Regulatory T cells and lymphocyte migration into intestinal tumors

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UNIVERSITY OF GOTHENBURG
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Tumor-infiltrating lymphocytes (TIL) are crucial for anti-tumor immunity. However, regulatory T cells (Treg) often accumulate in tumor tissue and are able to reduce both lymphocyte activity and transendothelial migration and thereby reduce the local anti-tumor immunity. The aim of this thesis was to investigate the anti-tumor immune response in intestinal tumors *in vivo* with a special emphasis on Treg function and lymphocyte recruitment. First, the APC\(^{Min/+}\) mouse model of intestinal tumors was used to investigate tumor-associated lymphocyte subsets and their modes of accumulation into intestinal tumors. We could show that the tumors of APC\(^{min/+}\) mice harbour an increased number of Treg, which was also confirmed in human colon cancer and colon adenomas. Furthermore, a decrease of conventional T cells was observed.

By breeding APC\(^{min/+}\) mice with DREG mice, which harbour a high affinity diphtheria toxin receptor under the control of the FoxP3 promoter, we were able to deplete Treg in tumor-bearing mice. Treg depletion resulted in an accumulation of effector T cells in the intestinal tumors, as a consequence of both higher proliferation and increased migration into the tumors. Furthermore, an increase of the Th1 associated chemokine receptor CXCR3 on T cells and increased levels of IFN-γ was found in the absence of Treg. One important mechanism for TIL migration in the absence of Treg was the increased secretion of the CXCR3 ligands CXCL9 and 10. We could also demonstrate that CXCR3 is crucial for migration into intestinal tumors.

In conclusion, this thesis demonstrates that Treg inhibit a Th1 associated anti-tumor response in intestinal tumors partly by reducing effector T cell accumulation. Strong Th1 responses have been correlated to improved patient outcome in colon cancer. Therefore, the results of this thesis indicate that eliminating Treg or reducing their suppressive mechanisms would constitute a viable anti-tumor therapy, not only increasing effector T cell activity but also their recruitment into tumors.

**Keywords:** Treg, CRC, Tumor infiltrating lymphocytes, CXCR3

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SAMMANFATTNING PÅ SVENSKA


APC^{min/+} möss utvecklar spontant tumörer i hela tarmsystemet på grund av en mutation i apt supressorgenen. Sådana mutationer initierar tumörutveckling också i en majoritet av alla humana koloncancer patienter. I tumörerna hos APC^{min/+} möss upptäcktes en ansamling av Treg jämfört med närliggande normal tarmvävnad. En liknande ansamling bekräftades även i humana koloncancerprover och i humana adenom, ett förstadium till invasiva tumörer. Vidare var även effektor T-cellerna påverkade i mustumörerna, med lägre andel celler än i den normala tarmvävnaden. Detta indikerar att immunförsvaret är reducerat i tumörtumörer och att detta kan öka tumörtillväxten. Denna musmodell har i denna avhandling påvisats besitta specifika immunologiska skillnader enbart i tumörer som liknar de i human koloncancer och har därför vidare i arbetet använts som en modell för koloncancer.

För att undersöka om ansamlingen av Treg i tumörerna påverkar immunförsvaret introducerades DEREG-möss i vår APC^{min/+} avel.
DEREG-möss gör det möjligt att selektivt eliminera Treg med difteritoxin, då dessa möss har en receptor för difteritoxin uttryckt bara på Treg. Med hjälp av dessa APC\(^{\text{min}}/+\)/DEREG-möss möjliggjordes studier av hur tumörernas immunförsvar påverkas i frånvaro av Treg. När Treg eliminerades noterade vi en ökning av effektor T-cellernas antal i tumörene. En ökning av T-cellernas antal i tumörvävnaden kan ha flera olika orsaker, ökat inflöde, ökad celldelning eller större benägenhet att stanna i tumören. T-celler delade sig i högre utsträckning i frånvaro av Treg vilket delvis kan ha påverkat den ökade antalet. För att undersöka om T-cellernas dessutom lyckades ta sig in i tumören bättre i absaknad av Treg gjordes migrationsexperiment där inmärkta celler injicerades i tumörbärande möss och frekvensen av migrerande celler in i olika organ undersöcktes. En större andel T-celler tog sig in i tumören när Treg var borta vilket därmed också påverkade den högre frekvensen av T-celler i avsaknad av Treg. Därmed visar detta arbete att Treg kan minska ett effektivt antitumör försvar bestående av T-celler genom att både hämma T-cells migration in i tumören och även deras tillväxt.

För att kunna migrera in i tumörer behöver T-celler signaler som leder dem rätt, så kallade kemokiner. CXCR3 är en kemokinreceptor som sitter på T-celler associerade med ett effektivt antitumöröver. Vi kunde visa att CXCR3 är nödvändig för T-cellernas migration in i tumörvävnaden och specifikt till tumörer. T-celler med denna receptor var få i tumörerna, men när Treg eliminerades ökade frekvensen av CXCR3\(^+\) T-celler i tumören. De kemokiner som binder till CXCR3, CXCL9 och CXCL10, var också de som ökade i tumören när Treg var borta. En specifik ökad produktion av CXCL10 från endotelceller i tumören kunde också ses i frånvaro av Treg. Detta indikerar att Treg påverkar denna rekryteringsväg för att undvika att effektiva T-celler migrerar in i tumören.

Sammanfattningsvis, Treg ansamlas i tumörer och hjälper där till med att minska immunförsvar mot tumören. Att eliminera Treg eller påverka deras supressiva funktioner skulle därför kunna bli en effektiv immunterapi mot koloncancer.
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<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen producing cells</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DEREG</td>
<td>Depletion of regulatory T cells</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
</tr>
<tr>
<td>FC</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HEV</td>
<td>High endothelial venules</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<td>IEL</td>
<td>Intraepithelial lymphocytes</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>LPL</td>
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</tr>
<tr>
<td>MAdCAM-1</td>
<td>Mucosal addressin cell adhesion molecule-1</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Multiple intestinal neoplasia</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph node</td>
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<tr>
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<td>Natural killer cells</td>
</tr>
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</tr>
<tr>
<td>PNAd</td>
<td>Peripheral lymph node addressin</td>
</tr>
<tr>
<td>PP</td>
<td>Payers patch</td>
</tr>
<tr>
<td>pTreg</td>
<td>Peripheral induced Treg</td>
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<tr>
<td>RT-PCR</td>
<td>Real time PCR</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TIL</td>
<td>Tumor infiltrating lymphocytes</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>tTreg</td>
<td>Thymus derived Treg</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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INTRODUCTION

Brief introduction to the immune system

All living organisms are constantly exposed to pathogens, bacteria, viruses and foreign substances. The immune system is a crucial player to defeat invaders and to keep a balanced reaction. It comprises an intricate organisation of cells and organs that protect its host against pathogens and can be divided into innate or adaptive immunity. The innate immune system is the first response that is always present to defeat an intruder. It consists of both the epithelial border creating a physical barrier but also phagocytes, antigen presenting cells (APCs), and proteins of the complement system\(^1\). Adaptive immunity consists of lymphocytes recognizing antigens on infectious agents and provides a secondary response when innate immunity is insufficient. This is a much stronger and specialized response that provides a memory for future infections\(^2\).

Lymphocytes arise from stem cells in the bone marrow and further mature in either bone marrow for B cells or thymus for T cells. Naïve lymphocytes leave their generative lymphoid organ and circulate between the blood and peripheral lymph nodes where they can encounter APCs that present antigens from digested microbes. If naïve lymphocytes encounter their antigen and receive stimulation from APCs a differentiation into effector cells or memory cells occurs\(^2\).

The course of an infection usually starts with an infectious microbe penetrating the epithelial border such as the gastrointestinal (GI) tract or the skin to invade the host. The first response is the innate immunity where phagocytes, including neutrophils and monocytes ingest the microbes in order to neutralize them, cell death initiated by Natural killer (NK) cells and cytokines secreted by APCs, such as macrophages and dendritic cells (DC) to initiate inflammation and a lymphocyte response. DC capture protein antigens and process them in order to display peptides bound to Major histocompatibility complex (MHC) on the surface and migrate into peripheral lymph nodes to activate the adaptive
immunity. Two MHC complexes exist, MHC I and II. The peptides presented by MHC class I molecule are generated in infected cells by proteasome-mediated protein degradation in the cytosol and transported into the endoplasmic reticulum (ER) where they will bind to MHC class I molecules. Infected cells can also phagocytose antigens leading to peptides residing in endosomes that can be presented by MHC class II, assembled in the ER.

Co-stimulatory molecules on the APCs and secreted cytokines enable T cells to proliferate and differentiate into effector cells, either T helper (Th) cells or cytotoxic T lymphocytes (CTL) and these can then be transported through the circulation to the site of infection. T cells exhibit different ways of combating the microbe, Th cells secretes cytokines in order to activate innate cells to phagocytos microbes and to kill infected cells, while CTL kills infected cells in a cell-contact dependent manner. B cells are also activated in peripheral lymph nodes but will need help from Th cells to gain full response. Differentiated B cells are called plasma cells and secretes antibodies that bind to extracellular microbes and prevent them from infecting host cells, promoting their ingestion and destruction. When a pathogen is eliminated, the majority of activated lymphocytes die by apoptosis and the immune system returns to homeostasis. However, memory cells remain for a long period of time and respond rapidly in case the same pathogen invades the host again.

**Gastrointestinal tract**

The GI tract is an intricate organ system involved in food transportation and uptake, and is in constant contact with the environment. The anatomy of the GI tract consists of all structures involved in the process from food intake to waste, with the main parts being stomach, small intestine and colon. Not only is the GI tract part of absorptive, digestive and secretory processes but also an intricate part of the host defence against foreign antigens and pathogens. A mucosal surface throughout the GI tract forms a barrier between the host and the environment and
consists of one layer of epithelial cells and the underlying lamina propria (LP). The epithelium consists of mainly absorptive enterocytes but also antimicrobial peptide-secreting Paneth cells, mucus producing goblet cells, neuroendocrine cells\(^\text{12}\) and intraepithelial lymphocytes (IEL). The LP consists of mainly connective tissue that supplies the mucosa with blood vessels, lymphatic drainage and nerves but also many different types of immune cells. In the small intestine the mucosal surface forms villi that stretch out into the lumen and to further increases the surface area microvilli are present on epithelial cells. The crypts between villi contain the stem cells that give rise to the different types of mature epithelial cells\(^\text{13}\).

**Figure 1.** The intestinal mucosa consists of one layer of epithelial cells and the underlying lamina propria. The small intestine contains villi that stretches out into the lumen while the colon present a smooth surface with crypts. IEL: Intra epithelial lymphocytes, LPL: lamina propria lymphocytes, MLN: Mesenteric lymph node.

The small intestine receives digested food from the stomach and further digests it into disaccharides, peptides and fatty acids and absorbs the nutrients. Liquid residue and non-digestive material continue into the colon, which main function is to reabsorb water released in the small intestine\(^\text{14}\). The colon contains more bacteria than there are cells in the entire human body. Non-pathologic bacteria are called commensals as they live in symbiosis with the host and exert help with synthesizing vitamins and digesting polysaccharides in pathways that humans lack.
enzymes for\textsuperscript{15}. The colon do not form villi and instead form a smooth surface with crypts like the small intestine, however lacking Paneth cells\textsuperscript{10}.

Due to the constant interaction between host and environment the GI tract needs several barrier mechanisms to protect from foreign bacteria and substances\textsuperscript{16}, such as the chemical barrier of anti-bacterial peptides secreted by paneth cells\textsuperscript{17} and the physical barriers of epithelial cells and mucin, secreted by goblet cells, which creates a mucus layer reducing the direct contact between lumen and epithelium\textsuperscript{10}. To discriminate between pathogenic agents, commensal bacteria and food nutrients the immune system is important. Through oral tolerance APCs and T cells exert a state of unresponsiveness towards non-pathogenic antigens in the intestine\textsuperscript{18}. In the absence of a danger signal homeostatic gut APCs migrate to the lymph nodes where they contribute to the oral tolerance against gut content\textsuperscript{19}.

Gut-associated lymphoid tissue (GALT) is a network of highly organized immune structures in the intestine and consists of Peyer’s patches (PP), appendix and isolated lymphoid follicles. PP are lymphoid follicles residing in the small intestine and are responsible for T cell priming\textsuperscript{20}. Some of the epithelial cells covering PP are M cells that transport antigens from the lumen into the PP and to the APCs\textsuperscript{21}. Mesenteric lymph nodes (MLN) are another major site of antigen presentation\textsuperscript{22} and oral tolerance induction in the gut\textsuperscript{23}. Together with the gut draining MLN, GALT provides antigen sampling from the entire GI tract to optimize opportunities for naïve lymphocytes to encounter antigens and thereby activate them or induce tolerance\textsuperscript{24}. The effector cells can thereafter exit GALT and MLN via efferent lymphatic and enter the systemic circulation to home to the gut to exert their effector functions\textsuperscript{25}.

**Intestinal lymphocytes**

The majority of the human body’s lymphocytes reside in the intestine and have an important task to balance the constant interaction of microbes, both pathogenic and commensals in the lumen\textsuperscript{26}. The immune cells in the intestine are mainly present in two major sites, the GALT where initial
antigen presentation to adaptive immunity cells takes place and the LP and epithelial layer where effector functions are executed. IEL residing in the epithelial layer, are antigen experienced T cells that balance a protective immunity while keeping the integrity of the epithelial barrier.

Both cells of innate and adaptive immunity co-exist in the LP. T cells are a major player orchestrating immune responses, both by activation of other cells but also by direct killing of foreign pathogens. Naïve T cells leave the thymus as immature T cells with a broad range of T cell receptors (TCR) and can be activated in the periphery after antigen encounter. T cell activation requires at least two signals to become activated, first TCR engagement with the antigen presented by MHC complexes and secondly engagement of the co-stimulatory molecule CD28 by CD80 and CD86 on APCs. A third signal includes cytokines secretion from the APCs, potentially initiated by adjacent innate immune cells.

Mucosal T cells form a complex and heterogeneous population of lymphocytes, which are all antigen experienced but have a wide repertoire of antigen recognition and mode of action. Several different effector T cells exist and they are commonly divided into CD4+ Th cells and CD8+ CTL. CD4+ Th cells mediates a response in inflammation, autoimmunity, asthma, allergy and tumor immunity and their TCR are able to bind to MHC class II. Th cells can be further divided into different subsets depending on cytokine secretion and transcription factors. The differentiation pathway of an individual T cell is dependent on the cytokine milieu during activation. The different subsets have also been shown to be plastic leading to a possibility of evolvement of subset depending on the cytokine milieu. Another CD4+ subset is the regulatory T cell (Treg) that regulates and maintains self-tolerance and can suppress other T cells in order to control immune responses. CTL on the other hand are CD8+ T cells that can kill infected cells by releasing perforin and granzymes and by Fas-FasL interaction.
Differentiation of naïve T cells into different subsets is dependent on cytokine milieu.

**Th1 cells**

Th1 cells are critical for cell-mediated immune responses against intracellular pathogens and tumor cells, and are characterized by secretion of IFN-γ and IL-2 and expression of the transcription factor T-bet. A cytokine milieu consisting of IL-12, TNF-α and IFN-γ followed by activation of the transcription factor STAT1 and STAT4 induces Th1 cell differentiation. Activation by IL-12 increases IFN-γ expression through STAT1 signalling to induce the transcription factor T-bet. This further increases the signature cytokine IFN-γ expression in a positive feedback loop.

IFN-γ is a cytokine mediating innate and adoptive immunity against both viral and bacterial infections and tumors. The main functions of IFN-γ signalling are regulation of host defence, immune system, cell cycle, apoptosis, inflammation and cell proliferation but also maintenance of the intestinal epithelial barrier integrity. IFN-γ produced by Th1 cells can activate macrophages to phagocytose and digest intracellular bacteria and stimulate the microbicidal activities of phagocytes thereby promoting the intracellular destruction of phagocytosed microbes. IFN-γ can also suppress T cell differentiation into Th2 and Th17 cells, creating a favourable milieu for Th1 cells.
Th2 cells

Th2 cells are responsible for humoral-mediated immunity against extracellular parasites but also eosinophilic inflammation involved in allergies and atopic illnesses. Their main functions are to enhance barrier mechanism and expulsion and/or killing of parasites. IL-4 and IL-2 are primarily accountable for the differentiation of Th2 cells through transcription factors STAT6 and GATA-3. Th2 cells mediate their functions by secretion of IL-4, IL-5, IL-9 and IL-13 and often in epithelial tissues in the intestinal tract and lungs.

Th17 cells

Th17 cells are a pro-inflammatory T cell subset defined by secretion of IL-17 and expression of the transcription factor ROR-γt. Th17 cells are important in host defence against extracellular bacteria and fungi but are also involved in tissue inflammation and autoimmunity. The signature cytokines of Th17 cells are IL-17A, IL-17F, IL-22, IL-6 and TNF-α. Differentiation of Th17 cells is induced by cytokines IL-6, TGF-β and IL-1β while IL-23 provides maturation, expansion and pathogenicity for Th17 cells.

ROR-γt induces transcription of the IL-17α gene, allowing production of IL-17 which is a pro-inflammatory cytokine. In the gut IL-17 promotes epithelial barrier functions by stimulating tight junction protein, microbial peptides and mucin secretion. Secretion of IL-17 leads to recruitment of neutrophils, activation of innate immune cells, enhanced B cell function and release of pro-inflammatory cytokines TNF-α, GM-CSF, and IL-1β.

Pro-inflammatory Th17 cells can be re-programmed in the gut to take on a regulatory function. These cells secrete less pro-inflammatory cytokines and exert their suppressive capacity by secretion of IL-10, TGF-β and expression of CTLA4. In the presence of IL-6, Treg can be converted into a Th17 cell and even regulatory IL-17+FoxP3+ cells have been observed in colitis. In light of this, Th17 can have both a pathogenic and a tissue-protective role in the intestine.
Regulatory T cells

Treg is a CD4+ T cell subset involved in maintaining immune homeostasis, preventing autoimmunity and limiting immunopathology. Development and maintenance of Treg is dependent on IL-2, IL-10 and TGF-β. Treg are usually identified as expressing the transcription factor forkhead box P3 (FoxP3), which is considered as the “master regulator” of Treg development and function. Treg lacking FoxP3 have been found in autoimmune diseases, however they are not as potent suppressors as FoxP3+ Treg. Other markers used to distinguish Treg are CD25, CTLA-4, GITR, Programmed cell death protein-ligand 1 (PD-L1), CD127low and Nrpl-1, however neither marker is unique for Treg. CD25, the α-chain of the IL2 receptor, was the first marker used to identify T cells that were able to suppress autoimmunity, but has since then also been reported to be expressed on activated conventional T cells in humans. CTLA-4, a co-inhibitory molecule and GITR, a co-stimulatory molecule is expressed on both Tregs and activated T cells and are therefore not unique Treg markers. CD39 has gained interest as a new functional Treg marker, however only in mice all Tregs express CD39 while in humans only a fraction of Tregs express CD39.

Two subsets of Tregs exist, thymus derived Treg (TdTreg) and peripherally induced Treg (pTreg). pTreg are induced in the periphery in response to environmental signals from tumors, infections and other pathological inflammatory conditions where mucosal sites are known to be a preferential site for peripheral induction. While TdTreg only need TCR engagement and IL-2 signalling to develop, pTreg need additional signalling such as TGF-β and retinoic acid. The ability to differentiate between these two subset is however difficult since no specific marker has been identified.

Treg migrate to both sites of inflammation and the draining lymph nodes during an immune response and suppress effector cells such as Th1, Th2, Th17, T follicular cells, NK cells and natural killer T (NKT) cells, and also induce tolerogenic properties in DC and macrophages. Several
different modes of suppression have been identified for Tregs, including cytokine secretion, surface molecule signalling, cytolysis and adenosine production. Treg have the ability to suppress via cell contact-independent mechanisms by production of the inhibitory cytokines IL-10, IL-35 and TGF-β, which regulates proliferation and cytokine production of both Treg and conventional T cells. IL-10 secretion can down-regulate pro-inflammatory cytokine production and expression of co-stimulatory molecules on APCs, which decreases T cell activation and differentiation. TGF-β inhibits cytotoxic activity of NK cells, macrophages and CD8+ T cells and inhibit proliferation and differentiation of effector T cells. IL-35 is secreted by Tregs, non-effector cells and tumor cells and is able to suppress T-cell proliferation and effector functions. Another mode of action for IL-35 is suppressing Th17 response and inhibiting differentiation into Th17 cells.

Treg can also use cell-cell contact dependent signalling through surface receptors, CD25, CTLA-4 and PD-L1 to suppress immunity. CTLA-4 inhibits effector T cell activation while PD-L1 regulates on-going immune responses. High expression of the inhibitory molecule CTLA-4 creates a competition for the co-stimulatory marker CD80/86 on DCs and leads to a down regulation of these co-stimulatory molecules and reduces T cell activation. CTLA-4 can also increase the TCR signal strength required for naïve T cell activation thereby preventing activation of T cells by low levels of TCR stimulation. PD-1 exerts two mechanisms of peripheral tolerance, promotion of Treg development and inhibition of self-reactive T cells. PD-L1 expressed on Treg interact with PD-1 on T cells, and will cause inhibition of TCR signalling leading to reduced T cell proliferation, cytokine production and cytolytic function and impaired T cell survival. Another way Treg could dampen T cell activation is by their expression of CD25, which is part of the IL-2 receptor. IL-2 is crucial for T cell activation, and Treg consumption of IL-2 prevent effector T cell access to IL-2 and can result in cytokine deprivation induced apoptosis of effector T cells.
Other cell-cell contact mechanism includes cytolysis through secretion of perforin and granzyme B, with the ability to kill effector T cells and APCs. However, mucosal Treg might not use this mechanism since a study has shown absence of granzyme expression in Treg in human gastric mucosa. Generation of the anti-inflammatory mediator adenosine from ATP through the ectonucleotidas CD39 and CD73 is identified as another way for Treg to suppress proliferation and cytokine secretion. Binding to the A2A adenosine receptors on effector T cells results in increased cAMP levels that inhibit cytokine responses and suppresses effector T cells and DCs.

**CD8$^+$ T cells**

Naïve CD8$^+$ T cells give rise to CTL that can eliminate tumor cells and cells infected with intracellular pathogens, mainly viruses. CD8$^+$ T cells are able to recognize pathogen-derived peptide complexes on MHC class I molecules present on the surface of infected cells. The peptides are derived from cytosolic proteins. In a cell contact dependent manner CTLs kill infected cells through two mechanisms, release of cytolytic granules or by engaging cell-surface death receptors with both leading to apoptosis. Cytolytic granules released from CTLs contain perforin and granzyme A and B. Perforin causes pore-like structures analogous to the C9 component of the complement system, enabling granzyme passage into infected cells. Granzymes subsequently induce apoptosis through caspase activation. The death-receptor pathway includes FasL-Fas and TNF-α interaction that activates the caspase pathway of apoptosis.
Homing and recruitment

Lymphocytes circulate in the blood stream in search for their antigen and for their site of effector response during a process of recirculation and homing\(^\text{14}\). Migration of naïve T cells from the blood to secondary lymphoid tissues occurs through high endothelial venules (HEVs). HEVs associated with PPs contain mucosal adressin cell adhesion molecule-1 (MAdCAM-1) whereas HEVs within MLN express both MAdCAM-1 and peripheral lymph node adressin (PNAd)\(^\text{25}\) in order to guide the cells to the right location. In the MLN and PPs effector T cells are activated, differentiated and imprinted to express specific chemoattractants, receptors for endothelial adhesion molecules thus enabling them to migrate into the intestine from the circulation\(^\text{14}\).

Transendothelial migration into peripheral tissue is an intricate multistep process allowing cells to migrate from blood vessels into sites of infection and it includes several steps; tethering, rolling, activation, arrest and finally transmigration\(^\text{14}\). Endothelial cells are able to express P- and E-selectin during inflammation and will recognize their ligands on leukocytes. A weak reversible adherence to the vessel wall will occur leading to rolling of the cells. The second step includes integrins interacting with adhesion molecules on endothelium slowing down the rolling. By stimulation by chemokines, an increased integrin activation and strong adhesion to the endothelial cell surface will occur leading to firm arrest of the cell on the endothelium. Finally the cell can enter, through the endothelium by diapedesis, guided by chemokines\(^\text{90,91}\).

Transendothelial migration can however be hampered by suppressive mechanisms. Tregs from cancer patients have been found to inhibit in vitro transendothelial migration\(^\text{92}\) and this may be a mechanism to regulate an infiltrating immune response. However, the role of Treg in migration of lymphocytes in vivo has not been fully investigated and this is a major focus in this thesis.
Chemokines play a vital role in mediating migration and homing. Chemokines are small (8-10kDA) chemoattractants secreted by leukocytes, epithelial cells and tumor cells in order to mediate migration of immune cells\textsuperscript{94}. More than 50 chemokines have been identified and they are grouped into two major sub-groups, CXC and CC chemokines and two smaller families C and CX\textsubscript{3}C, based on the position of the N-terminal cysteine residues\textsuperscript{95}. Several chemokine receptors and ligands are important for homing into the intestine, some of particular interest have been studied in this thesis, CXCR3, CCR6 and CCR9 and their ligands.

**CXCR3/CXCL9, 10, 11**

CXCR3 is a Th1 associated chemokine receptor responsible for recruiting T cells to site of inflammatory\textsuperscript{96}. CXCR3 is absent on naïve T cells, but is quickly up-regulated after DC induced T cell activation\textsuperscript{97}. CXCR3 is also expressed on some Tregs\textsuperscript{98} and innate cells such as NK and NKT cells\textsuperscript{97-99}, plasmacytoid DCs\textsuperscript{100} and subsets of B cells\textsuperscript{101}. Both Th1 cells and CTL traffic into inflamed tissue is associated with CXCR3\textsuperscript{102}. However, if CXCR3 is needed for lymphocyte migration into intestinal tumor tissue is unknown and this is a question that this thesis will address.

Ligands of CXCR3 are the three IFN-γ inducible chemokines CXCL9, CXCL10 and CXCL11\textsuperscript{94}. In C57BL/6 mice a point mutation introduces a stop codon early in the CXCL11 gene leading to CXCL11 deficiency in these mice\textsuperscript{103}. In addition to chemotactic migration CXCR3 ligands can induce Th1 polarization, CD4\textsuperscript{+} and CD8\textsuperscript{+} expansion\textsuperscript{104}, apoptosis, regulation of growth and angiogenesis\textsuperscript{105}. Differences in both function and mechanism have been found between the different chemokines, for instance CXCL9 is completely depended on IFN-γ whereas CXCL10 and 11 are not. Also the binding affinity to CXCR3 differs among the ligands, where CXCL11 has the highest and CXCL9 the lowest\textsuperscript{102}.

Three different isoforms of CXCR3 exist in humans, CXCR3-A, -B and –alt. CXCR3-A mediates proliferation, chemotaxis, cell migration and invasion while CXCR3-B mediates antiproliferative, angiostatic and pro-apoptotic effects of the CXCR3 ligands. CXCR3-alt resembles CXCR3-A.
but displays a different carboxyl terminus and is only able to bind to CXCL11. In mice only CXCR3-A exists.\textsuperscript{106}

**CCR6/CCL20**

CCR6 and its sole ligand CCL20 are involved in many autoimmune and inflammatory processes such as Rheumatoid Arthritis, Multiple Sclerosis and inflammatory bowel disease.\textsuperscript{89} CCR6 is expressed on subsets of DC, B cells, memory T cells, Tregs and Th17 cells.\textsuperscript{107} A subset of IL17+ Tregs is induced from memory CCR6+ T cells.\textsuperscript{52}

CCL20 attract CCR6-expressing cells by inducing migration\textsuperscript{107} into inflammatory sites but not during homeostasis.\textsuperscript{14} However, the PP epithelium constitutively express CCL20 and is involved in recruitment of CCR6+ Tregs and Th17 cells.\textsuperscript{10} Th17 cell accumulation in LP is also dependent on CCR6/CCL20 signalling.\textsuperscript{51}

**CCR9/CCL25**

CCL25 is expressed by epithelial cells in the small intestine and by interaction with CCR9 directs T cells, IgA+ plasma blasts and plasmacytoid DC specifically to the small intestine.\textsuperscript{10} When CCR9 binds to CCL25 an interaction between Gβγ and PI3 kinase initiates a downstream cascade activating AKT kinase, which promotes T cell proliferation, anti-apoptosis and regulates intestinal inflammation.\textsuperscript{19,108,109}

**Colorectal cancer**

Colorectal cancer (CRC) is the third most common malignancy worldwide and is the second largest cause of cancer-related death in Europe and USA.\textsuperscript{111,112} CRC can metastasise, which decreases overall survival prognosis for the patient. Primary destinations for metastases are liver, lungs and lymph nodes.\textsuperscript{113,114} Genetic factors cause approximately 20% of all CRC\textsuperscript{110} but the majority of cases are due to sporadic mutations and risk factors including obesity, lack of exercise, alcohol, tobacco and a western diet with large amounts of red meat and fat and low amounts of
vegetables, fruits and fibre\textsuperscript{115}. Microbial infections and inflammation are also found to increase the risk of CRC\textsuperscript{94}.

Tumor progression is a multistep process involving genetic, molecular and pathological changes\textsuperscript{116}, driving the transformation from low-grade dysplastic adenoma to high grade dysplastic adenoma and ultimately carcinoma, transforming normal cells into highly dividing malignant cells\textsuperscript{117}. Cancer cells originate from a normal cell that accumulate driver mutations leading to full-blown cancer, which usually harbours 2-8 driver mutations\textsuperscript{118}. Mutation of the adenomatous polyposis coli (\textit{apc}) tumor suppressor gene, which is rate limiting and an initiator of CRC progression, triggers a majority of human CRC. Loss of \textit{apc} initiates a constant activation of the wnt/\beta-catenin pathway leading to formation of aberrant crypt foci and adenomas\textsuperscript{119,120}, which are defined as an accumulation of highly replicating cells\textsuperscript{94}. The subsequent progression into invasive cancer occurs by further mutations in different signalling pathways such as K-Ras, PIK3CA, TGFBR1, BRAF, TP53, DNA mismatch repair genes, FBXW7, NOTCH, SMAD, PI3 kinase and TP53\textsuperscript{109,121,122}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Colorectal adenoma-carcinoma sequence}
\end{figure}

Treatment of CRC consists of surgery, radiation or chemotherapy\textsuperscript{123}. Although surgery is the main treatment, radiation and chemotherapy aims to improve survival and reduce local recurrence\textsuperscript{124}. However, high dose chemotherapy has been shown to have a negative effect on the immune system causing neutropenia and lymphopenia\textsuperscript{125}.  

14
Cancer immunology

The immune editing hypothesis claims that the host immune response to a neoplastic transformation can be divided into three phases: elimination, equilibrium and escape\textsuperscript{126,127}. The elimination stage occurs early during tumor growth where a productive anti-tumor immunity can eradicate malignant cells\textsuperscript{127}. In equilibrium stage the expansion of transformed cells is held in check by the immune system\textsuperscript{128}. Finally, tumor cells evolve to escape the immune response, which enables tumor growth\textsuperscript{129}. Several studies show how the progression from adenoma to CRC is accompanied by an immune equilibrium turning into escape phase, where Th1 cytokines decreases\textsuperscript{130}, and IL-17\textsuperscript{116} and angiogenesis increases\textsuperscript{131}, which are all part of the progression into an aggressive cancer.

The immune system is not always able to eliminate the malignant cells during the elimination stage. This has been formulated as the Matzinger danger model where cancer might not appear dangerous to the immune system since they initially grow as healthy cells and therefore do not send out a distress signal to activate APCs\textsuperscript{132}. Unstimulated APCs continuously capture exosomes from living cells and apoptotic bodies and by presenting them without co-stimulation tolerance of effector cells will occur\textsuperscript{133}. T cells can therefore not be stimulated by APCs when encountering tumor associated antigens, leading to anergy or apoptotic death making the immune system unable to react towards tumors\textsuperscript{134}. Other mechanisms for tumor tolerance include tumor derived suppression through cytokine secretion including IL-10 and TGF-\beta and accumulation of suppressive Tregs and NKT cells\textsuperscript{134,135}.

After escaping an innate surveillance, tumors can take advantage of their genetic instability to evade an adaptive immune response\textsuperscript{134} by down regulation of MHC molecules or tumor antigens\textsuperscript{136,137}, or by secreting immunosuppressive cytokines\textsuperscript{138}. Tumors might directly kill lymphocytes by expression of apoptosis-inducing cell surface molecules such as FasL\textsuperscript{139}. Cancer cells can also express PD-L1 to help them evade an immune response by interacting with the PD-1 receptor on T cells\textsuperscript{140}, leading to down-regulation of effector functions. A strong correlation
between PD-L1 expression on tumors and poor prognosis in several cancers such as renal cell carcinoma, cervical carcinoma, pancreatic carcinoma, and melanoma\textsuperscript{141-144} has been observed.

The interaction between host immunity and cancerous cells is vital in tumor progression. For cancer development it is necessary that the tumor immune response shift from an immunosurveillance profile in the early adenoma stage to an immunosuppressive profile in invasive cancer\textsuperscript{145}. Consequently, the immune system has emerged as a crucial compartment in tumor progression both as an anti-tumor and pro-tumor agent, and extensive research is conducted in this field.

**Tumor infiltrating lymphocytes**

Tumor infiltrating lymphocytes (TIL) are the primary host immune response against solid tumors\textsuperscript{146}. Infiltration of CD8\textsuperscript{+} T cells and CD57\textsuperscript{+} NK cells, as well as a Th1-type immune response are all correlated to better prognosis in CRC patients\textsuperscript{147,148}. Through a strong TIL response metastasis can also be reduced\textsuperscript{149}. However, tumors can manage to escape an immune response by modulating the immune system. Functional T cells are indeed found in tumors, however they are usually not sufficient to defeat tumors because of insufficient amount of T cells, Treg suppression, exhaustion from constant stimulation, tumor cytokine secretion or tumor secreted enzyme indoleamine 2,3-dioxygenase (IDO) suppression\textsuperscript{150}.

CD4\textsuperscript{+} T cells are one of the main effector cells against tumors and can eradicate tumor cells through activation and recruitment of other effector cells\textsuperscript{151}. By recruitment of NK cells, that directly kills tumor cells, and macrophages, which may have an effect on tumor destruction through production of reactive oxygen/nitrogen species, CD4\textsuperscript{+} T cells can contribute to tumor defence\textsuperscript{152}. CD4\textsuperscript{+} T cells can also exert a direct anti-tumor effect by secretion of cytokines or by inducing tumor cell death by TNF-related apoptosis-inducing ligand (TRAIL)\textsuperscript{153} or Fas/Fas ligand pathway\textsuperscript{154}. Th1 cells in particular are essential for tumor rejection through several different ways, priming of CD8\textsuperscript{+} cell\textsuperscript{151}, activation of
innate antitumor cells\textsuperscript{151,152,155} and recognition of antigen on MHC class II on tumor cells leading to production of cytokines that prevent tumor growth or induce tumor cell death\textsuperscript{152}. Thereby is a strong Th1 response in CRC associated to prolonged diseases free survival. A Th17 response is on the other hand associated to poor prognosis\textsuperscript{148} possibly by contributing to an increased inflammation.

Tumors can express MHC class I and are therefore likely to be attacked by CD8\textsuperscript{+} cells\textsuperscript{151}. CD8\textsuperscript{+} T cells are crucial in anti-tumor immunity through direct killing by cytotoxic release of granzyme B and perforin and induction of NK cells and innate immune cells\textsuperscript{156}. In order to evade an immune response, cancer cells can down regulate the MHC class I to avoid a CD8\textsuperscript{+} T cells\textsuperscript{137} and also CD1D NK T cells response\textsuperscript{136}. However by secretion of IFN-\(\gamma\) from Th1 cells MHC expression is upregulated on tumor cells making them more susceptible for T cell recognition\textsuperscript{157}.

In colon cancer and many other cancer forms, such as ovarian, lung, breast, pancreatic, hepatocellular, head and neck, prostate, and anal carcinoma, an accumulation of Tregs is found\textsuperscript{146}. An increased Treg frequency in the periphery in later stages of CRC has also been observed\textsuperscript{117,158}. However, it is not known if Treg frequencies are increased already during early adenoma formation. The role of Treg in most solid sterile tumors, such as ovarian and pancreatic hepatocellular is protumorigenic helping the tumor evade an immune response by supressing an immune attack\textsuperscript{159}. However, in CRC it is still under debate whether the accumulation of Tregs is related to a good\textsuperscript{146,148,160-162} or bad\textsuperscript{60,159,163} prognostic outcome for the patient.

Treg can supress protective anti-tumor immune responses and thereby help the tumor escape an effective immune attack\textsuperscript{164}. One way Treg can help the tumor to avoid a strong immune response is by down-regulating the lymphocyte migration into the tumor\textsuperscript{22} or suppress otherwise potent T cells, NK cells and DC effector cells\textsuperscript{96}. Whether this is also true for intestinal tumors is investigated in this thesis. In the intestine a constant interaction with bacteria and the environment is in place leading to an
inflammatory milieu for tumors to grow in. Since Treg can control cancer associated inflammation in an IL-10 dependent manner an accumulation of Tregs might be beneficial for the host during the early stages of tumor formation\textsuperscript{165}. However, studies have shown that Treg can shift into an pro-inflammatory cell secreting IL-17 instead of IL-10 and thereby create a more inflammatory milieu helping the tumor to grow\textsuperscript{166}. This shift into a pro-inflammatory cell could be one reason for why Treg can be both of good and bad prognostic outcome for the patient, depending on cytokines secreted.

Recently, Saito et al. proposed a model where FoxP3\textsuperscript{+} T cells in colorectal tumors can either be non-suppressive FoxP3\textsuperscript{low} T effector cells or suppressive FOXP3\textsuperscript{high} Tregs and their proportions define the prognostic value since FoxP3\textsuperscript{low} cells can suppress tumor formation. Accordingly, CRC can be divided into type A and type B where type B have a higher frequency of FoxP3\textsuperscript{low} cells, presumably effector cells, and therefore a better patient survival\textsuperscript{167}.

**Chemokines in cancer**

Chemokines may promote anti-tumor immunity through lymphocyte migration or could act as tumor growth factors by increasing tumor metastasis, angiogenesis and tumor cell proliferation\textsuperscript{168,169}.

CXCR3 has a dual role in tumor progression, either by favouring tumor growth and metastasis when expressed on cancer cells, as observed in glioma\textsuperscript{170}, osteosarcoma\textsuperscript{171}, breast cancer\textsuperscript{172} and CRC\textsuperscript{173} or by limiting tumor growth by enhancing anti-tumor immunity when expressed on effector cells. CXCR3 is required for optimal generation of IFN-\gamma secreting Th1 cells\textsuperscript{174} and are thereby an crucial part of anti-tumor immunity. In humans, the three different isoforms of CXCR3 exert different effects on tumor immunity and affect patient survival. The relative expression of CXCR3-A and B isoforms on tumor cells might be important in regulating tumor proliferation and survival\textsuperscript{106}. CXCR3-A expression on breast\textsuperscript{172,175} and glioma\textsuperscript{170} tumor cells is associated with increased metastasis and poor survival, while CXCR3-B expression is
associated with reduced invasion and growth\textsuperscript{176,177}. Depending on which isoform expressed by tumor cells, CXCR3 expression could be of positive or negative prognostic value.

Lack of the CXCR3 ligands CXCL9 and CXCL10 has been associated with limited infiltration of T cells into tumors\textsuperscript{178,179}. In several different mouse studies expression of CXCL10 in tumor tissue leads to increased tumor-specific T cell infiltration and increased survival\textsuperscript{180,181} and in this thesis we will also extend these studies and investigate the effect of these chemokines in intestinal tumors. In human breast cancer, however, CXCL10 expression is associated with increased frequencies of CXCR3\textsuperscript{+} Tregs\textsuperscript{175}. CXCL9, 10 and 11 are thought to participate in anti-tumor immunity by blocking tumor angiogenesis\textsuperscript{182-184} and by recruiting Th1 cells, CD8\textsuperscript{+} T cells and NK cells. However, these chemokines have also been observed to be involved in metastasis\textsuperscript{104}. Furthermore, CXCL10 has also been shown to reduce tumor progression both by increasing the apoptotic rate of cancer cells\textsuperscript{185} and by inhibiting endothelial cell proliferation independently of CXCR3\textsuperscript{186}.

Several other chemokines are also involved in tumor progression. CCL22 helps tumors to escape an anti-tumor response by accumulation of Tregs in tumor tissue\textsuperscript{187,188} and by metastasis of colon cancer\textsuperscript{111}. CCR6 recruits macrophages and stimulates epithelial proliferation helping the tumor grow\textsuperscript{93} and CCR9 is thought to be involved in tumor chemoresistance and metastasis\textsuperscript{189}.

**Mouse models of colon cancer**

In order to investigate CRC \textit{in vivo} the use of mouse models have emerged. As previously described, CRC results from a cascade of genetic changes and mutations and several of them have been pinpointed as important steps in tumor progression. Mutation in the tumor suppressor \textit{apc} gene is the earliest alteration in the genesis of CRC and is found already in the first stage of CRC, the formation of aberrant crypt foci\textsuperscript{16}. In human 85\% of all CRC have mutations in the \textit{apc} gene\textsuperscript{112}, both in sporadic cases but also in hereditary disease such as Familial...
Adenomatous Polyposis (FAP), an autosomal, dominant disease caused by truncation of the \textit{apc} gene\textsuperscript{190}. Therefore, the use of \textit{apc} mutations in mice is well established in order to investigate intestinal tumors. The most used model is the APC\textsubscript{\textit{min}}/+ mice but several other exist, such as Apc\textsubscript{F/wt} mice, harbouring a Cdx2-Cre transgene with tumors primarily in the colon\textsuperscript{121}, apc\textsuperscript{Δ468} with systemic inflammation\textsuperscript{191} and \textit{apc}\textsuperscript{+/1638N} reassembling FAP with other extraintestinal manifestations\textsuperscript{120}.

**APC\textsubscript{\textit{min}}/+ mice**

The APC\textsubscript{\textit{min}}/+ model was developed by Moser \textit{et al.} almost 30 years ago\textsuperscript{192} as a mouse model of intestinal adenomas. APC\textsubscript{\textit{min}}/+ mice harbour a multiple intestinal neoplasia (min) mutant allele of the \textit{apc} gene, and loss of the remaining normal \textit{apc} allele leads to continuous \textit{wnt} signalling. In the normal setting, \textit{wnt} signalling is controlled by \textit{wnt} initiators and inhibitors (Figure 5). When \textit{wnt} signalling is resting, the \textit{apc} protein forms a complex with several other molecules, which destabilize and degrades β-catenin. However during \textit{wnt} signalling the complex can’t be formed and β-catenin can translocate into the nucleus and activate transcription of genes involved in cell proliferation such as cyclin-D1 and c-Myc\textsuperscript{16,193}.

![Figure 5. \textit{wnt} signaling in the absences of \textit{wnt} molecules and during ongoing signaling.](image-url)
APC<sup>min/+</sup> mice develop approximately 15-30 adenomas<sup>194</sup> throughout the small intestine mostly in the ileum, but colonic tumors are also present<sup>93</sup>. Besides tumor growth in the intestine, the <i>apc</i> mutation in these mice causes impairment of development and differentiation in proliferative tissues such as the reproductive tract, and in the hematopoietic and lymphoid system<sup>195</sup>. Because of extensive splenic haematopoiesis APC<sup>min/+</sup> mice have enlarged spleens<sup>93</sup> and anaemia as tumorogenesis progresses<sup>195</sup>. Furthermore, splenic lymphoid follicles and PP are diminished in these mice<sup>195</sup>. Female APC<sup>min/+</sup> mice can also develop tumors in the mammary glands<sup>196</sup>.

Several studies have been conducted on these mice and have helped expand the knowledge of tumor progression in the intestine. An important role for the gut microbiota in cancer progression has been found, where germfree APC<sup>min/+</sup> mice exhibit lower tumor numbers and smaller spleens. Increased intestinal permeability is seen in APC<sup>min/+</sup> mice and might therefore help the bacteria to accelerate tumor growth<sup>197</sup>. The immune system is partly characterized in these mice and a lymphocyte accumulation in the tumors are found consisting of T cells which are mainly Tregs<sup>164</sup>. A further characterisation is conducted in this thesis. In the epithelium, CD3<sup>+</sup> IEL are increased and express a reduced cytotoxicity, which might possibly help promote tumorgenesis<sup>198</sup>. Also the importance of cytokines in CRC development has been found in these mice, were for instance IFN-γ signalling is a rate-limiting step in the development of adenocarcinoma<sup>37</sup> and IL-17 instead promotes tumor development<sup>199</sup>.

![Figure 6. Small intestine of APC<sup>min/+</sup> mice. A. Intestine from an 18-week-old APC<sup>min/+</sup> mouse. Arrows indicate tumors. B Hematoxylin staining of small intestine from an APC<sup>min/+</sup> mouse. T indicates a tumor.](image)
AIM

In order to defeat intestinal tumors a strong anti-tumor response is needed. However, previous studies have shown that this response can be hampered by various suppressive mechanisms. The aim of this thesis was to investigate how an effective T cell response can accumulate in intestinal tumors and define the role of regulatory T cells as a suppressor of anti-tumor immunity.

Specific aims:

- Determine the suitability of APC^{min/+} mice as an *in vivo* model to study immune responses in intestinal cancer.
- Define the role of Treg in lymphocyte accumulation and migration into tumors.
- Investigate the importance of Th1 associated chemokine receptor CXCR3 in lymphocyte migration into tumors.
MAIN METHODS

APC\textsubscript{min/+} and DEREG mice

APC\textsubscript{min/+} mice on a C57BL/6 background with a mutation in the \textit{apc} gene, resulting in intestinal tumor formation were used in this thesis in order to investigate lymphocyte migration and distribution during tumorgenesis as a model for colon cancer.

In order to investigate Treg in intestinal tumors depletion of regulatory T cells (DEREG) mice where used since they enable selective depletion of Tregs. DEREG mice are a bacterial artificial chromosome (BAC) transgenic mouse line that carries a DTR-eGFP transgene under the control of the FoxP3 promoter, which allows Tregs to be depleted by diphtheria toxin (DT) injections. Rodents have very low affinity DT receptors compared to humans and are less susceptible to DT induced cell death. However, BAC transfection does not cause a complete genetic shift such as knock-in mice. Hence only 95-98\% depletion of Tregs occurs in DEREG mice. Another drawback with DEREG mice is that the depletion is only temporary since transgene negative FoxP3\textsuperscript{+} Tregs starts to appear within a week after depletion\textsuperscript{200}.

APC\textsubscript{min/+} males were bred together with DEREG females to generate APC\textsubscript{min/+}/DEREG and APC\textsubscript{min/+} mice for paper II and III. A second breeding with APC\textsubscript{min/+} males and wild-type (WT) females generated APC\textsubscript{min/+} and WT mice for paper I. At weaning the mice were genotyped for the \textit{apc} mutation by PCR of the tail tip and blood was taken for typing of GFP\textsuperscript{+} Tregs by flow cytometry (FC) to identify DEREG type.

\textit{In vivo} Treg depletion

FoxP3\textsuperscript{+} Tregs in APC\textsubscript{min/+}/DEREG mice were transiently depleted by intraperitoneal (i.p.) injections of 0,5 mg DT at four time points, day 1, 2, 8 and 9 (Figure. 7). Control APC\textsubscript{min/+} mice were equally treated.
Day 3 after the first DT injection, 95% of Tregs in blood were depleted. However, Tregs are rapidly reappearing, demanding several injections to reduce the frequencies of Tregs but within two weeks, Treg frequencies are back to baseline and no efficient depletion can occur because of outgrowth of transgene negative FoxP3+ Treg (Figure 8).

**Adoptive transfer**

To examine the migration of lymphocytes in an *in vivo* system, Carboxyfluorescein succinimidyl ester (CFSE) labelled lymphocytes were injected into mice tail vein. For investigating specifically CXCR3 migration, both anti-CXCR3 treated lymphocytes and untreated cells were labelled with either CFSE or FARred and co-injected into the tail. After two days the mice were sacrificed and migrated cells were identified with FC analysis. This method was used in paper II and III to investigate lymphocyte migration into murine intestinal tumors.
**Intestinal lymphocyte isolation**

For investigation of the immune cells in tumors and normal tissue, the intestine needs to be digested to create a cell suspension. This was conducted by EDTA treatment to remove epithelial cells followed by collagenase treatment for LP or tumor digestion to yield a single cell suspension. The cells were counted and used for FC analyses or stimulation assays in all three papers.

**Human sample collection**

Human samples were collected during curative surgery from patients previously diagnosed with colon cancer with the permission of the Regional Research Ethics Committee of West Sweden and all patients gave an informed consent. None of the patients had undergone radiotherapy or chemotherapy for at least 3 years prior to colectomy, and none suffered from autoimmune diseases. Single cell suspensions were prepared by collagenase/DNase enzymatic digestion after removal of epithelial cells and used for FC analysis (paper III).

**Treg suppression assay**

To evaluate Treg suppressive capacity a co-culture system is the usual approach. By isolating Tregs from either mouse spleen in Paper II or from human peripheral blood in Paper III their suppressive capacity on target cells can be examined. In Paper II, Tregs were cultured together with conventional T cells and irradiated APCs to study suppression of effector T cells. After 3 days of culture, proliferation was measured by thymidine incorporation. In Paper III, we investigated if Treg directly affects endothelial production of CXCR3 ligands. Human peripheral blood was used to isolate Tregs, which were cultured together with human umbilical vein endothelial cells (HUVEC). Culture media was analysed for secreted CXCL9, 10 and 11 by multiplex analyses.
Flow cytometry

FC enables recognition of antigens either on cell surfaces or intracellular through cell permeabilisation by antigen-binding to fluorescence tagged antibodies. Cells are suspended in a stream of fluid and beamed by lasers at different wavelength. By detection of emitted light of different wavelengths from the cell the antibody can be identified. In this thesis cells prepared from spleen, MLN, blood, peripheral lymph node (paper I & III), intestine and tumors (paper I, II and III) were used to identify lymphocyte subsets, chemokine receptors, selectins, cytokine production, activation stage and cytotoxicity.

PrimeFlow® RNA assay was used in Paper III to determine the source of CXCL10 production. This method allows FC analysis of RNA transcripts in single cells when FC antibodies are not available. This is a unique approach to RNA detection and signal amplification by amplifying the reporter signal rather than the target sequence, as usually done by PCR. The assay is conducted by addition of target specific probes that hybridize to the target RNA transcript in fixed cells. Signal amplification and binding with fluorescence-labelled probes allows the RNA transcript to be detected by flow cytometry.

Immunofluorescence

Distribution of cells in the intestinal tissue can be visualized by fluorescence microscopy. The intestine is embedded and sectioned into thin sections. Thereafter antigens of interest are labelled with a primary antibody followed by a secondary antibody carrying a fluorochrome. In this thesis, immunofluorescence (IF) was used to examine the distribution of T cells and adhesion molecules within tumor and intestine in both humans and mice (paper I and II).
Real time PCR
Real time (RT) PCR is a common method to quantify gene expression at the RNA level. cDNA prepared from RNA in tissue lysates are amplified each thermal cycle by polymerase reactions. Fluorescence signals are sent out each cycle and while in the beginning the fluorescence is too low to be detected, at a certain point enough amplified products accumulate to yield a detectable signal. The cycle that gives rise to this signal gives a semi quantiative number on the amount of mRNA from a certain gene of interest, compared between samples. In paper I, II and III RT-PCR was used to examine expression of adhesion molecules, chemokines and cytokines.

Multiplex analyses for protein detection
In this thesis mRNA expression levels have been complemented by investigating protein levels in tissue by multiplex analyses. Cytokine secretion into culture media has also been determined in the same type of assay. In multiplex analysis, analytes of interest bind to beads pre-coated with analyte-specific capture antibodies. Through adding detection antibodies and a fluorochrome the beads can be analysed on a dual-laser flow-based detection instrument. Several different analytes in the same suspension can be measured at the same time since the first laser classifies the bead and the second determines the magnitude of the fluorochrome signal. This method has been used to quantify cytokine and chemokine levels in intestinal tissue (Paper II) and culture media (Paper III).

Statistics
To evaluate significant differences between organs or treatment groups Mann-Whitney test was used. Since we had relatively small sample groups and a non-normally distribution we choose non-parametric tests. \( p \) values <0.05 were considered significant.
RESULTS AND DISCUSSION

In this thesis an extensive attempt has been conducted in order to investigate both \(\text{APC}^{\text{min/}+}\) mice as a model for CRC and to determine the effect of Treg on lymphocyte migration in CRC. Several interesting findings have been made during this study, as summarized in this chapter.

**\(\text{APC}^{\text{min/}+}\) mice exhibit similar immunological properties as WT mice**

To investigate CRC in an *in vivo* system we used \(\text{APC}^{\text{min/}+}\) mice, a mouse model of spontaneous intestinal cancer. These mice have a mutated \(\text{apc}\) gene leading to tumor formation due to uncontrolled wnt signalling. The wnt signalling pathway has also been implicated in other biological systems, such as development of haematopoietic and lymphoid cells\(^{195,201-203}\). To be able to use \(\text{APC}^{\text{min/}+}\) mice as a model for CRC and to rule out the possibility that the \(\text{apc}\) mutation and increased adenoma formation affects systemic migration and immune cell frequencies, the peripheral immune cell composition was examined. When comparing WT mice with \(\text{APC}^{\text{min/}+}\) mice at different ages, no differences were found between frequencies of T cells, Tregs and activated T cells in lymph nodes, spleen and blood (Paper I, Figure 1). This concludes that the systemic immune system remains largely intact regardless of age and increased tumor load and that the \(\text{apc}\) mutation does not influence the peripheral immune system and therefore is \(\text{APC}^{\text{min/}+}\) mice suitable to use as a model for intestinal tumors.

**Treg in \(\text{APC}^{\text{min/}+}\) mice suppress conventional T cells equally well as Treg from WT mice**

As investigations of Treg activity in tumor-bearing mice was one of the major objectives of these studies, the suppressive capacity of Treg from \(\text{APC}^{\text{min/}+}\) mice was investigated. A co-culture system was established were Tregs were cultured together with equal numbers of conventional T cells and irradiated APCs and T cell stimulation. Tregs from both \(\text{APC}^{\text{min/}+}\)
mice and WT mice were able to suppress proliferation in a similar manner, regardless of age and tumor load (Paper II, Figure 1). This concludes that Tregs in the APC^{min/+} mice are still able to suppress an immune response to the same extent as Tregs from WT mice.

Co-culture experiments further revealed that Treg from both WT or APC^{min/+} mice suppressed IFN-γ and TNF-α secretion from WT responder cells equally well, while they had no effect on IL-13 secretion (Figure 9) or IL-10 (data not shown). In contrast, IL-17A secretion was actually increased in the presence of Treg (Figure 9). Isolated APC^{min/+} Treg alone secreted some IL-17A (108±124 pg/ml) after stimulation, but these levels do not account for the whole increase detected in co-cultures, and IL-17A levels may have been influenced by the suppression of IFN-γ in the co-cultures. IFN-γ, TNF-α, IL-10 and IL-13 levels were low or undetectable in all cultures of Treg alone (data not shown).
It is interesting to note that the presence of Treg clearly increased the secretion of IL-17A in *in vitro* cell cultures, as IL-17 has been demonstrated to promote tumorigenesis in APC^{min/+} mice\(^{199,204}\). Also in humans, infiltration of Th17 cells into colon tumors predicts a poor outcome\(^{148}\). Th17 cells may enhance tumor growth by angiogenic effects, and by expression of the adenosine-generating ectonucleotidases CD39 and CD73\(^{205}\). Further, it has been suggested that CD4^{+}FoxP3^{+} putative Treg in APC^{min/+} tumors secrete IL-17 but lack the IL-10 production seen in WT intestinal Treg. The shifted IL-17/IL-10 balance promoted mast cell accumulation and thereby tumor progression\(^{164}\). Adoptive transfer of WT Treg to APC^{min/+} mice delayed tumor formation and also induced apoptosis in established tumors in the intestine, an effect that was dependent on IL-10\(^{164,206}\). Thus, a shift in Treg phenotype from IL-10 to IL-17 secreting cells during the course of tumorigenesis was predicted, and would indicate that tumor infiltrating Tregs in APC^{min/+} mice have the ability to suppress Th1 responses and promote tumor progression due to on the cytokines secreted. Indeed, APC^{min/+} Tregs suppressed production of the Th1 cytokines IFN-γ and TNF-α *in vitro* and promote IL-17 secretion.

**Higher frequencies of Treg in tumors of APC^{min/+} mice**

After stating that the *apc* mutation does not affect the peripheral distribution of lymphocytes or Treg suppression and thus that the APC^{min/+} model can be used to study the immune response in intestinal tumors, we directed our interest to the tumors to evaluate the intratumoral immune reactions. The mice show established tumors around 15 weeks of age and after 20 weeks the tumors are often large and prevent the passage of food leading to death. However, differences exist between animal facilities depending on inflammation and bacterial flora. We chose to investigate the tumors at 18 weeks of age, at which time we collected the intestine and used both tumors and unaffected intestinal tissue for FC, RT-PCR and IF examination.
The cell composition in the tumor was evaluated and lower frequencies of conventional T cells were found in tumors compared to surrounding unaffected tissue (Paper I, Figure 2). The tumor microenvironment can be hostile to T cells and their effector functions due to presence of enzymes that depletes the amino acids tryptophan and arginine, high concentration of tumor secreted lactate and accumulation of suppressive cells such as Treg\(^{207}\). TIL are crucial for anti-tumor immunity\(^{151,152}\), high frequencies of CD3\(^{+}\) and CD8\(^{+}\) cells in tumor tissues have been correlated to positive clinical outcome\(^{208,209}\). The decrease of T cells in tumors of the APC\(^{\text{min/}+}\) mice indicates that intestinal tumors are able to affect the immune system in order to avoid an anti-tumor response.

Tumors of the APC\(^{\text{min/}+}\) mice had a higher frequency of Treg compared to unaffected tissue (Paper I, Figure 4) and this could be one reason for the decreased conventional T cell numbers. Treg could decrease the number of conventional T cells through several mechanisms, such as IL-2 consumption, cytolysis and cytokine secretion\(^{210}\). Tumors are able to accumulate Tregs and this would then help them evade an anti-tumor attack\(^{211}\).

Altered chemokine expression in APC\(^{\text{min/}+}\) mice

A shift in the lymphocyte composition in the intestinal tumors may result from altered recruitment mechanisms. Therefore, chemokines and their receptors important for T cell migration were investigated (Figure 10 and Paper I, Figure 5). CCL17, which attracts CCR4\(^{+}\) Tregs and effector T cells\(^{212}\), was significantly up-regulated in tumors of APC\(^{\text{min/}+}\) mice compared with unaffected tissue. There was also a trend of higher levels of CCL22, another CCR4 ligand, in the tumors although this was not significant. The expression of CCR4 by Tregs and conventional T cells were however similar when comparing tumor and unaffected tissue indicating that the increase of CCR4 ligands in tumors is not sufficient to increase effector T cell migration.
Liu et al. have shown that increased levels of CCR6 expression are responsible for accumulation of Treg in an N-methyl-N-nitrosourea and Helicobacter pylori induced CRC mouse model\textsuperscript{13}. In their model, almost all tumor infiltrating Treg expressed CCR6 and interaction with the ligand CCL20 was the mechanism behind their increased Treg infiltration. However, the level of CCL20 was unchanged in tumors compared to normal tissue in our \(\text{APC}^{\text{min/+}}\) mice (Paper I, Figure 5), but Tregs did express higher levels of CCR6 in the tumors of \(\text{APC}^{\text{min/+}}\) mouse (Paper I, Figure 5) and this might be one mechanism for Treg infiltration into tumor tissue.

The intestinal chemokine CCL25 is produced by intestinal epithelial cells\textsuperscript{108} and is crucial for T cell recruitment to intestinal LP. Intestinal \(\text{APC}^{\text{min/+}}\) tumors produced less CCL25 than unaffected tissue (Paper I, Figure 5). This is in analogy with another epithelial mucosal chemokine, CCL28, which is also produced in lower amounts in human colon adenocarcinomas than unaffected tissue\textsuperscript{214}. The lack of CCL25 may benefit the tumor by reducing recruitment of effector lymphocytes to the tumor but also by allowing extra intestinal metastasis of CCR9\textsuperscript{+} tumor cells. As recently demonstrated by Chen et al.\textsuperscript{109}, CCR9-CCL25 interaction prevent the spread of colon cancer cells from the intestine, and therefore, reduced CCL25 levels would be a mechanism for the tumors to metastasize and leave the primary tumor. The expression of CCR9 on
infiltrating T cells (Paper I, Figure 5) was however not changed between tumor and unaffected tissue in the APC$^{min/+}$ mice.

Tumor associated Tregs expressed lower levels of CXCR3 than Tregs in the unaffected tissue, indicating that this chemokine is not accountable for Treg accumulation in tumors (Paper I, Figure 5). Instead, Treg express CCR9, although the chemokine ligand was not increased. Therefore, Treg could have used CCR6 to enter the tumors and accumulate in the tissue. Overall, several changes in expression of chemokine and chemokine receptors were observed between intestinal tumor and unaffected tissue and this could be accountable for the changes in tumor immune cell composition.

**Increased frequencies of Treg in human intestinal adenoma**

Human adenomas, which mainly correspond to the tumors in our APC$^{min/c}$ mice, have not been examined to the same extent as invasive CRC. To investigate if the APC$^{min/+}$ mouse model for intestinal tumors corresponds to human adenomas, the T cell composition in human intestinal adenomas was investigated by IF. Already in this early tumor formation stage, an accumulation of Treg was found (Figure 11 and Paper I, Figure 4). However, the increased Treg frequencies did not affect the adenoma frequencies of conventional T cells, which remained unchanged compared to unaffected intestinal tissue (Figure 11).

A decrease in the expression of the adhesion molecule MAdCAM-1 was observed in human adenomas (Figure 11) but a corresponding decrease was not found in the APC$^{min/+}$ mice. In later stages of CRC a decrease of MAdCAM-1 has previously been documented by our group$^{215}$ and this new finding demonstrates that this decrease is consistent throughout the adenoma CRC progression and indicates a difference in lymphocyte homing mechanisms to intestinal malignancies between mice and human.
Depletion of Treg in APC\textsuperscript{min/+} mice leads to increased frequencies of conventional T cells

In order to investigate the function of Treg in intestinal tumors we introduced the DEREG mice and bred them together with APC\textsuperscript{min/+} mice to generate APC\textsuperscript{min/+}/DEREG mice. This allowed us to selectively deplete Treg by DT injections and study the impact on the immune cell composition in intestinal tumors.

An accumulation of conventional T cells was found after Treg depletion in the intestinal tumors. This was true for both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells examined with IF and FC (Paper II, Figure 2). Accumulation of Treg is thereby one mechanism tumors use to decrease the frequencies of conventional T cells in the tumor. By suppression of a potent anti-tumor
response Treg can contribute to tumor progression. Previously, increased T cell frequencies have been observed in experimental thymomas, melanoma and chemically induced sarcomas after Treg depletion and these new data for spontaneous intestinal tumors are in line with these findings\textsuperscript{216-218}.

By using the crossing of APC\textsuperscript{min/+} mice with DEREG we reach at least 90\% depletion of Treg, which is sufficient to gain an increased T cell frequency in tumors. Other studies using a CD25 antibody only achieved a 70\% Treg depletion and no differences in lymphocyte numbers, indicating that even in low numbers Treg are strong enough to reduce lymphocyte accumulation\textsuperscript{219}.

**Increased T cell proliferation and migration after Treg depletion**

The increased frequency of conventional T cells may have different causes, such as increased migration into the tumor, increased local proliferation or that the T cells are less prone to leave the tumor. Therefore we aimed to study some of these potential causes. An increased proliferation of conventional T cells was observed in the tumor after Treg depletion, based on expression of Ki67 (Paper II, Figure 7). This would partly contribute to the increased frequency of conventional T cells seen in Treg depleted tumors.

Furthermore, migration was studied through adoptive transfer experiments where CFSE labelled MLN cells were injected into Treg depleted APC\textsuperscript{+/min+}/DEREG mice and control APC\textsuperscript{+/min+} mice. After Treg depletion more lymphocytes migrate into the tumors (Paper III, Figure 1) and that confirms that Treg also prevent migration into the tumor. This is in line with previous studies in our group were human Tregs inhibited transendothelial migration in an \textit{in vitro} system\textsuperscript{92}. Furthermore, a specific increase of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell migration into tumors was seen in the adoptive transfer experiments (Paper III, Figure 1) stating that Treg specifically reduce migration of effector T cells. However, the T cells
migrating into Treg depleted tumors were not more active or proliferative than cells migrating into Treg sufficient tumors (Paper III, Figure 6) indicating that activation of T cells may occur in the tumors by intra-tumoral antigen-presenting cells, rather than in the draining lymph nodes.

**Depletion of Treg leads to a Th1-associated cell infiltration**

To define the mechanisms behind the increased migration several important chemokines and chemokine receptors for T cell migration were investigated. Of great interest, CXCR3, which is associated with a Th1 cell response, was significantly higher on conventional T cells in Treg depleted tumors than Treg sufficient tumors (Paper II, Figure 4). This also corresponded to a higher expression of the CXCR3 ligands, CXCL9 and CXCL10 in Treg depleted tumors (Paper II, Figure 3). Besides attracting effector T cells, CXCL10 has anti-tumor potential by reducing angiogenesis through inhibiting proliferation of endothelial cells and inducing apoptosis of cancer cells\(^{220}\) thus reducing tumor growth. In CRC higher expression of CXCR3 ligands is associated with increased TIL accumulation and patient survival\(^{221-223}\). Tumor promoting effects of CXCR3 ligands have also been observed in several other cancer types\(^{224}\), and this can possibly be explained by metastasis of CXCR3\(^+\) tumor cells or tumor infiltration of suppressive CXCR3\(^+\) Tregs.

Several different cell types in the intestine of APC\(^{min/+}\) mice, T cells, monocytes and epithelial cells produce CXCR3 ligands (Paper III, Figure 4). However, after Treg depletion, only endothelial cells increase their expression of CXCL10 in intestinal tumors, possibly highlighting them as targets for Treg suppression to reduce an anti-tumor response. This is a possibility that we will address in future studies.

The Th1 associated cytokine IFN-γ was increased in tumor tissue after Treg depletion (Paper II, Figure 5). IFN-γ is crucial for anti-tumor immunity. For example, Zhang *et al.* have demonstrated that APC\(^{min/+}\) mice lacking IFN-γ had a more severe tumor progression, which could
possibly lead to an invasive carcinoma\(^{225}\). Other studies have also pointed out the possible anti-tumor effects of IFN-γ by inducing T cell proliferation\(^{226,227}\), upregulate MHC expression on tumor cells\(^{157}\) and blocking of angiogenesis\(^{228,229}\).

Altogether, an increased expression of CXCR3 on tumor infiltrating T cells, higher secretion of its ligands CXCL9 and 10 specifically in the tumors and increased IFN-γ levels indicates that when Treg are depleted a Th1 associated response is reinstated which could lead to an anti-tumor response.

**CXCR3 is crucial for intestinal migration**

Chemokines guide lymphocytes into tissues and may be responsible for the increased T cell migration. We reasoned that CXCR3-mediated chemotaxis may be an important mean of T cell recruitment into intestinal tumors as Treg depletion increased the production of CXCL9 and CXCL10 selectively in the tumors. Therefore, we examined the effect of CXCR3 blocking on lymphocyte migration into APC\(^{−/−}\) tumors in an adoptive transfer system. The small molecule CXCR3 antagonist AMG487, was used for blocking CXCR3\(^{171}\). This CXCR3 blocking agent has been used in clinical trials\(^{230}\), since it is shown to inhibit Th1 cell migration into sites of inflammation\(^{231}\). However, a phase II trial was halted due to lack of efficacy in treating psoriasis\(^{232}\). In tumor mouse models of breast cancer\(^{233}\) and CRC\(^{234}\), the CXCR3 antagonist has been used to study lung metastasis of CXCR3\(^{+}\) tumor cells.

Pre-treatment of lymphocytes with the specific CXCR3 antagonist AMG487\(^{233}\) before transfer potently reduced lymphocyte migration into both small intestine and tumor in our APC\(^{−/−}\) mice (Paper III, Figure 3). Furthermore, CD4\(^{+}\)T cells had an even more profound CXCR3 mediated migration into tumors. Li et al. show that increased frequencies of CXCR3\(^{+}\) T cells in the tumors is associated with prolonged survival in gastric cancer\(^{235}\), indicating the importance of CXCR3 mediated migration into tumors.
Human CRC immunological composition

To verify some of the mouse model data in human CRC, Treg frequencies and chemokine composition was investigated. An increase of Tregs examined by RT-PCR was found in the tumors compared to surrounding unaffected tissue (Figure 12), which confirms our previous FC analysis. An increased Treg accumulation in tumors has also been observed in other mouse and human studies.

In invasive CRC, an increase of the CXCR3 ligands CXCL9, 10 and 11 was observed (Paper III, Supplementary figure 2). Furthermore, several different cell types such as T cells, monocytes and endothelial cells produced these ligands (Figure 13 and Paper III, Figure 5) confirming our data from the APC\textsuperscript{min+} mouse model.

Figure 12. Relative expression of FOXP3\textsuperscript{+} mRNA in human CRC and unaffected tissue.

Figure 13. Human cells expressing CXCR3 ligands in colon tumors and unaffected tissues.
An increase of CXCR3 ligands has been observed in several cancer types such as basal cell carcinoma\textsuperscript{237}, ovarian cancer\textsuperscript{238}, B-cell lymphoma\textsuperscript{239} and melanoma\textsuperscript{240}, and has been postulated to contribute to tumor growth. Human colon cancer cells express the CXCR3-B isoform\textsuperscript{241}, which has growth-inhibitory properties and inhibits chemotaxis\textsuperscript{105}. Over-production of CXCR3 ligands can lead to a down regulation of CXCR3-B, thus promoting tumor growth and metastasis\textsuperscript{242}. By this mechanism advanced CRC may possibly secrete CXCR3 ligands in an autocrine manner, in order to grow and may not affect CXCR3 mediated lymphocyte infiltration. Actually, a decrease of CXCR3\textsuperscript{+} T cell frequencies have been observed in CRC compared to the unaffected mucosa\textsuperscript{215} thus strengthening the concept that the increase of CXCR3 ligands do not affect lymphocyte infiltration but tumor progression in invasive CRC.
CONCLUSION
AND FUTURE PERSPECTIVES

Figure 14. Treg hinders an anti-tumor response. In this thesis Treg suppressive capacity on several different anti-tumor mechanisms have been examined. Treg can both inhibit migration and proliferation of effector T cells. Furthermore, Treg decrease secretion of CXCL9 and 10 thereby hindering migration of CXCR3+ T cells. Also secretions of cytokines are affected. Altogether this indicates that Treg hinders an effective Th1 associated immune response against tumor cells.

This thesis postulates that Treg are able to reduce an anti-tumor response through several different mechanisms that ultimately diminishes the amount of effector T cells in the intestinal tumor tissue (Figure 14). The APC\textsuperscript{min/+} mouse model of intestinal tumors was used for this work since we could show that the \textit{apc} mutation manifest immunological changes specifically in the tumors. Through depletion experiments the consequence of a Treg deficient tumor environment has been thoroughly examined and formed our proposition. In particular, Treg reduces a Th1 response, as demonstrated by lower levels of IFN-\(\gamma\), CXCR3+ T cells and decreased T cell migration. The Th1 associated chemokine receptor CXCR3 was shown to be specifically important for migration into the tumor tissue. After Treg depletion the CXCR3 ligands CXCL9 and 10
were increased in the tumor tissue and specifically endothelial cells increased their ligand production indicating that Treg may reduce chemokine signalling to hinder an effective anti-tumor response. A Th1 response is particularly beneficial in tumor regression since it activates CTL and NK cells, which both can attack cancer cells and defend against tumor progression. Furthermore, a strong Th1 response has been correlated to improved anti-tumor response and overall patient survival. In CRC, reduced levels of both Th1 cells and their cytokines have been observed in several studies indicating a suppressive milieu for an anti-tumor response. Therefore, Treg blocking could be a treatment that may reduce intestinal tumor development by accumulation of a Th1 response.

Today several Treg depletion studies have been conducted in both human and mice by targeting CD25 with anti-CD25 mAbs or IL-2-toxin fusion proteins. In some studies tumor regression has been observed following Treg depletion. However, these strategies also affect activated CD25+ effector T cells thereby limiting a positive effect. Other Treg depletion strategies have involved affecting Treg function by blocking of CTLA-4, OX-40 a co-stimulatory molecule of the TNF receptor family, and GITR, which all have resulted in an increased anti-tumor response, strengthen the concept of Treg suppressive capacity of an anti-tumor response. However, Treg depletion is also usually accompanied by autoimmune reactions, thereby limiting its usefulness.

Treg depletion has not been extensively investigated in intestinal tumors and in our short-term Treg depletion protocol no effect on tumor size was observed. DEREG mice outgrow transgene negative Treg, not susceptible to DT depletion, and can not be used for long term experiments. Possibly only a short-term Treg depletion is not sufficient to affect tumor size in the APC-/- mice. However, it can also indicate that Treg depletion as a single treatment is not effective enough in intestinal tumors and other strategies might be needed.

PD-1 is an inhibitory immune receptor up-regulated on T cells after activation. Tumors can express PD-L1 to create an immunosuppressive
milieu for T cells\textsuperscript{227} thereby preventing an immune response. Peng \textit{et al}, have shown that through blocking of PD-1, both CXCL10 and IFN-\textgamma increased at the tumor site without decreasing Treg frequencies\textsuperscript{227}. Several clinical trials conducted with PD-1 monoclonal antibodies to treat cancer have nevertheless had a relatively low complete response rate\textsuperscript{140,259,260}. However, Treg in intestinal tumors manifest a higher suppressive capacity by e.g. higher expression of PD-L1, CD39 and CTLA-4 (Ahlmanner \textit{et al}, unpublished). Perhaps blocking of only PD-1 is not efficient since Treg still can exert an immunosuppressive effect on T cells. This implies a need for dual treatments. By depleting Treg we may be able to increase migrating T cells but due to other immunosuppressive mechanism from tumor cells a joint strategy to both inhibit the immunosuppressive milieu in the tumor by PD-1 or PD-L1 inhibition together with Treg depletion may be a viable strategy for tumor regression.

Another possible mechanism for tumor regression could be to increase the availability of CXCR3 ligands locally in the tumors to optimize migration of effector T cells into the tumors. In breast cancer and ovarian cancer models, suppression of endogenous PGE\textsubscript{2} synthesis by cyclooxygenase has been shown to reduce tumor growth through increase of intratumoral CXCR3 ligands and increased Th1 responses\textsuperscript{261-263}. Also transfections of the IL-12 gene in a model of head and neck cancer up-regulated CXCL9 expression\textsuperscript{264}. However, both Treg and cancer cells express CXCR3\textsuperscript{241} and this might not be an effective single treatment since it could increase Treg accumulation and tumor metastasis. But combining promotion of CXCR3 ligands with Treg depletion might be an effective way to increase an immune response against tumor progression.

Intestinal cancer is an increasing disease in the western world and is still a major obstacle to be dealt with. Hopefully this thesis will help elucidate one hurdle the anti-tumor immune system has to overcome, namely the Treg. By transiently eliminating Treg or modulating their effector functions there might be an opportunity for tumor regression.
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