become increasingly clear that CD39-mediated hydrolysis of ATP to adenosine is an important effector mechanism for human Treg even though they lack CD73 [182]. $CD39^+$ Tregs seem to be particularly important for suppression of Th17 immune responses in autoimmunity, but have also been detected in patients with head and neck carcinoma [182]. Interestingly, in a mouse model of delayed type hypersensitivity, Tregs were shown to mediate suppression of T cell migration to the skin via adenosine [253].

Adenosine potentially mediates impaired TEM of conventional T cells from CRC patients

To evaluate the immediate effects of adenosine on TEM, we simultaneous added exogenous adenosine and T cells to transmigration assays, which demonstrated that adenosine strongly reduced TEM of both $CD4^+$ and $CD8^+$ from healthy individuals through LPS activated endothelium ($p<0.05$, Paper III, Fig 7A). To further confirm the inhibitory effect by adenosin we instead added the general adenosine receptor

antagonist 1,3-dipropyl-8-(p-sulphophenyl) xanthine (DPX) to the TEM assays together with T cells from patients with colorectal cancer. Blocking of adenosine receptors during transmigration more than doubled TEM by conventional T cells from cancer patients compared to untreated migration in preliminary experiments, as evident by increased migration of both $CD4^+$ and $CD8^+$ T cells (Paper III, Fig 7B). Adenosine hydrolysis by intratumoral Tregs probably contribute to tumor progression by mediating direct immunosuppressive effects, but can also stimulate angiogenesis [254-257]. Moreover, murine studies show that blocking of $A_{2A}AR$ signalling in cytotoxic T cells enhance their recognition and binding to tumor cells [250]. Further supporting a role for CD39 induced adenosine is that deletion of CD39 on Tregs promote antitumor cytotoxicity in experimental systems [258].

Lymphocyte distribution in $APC^{Min/+}$ mice

To examine the effect of Tregs on conventional T cell migration *in vivo*, we used the $APC^{Min/+}$ mouse model of spontaneous intestinal cancer. Tumor formation due to deleted APC is a result of uncontrolled wnt-signaling [259,260], a pathway also involved in immunological processes, not only during lymphocyte development but also in antigen responses [261,262]. Therefore, we first determined lymphocyte distribution and frequencies as well as functional properties in regulatory and conventional T cells in $APC^{Min/+}$ mice compared to wt controls. In our animal facility, $APC^{Min/+}$ mice start to present visible tumors in the small intestine (SI) at 12 weeks of age, and at week 18 all animals have formed multiple tumors along the SI. At week 22 tumors are large and more numerous and at this age some mice have to be euthanized due to blocking of the intestine, commonly the colon. To include both early events in tumor development as well as established tumors we studied four different time poins: week 10, 14, 18 and 22. Over time, frequencies of $CD4^+$ T cells in the SI were similar between $APC^{Min/+}$ and wt mice whereas $CD8^+$ T cells seemed to decrease with age in both groups (Paper IV. Fig 1). We observed that both splenic CD4 and CD8 T cells decreased with age in $APC^{Min/+}$ mice as previously reported by others [263]. This decrease of splenic T cells was not detected in wt animals (Paper IV, Fig 1). Circulating $CD4^+$ and $CD8^+$ lymphocytes were similar between $APC^{Min/+}$ and wt mice (Paper IV. Fig 1). When studying the subpopulations within the $CD4^+$ lymphocyte fraction more closely, we observed that $CD4^+Foxp3^+$ Tregs were clearly accumulated ($p<0.05$) in $APC^{Min/+}$ intestines compared to wt at week 22

(Paper IV. Fig 2A). At this time point, $APC^{Min/+}$ SI also harboured decreased frequencies of recently activated $CD4^+CD69^+$ lymphocytes. We also noted that the decrease in $CD4^+CD69^+$ T cells in the SI coincided with an increase of circulating $CD4^+CD69^+$ T cells in the same animals (Paper IV, Fig 2A). Tumors were not separated from unaffected lamina propria for these analyses. Still, while no difference in lymphocyte frequencies was observed at earlier time points, the shift in Treg/recently activated T cells probably reflects the high tumor load of the older mice.

A feature that is repeatedly reported to correlate to poor disease outcome in colorectal cancer is a low effector T cell to Treg ratio. Here we observed that SI adenomas in the $APC^{Min/+}$ mouse also harboured low frequencies of conventional T cells in relation to $Foxp3^+$ Tregs as mean ratios of CD4/Treg and CD8/Treg were only 50% to that detected in wt SI (Paper IV, Fig 2B). We used immunofluorescence to evaluate the infiltration of lymphocytes in SI tumors compared to that of unaffected lamina propria within the same mouse and wt lamina propria. Similar to what we have observed in human colon tumors, frequencies (expressed as cells/mm^2) of $CD4^+$ and $CD8^+$ T cells in colon adenomas were similar to that of unaffected lamina propria (Paper IV, Fig 3A). Instead, like also detected in human tumors, the B cells (CD220+) were significantly decreased in both SI and colon adenomas ($p<0.01$, Paper IV, Fig 2C). As already discussed in relation to human tumors, efficient tumor clearance is depending on the quality of the immune response but still, numbers of cells would probably also make a difference. Having similar numbers of $CD4^+Foxp3^-$ and $CD8^+$ T cells in tumors as in normal tissue might therefore indicate low immunogenicity and/or successful immune escape. In contrast to the findings in colon, SI adenomas displayed decreased infiltration of $CD4^+$ T cells as well as significantly decreased $CD8^+$ T cells ($p<0.05$, Paper IV, Fig 2A,B). The murine colon harbours low numbers of T cells, in particular $CD8^+$ T cells, in homeostatic conditions and therefore decreased numbers might be difficult to detect compared to the SI which is densely infiltrated by T cells. Further, we detected accumulation of $CD4^+Foxp3^+$ Tregs in colon adenomas ($p<0.01$), whereas the increase in $CD4^+Foxp3^+$ Tregs in SI adenomas was only modest (Paper IV, Fig 4A). However, the frequency of $CD4^+Foxp3^+$ Tregs in the $CD4^+$ fraction was strongly elevated in both SI and colon adenomas in $APC^{Min/+}$ mice ($p<0.01$ and $p<0.001$, Paper IV, Fig 4B). Consistently

throughout these analyses, no difference in lymphocyte infiltration between wt and unaffected $APC^{Min/+}$ lamina propria was observed (Paper IV, Fig 3A,B).

$APC^{Min/+}$ Tregs are functional and suppress the proliferation of conventional cells *in vitro*

To determine that $APC^{Min/+}$ Treg are functional, we evaluated their suppressive ability of splenic $CD4^+CD25^+$ Treg from both $APC^{Min/+}$ mice and wt controls in co-culture experiments. Responder $CD4^+CD25^-$ T cells were cultured with Tregs, antigen presenting cells and anti-CD3 at Treg:Tresponder ratios of 1:1, 1:2, 1:4 and 1:8. $APC^{Min/+}$ Treg had strong and similar suppressive effect as compared to wt controls as only 15-40 % proliferation was detected in responder cells at all time points (Paper IV, Fig 5A). The distribution of CD25 expression in splenic $CD4^+$ T cells is showed in Fig 4 (lower panel). The suppressive effect rapidly declined with lower concentrations of Treg and at the 1:4 ratio, the responder T cells proliferated almost as good as responder cells alone independent of age or tumor burden (Paper IV, Fig 5B). Still, it should be noted that important differences may exist *in vivo* as Tregs from $APC^{Min/+}$ animals do not prevent colitis equally well as wt Treg [264]. To examine $APC^{Min/+}$ responder cells compared to wt, we cultured responder cells from each of the genotypes together with $APC^{Min/+}$ Treg at a 1:1 ratio. $APC^{Min/+}$ responder T cells were consistently more resistant to suppression with 55-70% remaining proliferation compared to only 10-40% in the wt responder cells. This effect was particularly pronounced at week 18 ($p<0.01$, Paper IV, Fig 6). Our data suggests that impaired Treg function in $APC^{Min/+}$ mice might depend on low responsiveness in responder cells rather than defects in the Treg themselves. Actually, responder T cells isolated from joints in patients suffering from juvenile idiopathic arthritis have been reported to resist suppression from Tregs due to hyperactivation of protein kinase B, caused by inflammatory cytokines [265]. In addition, Smad7 dependent unresponsiveness in responder mononuclear cells to Treg suppression in the colon of patients with IBD has been reported [266]. Since the microenvironment in $APC^{Min/+}$ adenomas is not reported to harbour any strong inflammatory response and the fact that we detected low responsiveness also at early time points with no visible tumors, other mechanisms for inducing low responsiveness in T cells might exist. The *apc* mutation itself may induce low responsiveness in addition to other abnormalities

reported, such as decreased cytotoxicity of effector $CD8^+$ T cells and increased IL-17 secretion from CD4+ T cells [264,267].

When evaluating the cytokine production in responder T cells in co-culture with $APC^{Min/+}$ or wt Tregs, we could determine that independent of genotype, Tregs suppressed IFN-γ and TNF-α equally well, while there was only little effect on IL-13 secretion. In contrast, IL-17A levels from conventional T cells were increased in the supernatants where Tregs were present (Paper IV, Fig 7). Isolated Tregs alone produced some IL-17A (108± 124 pg/ml) after stimulation but were not responsible for the whole increase seen in co-cultures (data not shown). IL-17 has recently been shown to promote tumorigenesis in $APC^{Min/+}$ mice [263,264] and Th17 cell infiltration in human CRC predicts a poor outcome [102]. IFN-γ, TNF-α, IL-10 and IL-13 levels were low or undetectable in all cultures of Tregs alone (data not shown).

Treg depletion increase homing of $CD4^+$ and $CD8^+$ T cells to $APC^{Min/+}$ adenomas

After having demonstrated that $APC^{Min/+}$ Tregs are accumulated in intestinal adenomas and are functionally intact, we wanted to evaluate the effect on conventional T cell homing to tumors *in vivo*. We crossed $APC^{Min/+}$ mice with DEREG mice, to enable selective depletion of Treg in tumor bearing mice through DT administration. Efficient depletion of Tregs was observed on day five with 83% of circulating Treg depleted, after DT administration on four consecutive days (data not shown). Ten days after the start of Treg depletion, there was increased infiltration of both $CD4^+$ and $CD8^+$ conventional T cells into small intestinal tumors as compared to DT treated $APC^{Min/+}$ controls, assessed by flow cytometry and immunofluorescence (Paper IV, Fig 8A-B, D-E). Interestingly, Treg depletion has recently been reported to increase $CD4^+$ and $CD8^+$ T cell infiltration in experimental thymomas and chemically induced sarcomas [268,269]. We also noted that Tregs had started to reappear at the time of analysis (Paper IV, Fig 8C,F) but the frequency of Treg among $CD4^+$ lymphocytes was still lower in tumors from the DT-treated $APC^{Min/+}$/DEREG mice compared to DT treated $APC^{Min/+}$ controls (Paper IV, Fig 8G). To examine possible mechanisms for increased T cell infiltration to small intestinal tumors after Treg depletion, we analysed endothelial expression of the mucosal adhesion molecule MAdCAM-1 by immunofluorescence, as the expression is decreased in human gastric and colon adenocarcinoma [221,270]. We detected similar MAdCAM-1

expression in tumors and unaffected lamina propria both in DT treated $APC^{Min/+}$/DEREG and $APC^{Min/+}$ control animals, which was also confirmed by real time PCR (Paper IV, Fig 8D/ data not shown). Thus, it remains to be determined if depletion of Treg restores the expression of other endothelial adhesion molecules or chemokines to facilitate recruitment of T lymphocytes, or if the increased frequencies of T cells detected are rather caused by local expansion of T cells in tumors when the suppressive activity of Treg is removed.

Concluding remarks

Infiltration of lymphocytes to effector sites is crucial for mounting successful immune responses, but dysregulation of the lymphocyte homing process might also be harmful to the host and induce chronic inflammation, tumor development and resistance of tumor cells to anti-tumor immunity. Immunotherapy against tumors have had little success, as both vaccine induced immunity and administration of *in vitro* stimulated effector T cells result in poor infiltration of effector T cells into tumors. This thesis addresses the homing mechanisms of lymphocytes in colorectal adenocarcinoma as compared to surrounding unaffected tissue as well as possible effects from Tregs on conventional T cell homing.

Effector and in particular effector/memory T cell infiltration in colon tumors are beneficial for disease outcome whereas low ratios of $CD8^+$ effector cells or memory T cells to Tregs are not. However, the consequence of mere accumulation of intratumoral Tregs in CRC is not completely defined and seem to diverge from what is reported on other solid tumors, where accumulation of Tregs is associated with poor outcome. Instead, gathering data suggest a positive correlation between infiltrating Tregs and patient disease free and overall survival. One possible mechanism to why Tregs are beneficial in colorectal cancer, but not in other solid tumors, could be the balance of Th subsets in the microenvironment. Most solid tumors are sterile, whereas colon tumors grow in an environment enriched in commensal bacteria. Tumor tissue is characterized by necrosis and hence, tissue damage may result in constant bacterial insult known to drive Th17 differentiation. Th17 mediated inflammation seem to favour tumor progression and the presence of intratumoral Tregs may suppress Th17 inflammation

leading to a less favourable environment for tumor growth. There is little data on tumor-infiltrating Treg function in colorectal cancer, most likely due to difficulties to obtain sufficient numbers of primary cells for functional evaluation. However, one recent study proved the importance of Treg phenotype and function in predicting clinical outcome, since the suppressive ability of circulating Tregs from CRC patients with metastasis after tumor resection was higher than in patients who remained disease free [121]. We observed diverse phenotypes of Tregs between tumor and unaffected mucosa with accumulation of $Foxp3^{+}CCR4^{+}$ Tregs in colon tumors as well as elevated levels of the CCR4 ligand CCL22 that was not observed in surrounding unaffected tissue. Except for indications regarding the route of entry, we also detected elevated expression of CD39 and high expression of CTLA-4 in intratumoral Tregs, suggesting that their regulatory function is enhanced compared to Tregs in unaffected tissue. Indeed, suppressive function has been shown in Tregs isolated from human colon tumors as they suppress conventional T cell proliferation *in vitro*. Moreover, another finding suggesting that Tregs may have a negative impact on tumor progreesion is that tumor infiltrating Tregs correlates with Duke stage in CRC [117].

Therapeutic strategies to overcome immune suppression by intratumoral Tregs would probably be blocking of Treg function rather than depletion. Treg depletion with Diphteria toxin-conjugated anti-CD25 in patients with renal carcinoma did enhance DC vaccine induced anti tumor T cell responses [271]. However, the risk with prolonged anti-CD25 administration is depletion of activated T cells [272]. Interestingly, vaccination of mice with Foxp3 mRNA-transfected DCs induced cytotoxic T cells specific for Foxp3, which resulted in decreased density of intratumoral Tregs and enhanced protective immunity [273]. Whether this approach would be suitable in humans remains to be determined, but would probably be superior to CD25 based therapy in selectively depleting Tregs.

Our results suggest that homing of Tregs to colon tumors partly depends on CCR4/CCL22 as a $CCR4^{+}$ Tregs were more accumulated in tumors compared to unaffected tissue. Tumors also displayed elevated CCL22 levels in parallel with much decreased levels of CCL22 sequestring CDR D6. Together, our data indicate that colon tumors are protected from immune mediated rejection by CCL22 induced recruitment of

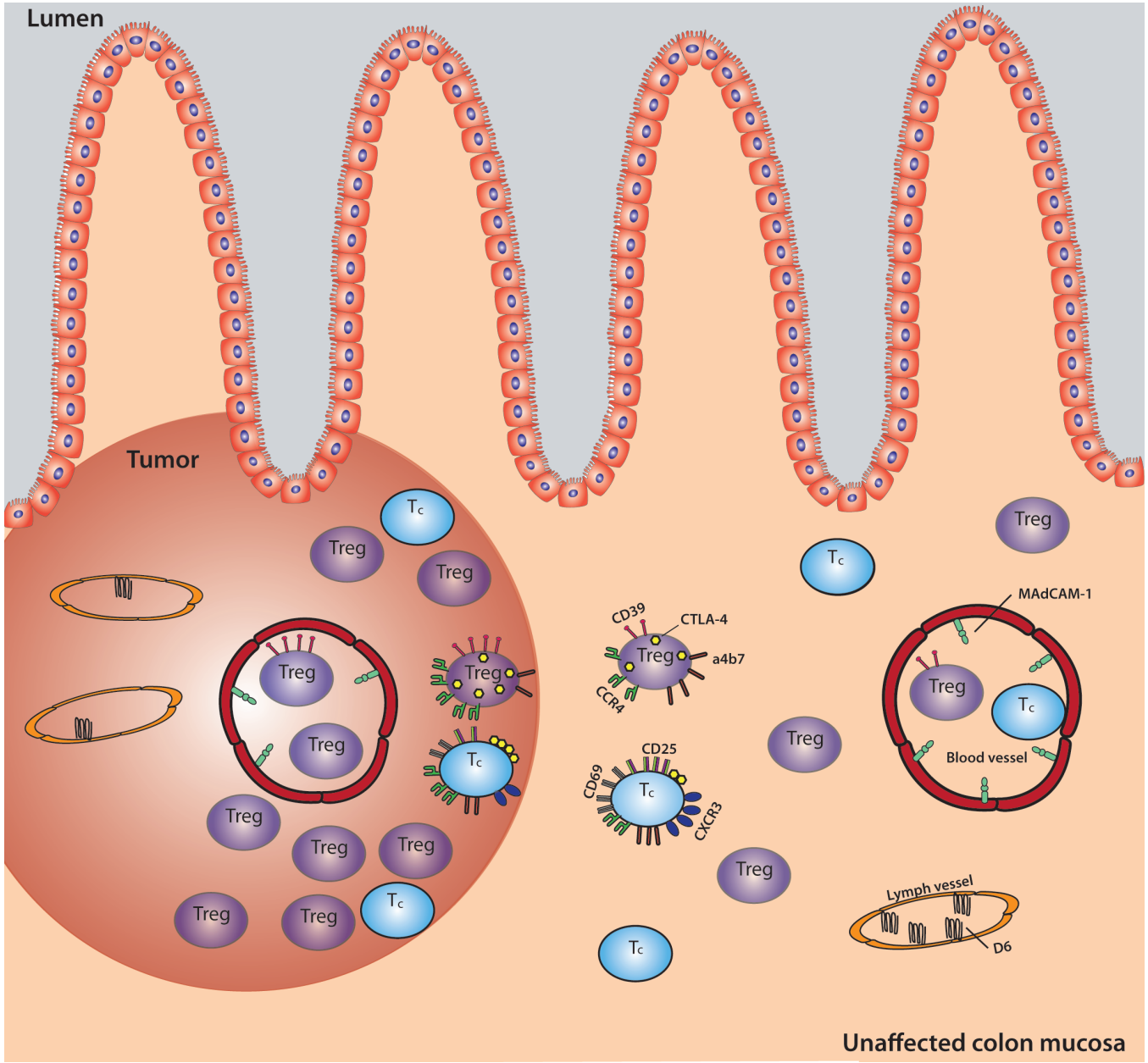
Tregs to the tumor site and might have therapeutic value. The forced expression of D6 may restore chemokine balance in the tumor microenvironment to facilitate proper entry of effector T cells. There is conflicting data on the importance of D6 in colon cancer as the study by Vetrano *et al* of IBD-associated colon cancer show that D6 expression in these tumors is elevated. Colon tumors in IBD-patients are probably detected earlier than spontaneous colon tumors due to regular medical check-ups within this category of patients. This means that colon tumors in IBD-patients are likely to be less advanced compared to spontaneous tumors at the time of diagnosis and might explain the discrepancy between the two studies as low D6 expression is correlated to tumor stage. We show here that the D6 expression is more decreased in advanced T3 and T4 tumors compared to T1 and T2 tumors. If IBD-associated tumors are detected at an early stage, D6 may still be up-regulated to scavange proinflammatory CC chemokines in response to IBD-induced inflammation. Still, as Th17 and Treg immune responses are intricately and inversely connected, there is a major risk that blocking of Treg migration to tumors may induce strong Th17 immunity, resulting in tumor progression. Thus, blocking entry of Tregs into tumors would probably demand the simultaneous blocking of Th17 differentiation in the treatment of CRC.

Blocking of Treg functions could perhaps reverse the impaired infiltration of activated conventional T cells into colon tumors. Our observation that $CD39^+$ Tregs are accumulated in colon adenocarcinoma in addition to blocking TEM of conventional T cells makes CD39 a potential molecular target for colon cancer immunotherapy. We detected less infiltration of activated $CD4^+$ and $CD8^+$ T cells in tumors compared to unaffected mucosa that might be a result of Treg mediated inhibition of migration as well as inhibition of effector functions via the production of adenosine. Indeed, when depleting Tregs in $APC^{Min/+}$ mice we detected enhanced infiltration of $CD4^+$ and $CD8^+$ T cells, supporting the role of Tregs in suppressing T cell migration. Hence, successful and non-toxic blocking of CD39 would abrogate immunosuppression by CD39 derived adenosine in the tumor in parallel to inducing effector T cell infiltration. Still, anti-CD39 therapy might also result in opposing effects as accumulation of ATP might induce excessive induction of inflammatory Th17 cells as well as impaired functions in DCs [274,275]. Adenosine receptors are expressed in several cell types involved in anti tumor activity including monocytes/macrophages, DCs, NK-cells and lymphocytes,

making them targets for Treg CD39 mediated suppression [276]. Hence, blocking of adenosine receptors would be a strategy to avoid suppressive effects of Treg derived adenosine and enhance effector cell infiltration into colon tumors and still circumvent local accumulation of ATP. Considering that intratumoral Tregs express high amounts of CTLA-4, indispensible for suppressive functions of Tregs, CTLA-4 qualifies as a candidate target in immunotherapy. Indeed, antibody mediated blocking of CTLA-4 in patients with melanoma, renal cell cancer and prostate cancer enhance tumor immunity, unfortunately there is also severe autoimmunity mediated side effects due to enhanced activation of conventional T cells [277,278].

This thesis contributes to the current knowledge on T cell homing to colon adenocarcinomas, by showing differential recruitment of Tregs compared to unaffected tissue, and that Tregs impair the migration of conventional T cells in colon cancer. Since therapy induced protection against tumors requires the infiltration of activated T cells into tumors, and possibly abrogation of Treg mediated suppression, results from this thesis will hopefully aid in the successful design of future immunotherapies.

This thesis proposes differential recruitment of Treg and conventional T cells to colon tumors compared to surrounding unaffected mucosa, partly depending on alterations of endothelial adhesion molecules, chemokine decoy receptors and accumulation of circulating and intratumoral Tregs.



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