Regulatory T cells and lymphocyte migration into intestinal tumors

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ABSTRACT

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 DEREG 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

En av de vanligaste cancerformerna i västvärlden är koloncancer, vilken ger upphov till en stor andel av alla cancerrelaterade dödsfall. De vanligaste riskfaktorerna för att utveckla koloncancer kan alla kopplas till den västerländska livsstilen, främst fetma, alkohol, tobak och en diet med mycket rött kött, fett och för lite frukt och fibrer. Något som däremot kan motverka de negativa följderna av en yttre livsstilspåverkan är kroppens immunförsvar där effektor T-celler, både cytotoxiska T-celler och T-hjälparceller, har en viktig roll för att bekämpa tumörcellerna. Men för att kunna döda cancer cellerna måste T-cellerna kunna ta sig in i tumören. En annan form av T-celler, regulatoriska T-celler (Treg), kontrollerar normalt att immunförsvaret inte överreagerar. I flera olika typer av cancer har det påvisats höga nivåer av Treg i tumörerna och man tror allmänt att detta kan ge ett minskat anti-tumörförsvar vilket då gynnar tumörtillväxten. I denna avhandling har vi därför undersökt Tregs påverkan på immunförsvaret i tumörer från tarmen för att förstå deras inverkan på rekrytering och funktion hos effektor T-celler.

$APC^{min/+}$ möss utvecklar spontant tumörer i hela tarmsystemet på grund av en mutation i *apc* 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 tarmtumörer 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 $APC^{min/+}$/DEREG-möss möjliggjordes studier av hur tumörernas immunförsvar påverkas i frånvaro av Treg. När Treg eliminerades noterade vi en ökning av effektor T-celler i tumörerna. En ökning av T-celler 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 det ökade antalet. För att undersöka om T-cellerna dessutom lyckades ta sig in i tumören bättre i avsaknad av Treg, gjordes migrationsexperiment där inmärkta celler injicerades i tumörbärande möss och frekvensen av migrerande celler in i olika organ undersöktes. 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 effektiv anti-tumör försvar bestående av T-celler genom att både hämma T-cell 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 anti-tumörförsvar. Vi kunde visa att CXCR3 är nödvändig för T-cellernas migration in i tarmvävnaden 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 immunförsvaret mot tumören. Att eliminera Treg eller påverka deras supressiva funktioner skulle därför kunna bli en effektiv immunterapi mot koloncancer.

LIST OF PAPERS

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

I. Akeus P, Langenes V, von Mentzer A, Yrlid U, Sjöling Å, Saksena P, Raghavan S, and Quiding-Järbrink M. "Altered Chemokine Production and Accumulation of Regulatory T Cells in Intestinal Adenomas of $APC^{Min/+}$ Mice." Cancer Immunology, Immunotherapy, 2014;63(8):807-819. doi:10.1007/s00262-014-1555-6.

II. Akeus P, Langenes V, Kristensen J, von Mentzer A, Sparwasser T, Raghavan S, and Quiding-Järbrink M. "Treg-Cell Depletion Promotes Chemokine Production and Accumulation of CXCR3(+) Conventional T Cells in Intestinal Tumors." European Journal of Immunology, 2015;45(6):1654-66. doi:10.1002/eji.201445058.

III. Akeus P, Ahlmanner F, Sundström P, Alsen S, Gustavsson B, Sparwasser T, Raghavan S, Quiding-Järbrink M, "Regulatory T cells control endothelial chemokine production and migration of T cells in intestinal tumors". Manuscript in preparation

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ABBREVIATIONS

APC	Adenomatous polyposis coli
APCs	Antigen producing cells
BAC	Bacterial artificial chromosome
CFSE	Carboxyfluorescein succinimidyl ester
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
DEREG	Depletion of regulatory T cells
DT	Diphtheria toxin
ER	Endoplasmic reticulum
FAP	Familial Adenomatous Polyposis
FC	Flow cytometry
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
HEV	High endothelial venules
HUVEC	Human umbilical vein endothelial cells
i.p.	Intraperitoneal
IDO	Indoleamine 2,3-dioxygenase
IEL	Intraepithelial lymphocytes
IF	Immunofluorescence
LP	Lamina propria
LPL	Lamina propria lymphocyte
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MHC	Major histocompatibility complex
min	Multiple intestinal neoplasia
MLN	Mesenteric lymph node
NK cells	Natural killer cells
NKT cells	Natural killer T cells
PD-1	Programmed cell death protein-1
PD-L1	Programmed cell death protein-ligand 1
PNAd	Peripheral lymph node addressin
PP	Payers patch
pTreg	Peripheral induced Treg
RT-PCR	Real time PCR
TCR	T cell receptor
TIL	Tumor infiltrating lymphocytes
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
tTreg	Thymus derived Treg
WT	Wild-type

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[3]. Infected cells can also phagocytose antigens leading to peptides residing in endosomes that can be presented by MHC class II, assembled in the ER[4].

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[5]. 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[6]. 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[7]. When a pathogen is eliminated, the majority of activated lymphocytes die by apoptosis and the immune system returns to homeostasis[8]. However, memory cells remain for a long period of time and respond rapidly in case the same pathogen invades the host again[9].

Gastrointestinal tract

The GI tract is an intricate organ system involved in food transportation and uptake, and is in constant contact with the environment[10]. 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[10]. 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[11]. 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[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[13].

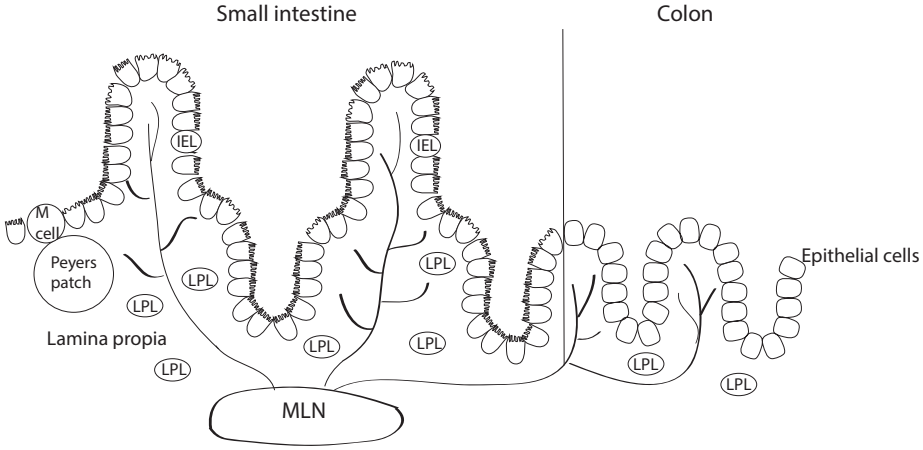

Figure 1. The intestinal mucosa consists of one layer of epithelial cells and the underlying lamina propria. The small intestine contains villi that stretches out into the lumen while the colon present a smooth surface with crypts. IEL: Intra epithelial lymphocytes, LPL: lamina propria lymphocytes, MLN: 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[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[15]. The colon do not form villi and instead form a smooth surface with crypts like the small intestine, however lacking Paneth cells[10].

Due to the constant interaction between host and environment the GI tract needs several barrier mechanisms to protect from foreign bacteria and substances[16], such as the chemical barrier of anti-bacterial peptides secreted by paneth cells[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[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[18]. In the absent of a danger signal homeostatic gut APCs migrate to the lymph nodes where they contribute to the oral tolerance against gut content[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[20]. Some of the epithelial cells covering PP are M cells that transport antigens from the lumen into the PP and to the APCs[21]. Mesenteric lymph nodes (MLN) are another major site of antigen presentation[22] and oral tolerance induction in the gut[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[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[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[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[11]. IEL residing in the epithelial layer, are antigen experienced T cells that balance a protective immunity while keeping the integrity of the epithelial barrier[27].

Both cells of innate and adaptive immunity co-exist in the LP[11]. T cells are a major player orchestrating immune responses, both by activation of other cells but also by direct killing of foreign pathogens[28]. 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[24]. T cell activation requires at least two signals to become activated, first TCR engagement with the antigen presented by MHC complexes[29] and secondly engagement of the co-stimulatory molecule CD28 by CD80 and CD86 on APCs[30]. A third signal includes cytokines secretion from the APCs, potentially initiated by adjacent innate immune cells[31].

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[10,11]. Several different effector T cells exist and they are commonly divided into $CD4^+$ Th cells and $CD8^+$ CTL[2]. $CD4^+$ Th cells mediates a response in inflammation, autoimmunity, asthma, allergy and tumor immunity[2,16] and their TCR are able to bind to MHC class II[4]. Th cells can be further divided into different subsets depending on cytokine secretion and transcription factors[28]. The differentiation pathway of an individual T cell is dependent on the cytokine milieu during activation[31]. The different subsets have also been shown to be plastic leading to a possibility of evolvement of subset depending on the cytokine milieu[24]. Another $CD4^+$ subset is the regulatory T cell (Treg) that regulates and maintains self-tolerance and can supress other T cells in order to control immune responses[32]. CTL on the other hand are $CD8^+$ T cells that can kill infected cells by releasing perforin and granzymes[33] and by Fas-FasL interaction[34].

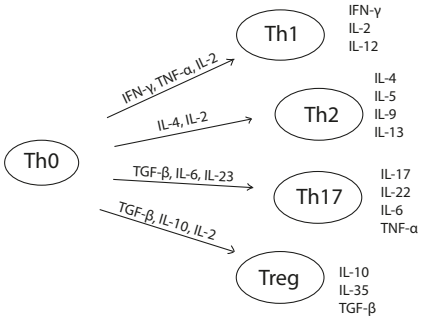

Figure 2. Differentiation of naïve T cells into different subsets is dependent on cytokine milieu.

Th1 cells

Th1 cells are critical for cell-mediated immune responses against intracellular pathogens and tumor cells[35], and are characterized by secretion of IFN-γ and IL-2 and expression of the transcription factor T-bet[28]. 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[28,36]. 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[28].

IFN-γ is a cytokine mediating innate and adoptive immunity against both viral and bacterial infections and tumors[37]. The main functions of IFN-γ signalling are regulation of host defence, immune system, cell cycle, apoptosis, inflammation and cell proliferation[37-39] but also maintenance of the intestinal epithelial barrier integrity[40]. 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[41]. IFN-γ can also supress T cell differentiation into Th2 and Th17 cells[24,42] 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[28,43]. 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[43]. 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[44].

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[45]. Th17 cells are important in host defence against extracellular bacteria and fungi but are also involved in tissue inflammation and autoimmunity[46]. 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-ß and IL-1β while IL-23 provides maturation, expansion and pathogenicity for Th17 cells[45,47,48].

ROR-γt induces transcription of the IL-17α gene, allowing production of IL-17[45] which is a pro-inflammatory cytokine[49]. In the gut IL-17 promotes epithelial barrier functions by stimulating tight junction protein, microbial peptides and mucin secretion[50]. 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-1ß[28].

Pro-inflammatory Th17 cells can be re-programed 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-ß and expression of CTLA4[51]. 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[52]. In light of this, Th17 can have both a pathogenic and a tissue-protective role in the intestine[48].

Regulatory T cells

Treg is a $CD4^+$ T cell subset involved in maintaining immune homeostasis, preventing autoimmunity and limiting immunopathology[53]. Development and maintenance of Treg is dependent on IL-2, IL-10 and TGF-ß[54,55]. 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[56-58]. Treg lacking FoxP3 have been found in autoimmune diseases, however they are not as potent suppressors as $FoxP3^+$ Treg[59]. Other markers used to distinguish Treg are CD25, CTLA-4, GITR, Programmed cell death protein-ligand 1 (PD-L1), $CD127^{low}$ and Nrpl-1[60,61], however neither marker is unique for Treg. CD25, the α-chain of the IL2 receptor[32], was the first marker used to identify T cells that were able to supress autoimmunity[57], but has since then also been reported to be expressed on activated conventional T cells in humans[62]. CTLA-4, a co-inhibitory molecule and GITR, a co-stimulatory molecule is expressed on both Tregs and activated T cells[63] and are therefore not unique Treg markers[62,64]. CD39 has gained interested as a new functional Treg marker[65], however only in mice all Tregs express CD39 while in humans only a fraction of Tregs express CD39[66].

Two subsets of Tregs exist, thymus derived Treg (tTreg) and peripherally induced Treg (pTreg)[67]. pTreg are induced in the periphery in response to environmental signals from tumors, infections and other pathological inflammatory conditions[65,68] where mucosal sites are known to be a preferential site for peripherial induction[69]. While tTreg only need TCR engagement and IL-2 signalling to develop, pTreg need additional signalling such as TGF-ß and retinoic acid. The ability to differentiate between these two subset is however difficult since no specific marker has been identified[70].

Treg migrate to both sites of inflammation and the draining lymph nodes during an immune response[71] and supress effector cells such as Th1, Th2, Th17, T follicular cells[60], NK cells and natural killer T (NKT) cells[56], and also induce tolerogenic properties in DC and macrophages[72]. Several

different modes of suppression have been identified for Tregs, including cytokine secretion, surface molecule signalling, cytolysis and adenosine production[73] (Figure 3). Treg have the ability to supress via cell contact-independent mechanisms by production of the inhibitory cytokines[71] IL-10, IL-35 and TGF-ß, which regulates proliferation and cytokine production of both Treg and conventional T cells[73]. 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[56]. TGF-ß inhibits cytotoxic activity of NK cells, macrophages and $CD8^+$ T cells and inhibit proliferation and differentiation of effector T cells[74]. IL-35 is secreted by Tregs, non-effector cells[75] and tumor cells[76] and is able to supress T-cell proliferation and effector functions[72]. Another mode of action for IL-35 is supressing Th17 response and inhibiting differentiation into Th17 cells[77].

Treg can also use cell-cell contact dependent signalling through surface receptors, CD25[60], CTLA-4 and PD-L1 to supress immunity[78]. CTLA-4 inhibits effector T cell activation while PD-L1 regulates on-going immune responses[73]. 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[56,71]. 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[79]. PD-1 exerts two mechanisms of peripheral tolerance, promotion of Treg development and inhibition of self-reactive T cells[80]. PD-L1 expressed on Treg interact with PD-1 on T cells, and will cause inhibition of TCR signalling[81] leading to reduced T cell proliferation, cytokine production and cytolytic function and impaired T cell survival[82]. 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[60] and can result in cytokine deprivation induced apoptosis of effector T cells[83].

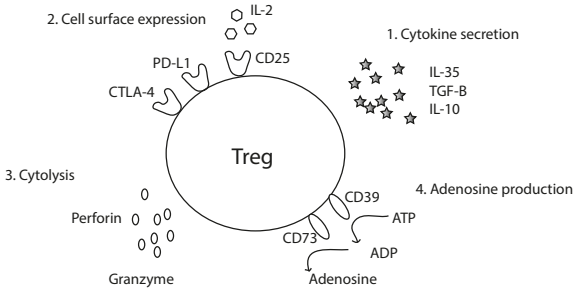

Figure 3. Suppressive mechanisms of Treg.

Other cell-cell contact mechanism includes cytolysis through secretion of perforin and granzyme B, with the ability to kill effector T cells[56] and APCs[73]. However, mucosal Treg might not use this mechanism since a study has shown absence of granzyme expression in Treg in human gastric mucosa[84]. Generation of the anti-inflammatory mediator adenosine from ATP through the ectonucleotidas CD39 and CD73 is identified as another way for Treg to supress 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[85,86].

$CD8^+$ T cells

Naïve $CD8^+$ T cells give rise to CTL that can eliminate tumor cells and cells infected with intracellular pathogens, mainly viruses[87]. $CD8^+$ 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[3]. 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[88]. 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[34] enabling granzyme passage into infected cells. Granzymes subsequently induce apoptosis through caspase activation[33]. The death-receptor pathway includes FasL-Fas and TNF-α interaction that activates the caspase pathway of apoptosis[89].

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[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 addressin cell adhesion molecule-1 (MAdCAM-1) whereas HEVs within MLN express both MAdCAM-1 and peripheral lymph node addressin (PNAd)[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[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[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[90,91].

Transendothelial migration can however be hampered by suppressive mechanisms. Tregs from cancer patients have been found to inhibit *in vitro* transendothelial migration[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[93] secreted by leukocytes, epithelial cells and tumor cells in order to mediate migration of immune cells[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_3C, based on the position of the N-terminal cysteine residues[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[96]. CXCR3 is absent on naïve T cells, but is quickly up-regulated after DC induced T cell activation[97]. CXCR3 is also expressed on some Tregs[98] and innate cells such as NK and NKT cells[97,99], plasmacytoid DCs[100] and subsets of B cells[101]. Both Th1 cells and CTL traffic into inflamed tissue is associated with CXCR3[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[94]. In C57BL/6 mice a point mutation introduces a stop codon early in the CXCL11 gene leading to CXCL11 deficiency in these mice[103]. In addition to chemotactic migration CXCR3 ligands can induce Th1 polarization, $CD4^+$ and $CD8^+$ expansion[104], apoptosis, regulation of growth and angiogenesis[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[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[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[93]. CCR6 is expressed on subsets of DC, B cells, memory T cells[107], Tregs and Th17 cells[52]. A subset of $IL17^+$ Tregs is induced from memory $CCR6^+$ T cells[52].

CCL20 attract CCR6-expressing cells by inducing migration[107] into inflammatory sites but not during homeostasis[14]. However, the PP epithelium constitutively express CCL20 and is involved in recruitment of $CCR6^+$ Tregs and Th17 cells[10]. Th17 cell accumulation in LP is also dependent on CCR6/CCL20 signalling[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[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[19,108,109].

Colorectal cancer

Colorectal cancer (CRC) is the third most common malignancy worldwide[110] and is the second largest cause of cancer-related death in Europe and USA[111,112]. CRC can metastasise, which decreases overall survival prognosis for the patient. Primary destinations for metastases are liver, lungs and lymph nodes[113,114]. Genetic factors cause approximately 20% of all CRC[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[115]. Microbial infections and inflammation are also found to increase the risk of CRC[94].

Tumor progression is a multistep process involving genetic, molecular and pathological changes[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[117]. Cancer cells originate from a normal cell that accumulate driver mutations leading to full-blown cancer, which usually harbours 2-8 driver mutations[118]. Mutation of the adenomatous polyposis coli (*apc*) tumor suppressor gene, which is rate limiting and an initiator of CRC progression, triggers a majority of human CRC. Loss of *apc* initiates a constant activation of the wnt/ß-catenin pathway leading to formation of aberrant crypt foci and adenomas[119,120], which are defined as an accumulation of highly replicating cells[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[109,121,122].

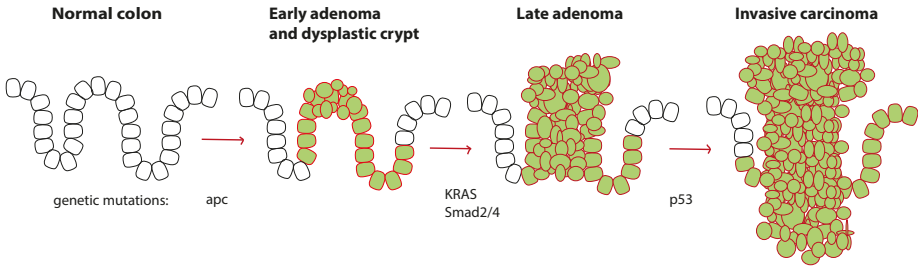

Figure 4. Colorectal adenoma-carcinoma sequence

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

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[126,127]. The elimination stage occurs early during tumor growth where a productive anti-tumor immunity can eradicate malignant cells[127]. In equilibrium stage the expansion of transformed cells is held in check by the immune system[128]. Finally, tumor cells evolve to escape the immune response, which enables tumor growth[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[130], and IL-17[116] and angiogenesis increases[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 were cancer might not appear dangerous to the immune system since they initially grow as healthy cells and therefor do not send out a distress signal to activate APCs[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[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[134]. Other mechanisms for tumor tolerance include tumor derived suppression through cytokine secretion including IL-10 and TGF-ß and accumulation of suppressive Tregs and NKT cells[134,135].

After escaping an innate surveillance, tumors can take advantage of their genetic instability to evade an adaptive immune response[134] by down regulation of MHC molecules or tumor antigens[136,137], or by secreting immunosuppressive cytokines[138]. Tumors might directly kill lymphocytes by expression of apoptosis-inducing cell surface molecules such as FasL[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[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[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[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[146]. Infiltration of $CD8^+$ T cells and $CD57^+$ NK cells, as well as a Th1-type immune response are all correlated to better prognosis in CRC patients[147,148]. Through a strong TIL response metastasis can also be reduced[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[150].

$CD4^+$ T cells are one of the main effector cells against tumors and can eradicate tumor cells through activation and recruitment of other effector cells[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, $CD4^+$ T cells can contribute to tumor defence[152]. $CD4^+$ 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)[153] or Fas/Fas ligand pathway[154]. Th1 cells in particular are essential for tumor rejection through several different ways, priming of $CD8^+$ cell[151], activation of

innate antitumor cells[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[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[148] possibly by contributing to an increased inflammation.

Tumors can express MHC class I and are therefore likely to be attacked by $CD8^+$ cells[151]. $CD8^+$ 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[156]. In order to evade an immune response, cancer cells can down regulate the MHC class I to avoid a $CD8^+$ T cells[137] and also CD1D NKT cells responses[136]. However by secretion of IFN-γ from Th1 cells MHC expression is upregulated on tumor cells making them more susceptible for T cell recognition[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[146]. An increased Treg frequency in the periphery in later stages of CRC has also been observed[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 pro-tumorigenic helping the tumor evade an immune response by supressing an immune attack[159]. However, in CRC it is still under debate whether the accumulation of Tregs is related to a good[146,148,160-162] or bad[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[164]. One way Treg can help the tumor to avoid a strong immune response is by down-regulating the lymphocyte migration into the tumor[92] or suppress otherwise potent T cells, NK cells and DC effector cells[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[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[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 $FoxP3^{+}$ T cells in colorectal tumors can either be non-suppressive $FoxP3^{low}$ T effector cells or suppressive $FOXP3^{high}$ Tregs and their proportions define the prognostic value since $FoxP3^{low}$ cells can supress tumor formation. Accordingly, CRC can be divided into type A and type B where type B have a higher frequency of $FoxP3^{low}$ cells, presumably effector cells, and therefore a better patient survival[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[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[170], osteosarcoma[171] , breast cancer[172] and CRC[173] or by limiting tumor growth by enhancing anti-tumor immunity when expressed on effector cells. CXCR3 is required for optimal generation of IFN-γ secreting Th1 cells[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[106]. CXCR3-A expression on breast[172,175] and glioma[170] tumor cells is associated with increased metastasis and poor survival, while CXCR3-B expression is

associated with reduced invasion and growth[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[178,179]. In several different mouse studies expression of CXCL10 in tumor tissue leads to increased tumor-specific T cell infiltration and increased survival[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 $CXCR3^+$ Tregs[175]. CXCL9, 10 and 11 are thought to participate in anti-tumor immunity by blocking tumor angiogenesis[182-184] and by recruiting Th1 cells, $CD8^+$ T cells and NK cells. However, these chemokines have also been observed to be involved in metastasis[104]. Furthermore, CXCL10 has also been shown to reduce tumor progression both by increasing the apoptotic rate of cancer cells[185] and by inhibiting endothelial cell proliferation independently of CXCR3[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[187,188] and by metastasis of colon cancer[111]. CCR6 recruits macrophages and stimulates epithelial proliferation helping the tumor grow[93] and CCR9 is thought to be involved in tumor chemoresistance and metastasis[189].

Mouse models of colon cancer

In order to investigate CRC *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 *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[16]. In human 85% of all CRC have mutations in the *apc* gene[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 *apc* gene[190]. Therefore, the use of *apc* mutations in mice is well established in order to investigate intestinal tumors. The most used model is the $APC^{min/+}$ mice but several other exist, such as $Apc^{F/wt}$ mice, harbouring a Cdx2-Cre transgene with tumors primarily in the colon[121], apcΔ468 with systemic inflammation[191] and $apc^{+/1638N}$ reassembling FAP with other extraintestinal manifestations[120].

$APC^{min/+}$ mice

The $APC^{min/+}$ model was developed by Moser *et al.* almost 30 years ago[192] as a mouse model of intestinal adenomas. $APC^{min/+}$ mice harbour a multiple intestinal neoplasia (min) mutant allele of the *apc* gene, and loss of the remaining normal *apc* allele leads to continuous wnt signalling. In the normal setting, wnt signalling is controlled by wnt initiators and inhibitors (Figure 5). When wnt signalling is resting, the apc protein forms a complex with several other molecules, which destabilize and degrades ß-catenin. However during wnt signalling the complex can´t be formed and ß-catenin can translocate into the nucleus and activate transcription of genes involved in cell proliferation such as cyclin-D1 and c-Myc[16,193].

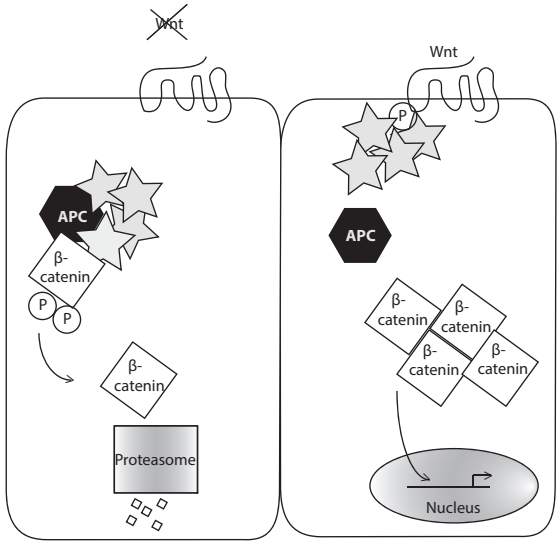

Figure 5. wnt signaling in the absences of wnt molecules and during ongoing signaling.

APC$^{min/+}$ mice develop approximately 15-30 adenomas[194] throughout the small intestine mostly in the ileum, but colonic tumors are also present[93]. Besides tumor growth in the intestine, the *apc* 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[195]. Because of extensive splenic haematopoiesis APC$^{min/+}$ mice have enlarged spleens[93] and anaemia as tumorogenesis progresses[195]. Furthermore, splenic lymphoid follicles and PP are diminished in these mice[195]. Female APC$^{min/+}$ mice can also develop tumors in the mammary glands[196].

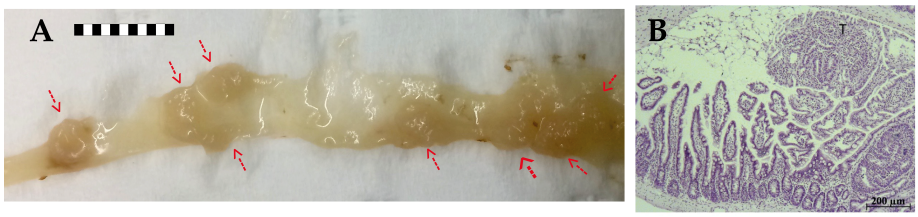

Figure 6. Small intestine of APC$^{min/+}$ mice. A. Intestine from an 18-week-old APC$^{min/+}$ mouse. Arrows indicate tumors. B Hematoxylin staining of small intestine from an APC$^{min/+}$ mouse. T indicates a tumor.

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$^{min/+}$ mice exhibit lower tumor numbers and smaller spleens. Increased intestinal permeability is seen in APC$^{min/+}$ mice and might therefore help the bacteria to accelerate tumor growth[197]. 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[164]. A further characterisation is conducted in this thesis. In the epithelium, CD3^{+} IEL are increased and express a reduced cytotoxicity, which might possibly help promote tumorgenesis[198]. 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[37] and IL-17 instead promotes tumor development[199].

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

$APC^{min/+}$ and DEREG mice

$APC^{min/+}$ mice on a C57BL/6 background with a mutation in the *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 *de*pletion of *reg*ulatory 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 $FoxP3^+$ Tregs starts to appear within a week after depletion[200].

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

In vivo Treg depletion

$FoxP3^+$ Tregs in $APC^{min/+}$/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 $APC^{min/+}$ mice were equally treated.

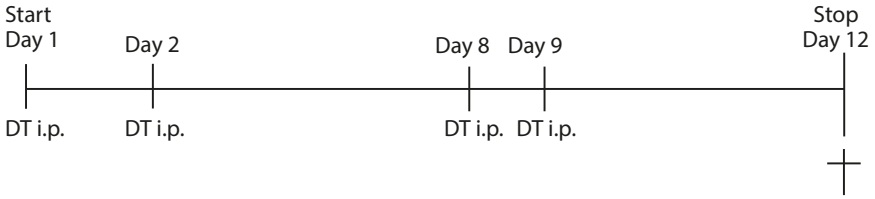

Figure 7. DT administration schedule.

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).

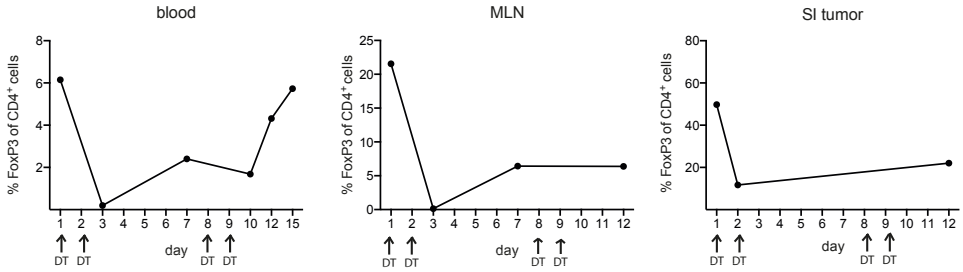

Figure 8. Treg frequencies during DT treatment in blood, MLN and tumor tissue.

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 quantitative 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 flourochrome 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 flurochrome 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 $APC^{min/+}$ 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.

$APC^{min/+}$ mice exhibit similar immunological properties as WT mice

To investigate CRC in an *in vivo* system we used $APC^{min/+}$ mice, a mouse model of spontaneous intestinal cancer. These mice have a mutated *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 $APC^{min/+}$ mice as a model for CRC and to rule out the possibility that the *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 $APC^{min/+}$ 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 *apc* mutation does not influence the peripheral immune system and therefore is $APC^{min/+}$ mice suitable to use as a model for intestinal tumors.

Treg in $APC^{min/+}$ mice supress 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 $APC^{min/+}$ 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 $APC^{min/+}$

mice and WT mice were able to supress 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 supress an immune response to the same extent as Tregs from WT mice.

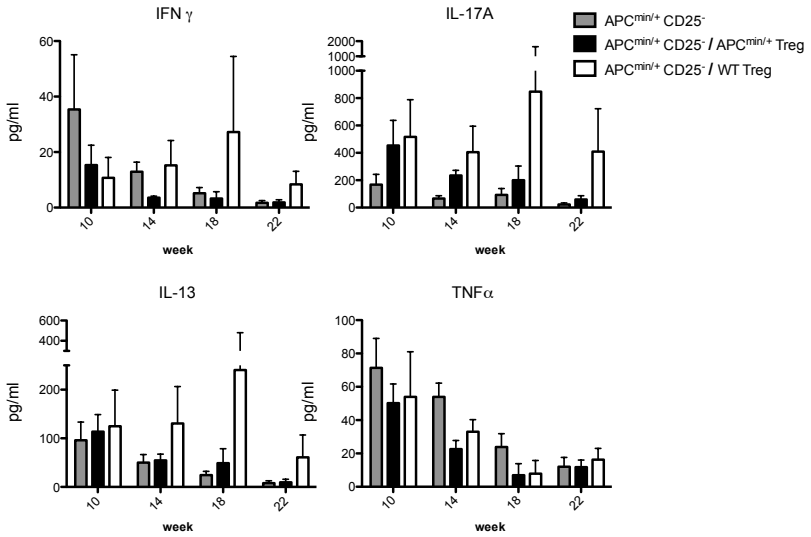

Figure 9. Effect on T cell cytokine secretion by Treg from $APC^{min/+}$ mice and WT controls. Splenic $CD4^+CD25^-$ T cells from WT mice were isolated and cultured with anti-CD3 and antigen-presenting cells alone (grey bars) with $APC^{Min/+}$ (black bars) or WT Treg (white bars) at 10, 14, 18, and 22 weeks for 3 days, n=2-6/time point. Supernatant cytokine concentrations were measured by multiplex assay. Bars represent mean and SEM.

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 $APC^{min/+}$ mice indicates that intestinal tumors are able to affect the immune system in order to avoid an anti-tumor response.

Tumors of the $APC^{min/+}$ 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 $APC^{min/+}$ 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 $APC^{min/+}$ 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.

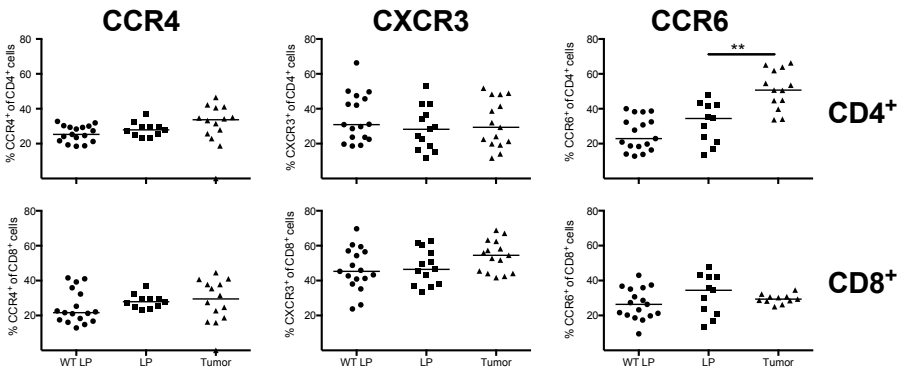

Figure 10. Chemokine receptor expression by conventional T cells in LP and tumors of $APC^{min/+}$ mice.

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[213]. 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 $APC^{min/+}$ mice (Paper I, Figure 5), but Tregs did express higher levels of CCR6 in the tumors of $APC^{min/+}$ 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[108] and is crucial for T cell recruitment to intestinal LP. Intestinal $APC^{min/+}$ 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 then unaffected tissue[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 $CCR9^+$ tumor cells. As recently demonstrated by Chen *et al.*[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 $APC^{min/+}$ 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.

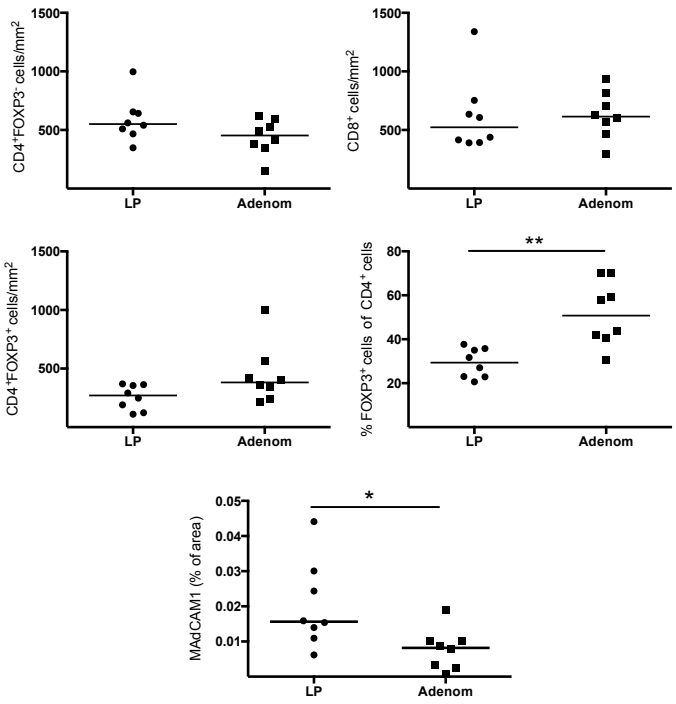

Figure 11. T cells frequencies and MAdCAM-1 expression in human intestinal adenomas and unaffected LP.

Depletion of Treg in APC$^{min/+}$ 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$^{min/+}$ mice to generate APC$^{min/+}$/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$^+$ and CD8$^+$ 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, melonoma and chemically induced sarcomas after Treg depletion and these new data for spontaneous intestinal tumors are in line with these findings[216-218].

By using the crossing of $APC^{min/+}$ 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[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. Therefor 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 $APC^{/min+}$/DEREG mice and control $APC^{/min+}$ 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 *in vitro* system[92]. Furthermore, a specific increase of $CD4^+$ and $CD8^+$ 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 then 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 $APC^{min/+}$ 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 $APC^{/min+}$ 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[215]. An increased Treg accumulation in tumors has also been observed in other mouse and human studies[160,164,215,236].

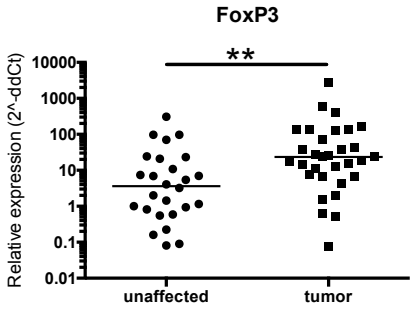

Figure 12. Relative expression of FOXP3+ mRNA in human CRC and unaffected tissue.

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$^{/min+}$ mouse model.

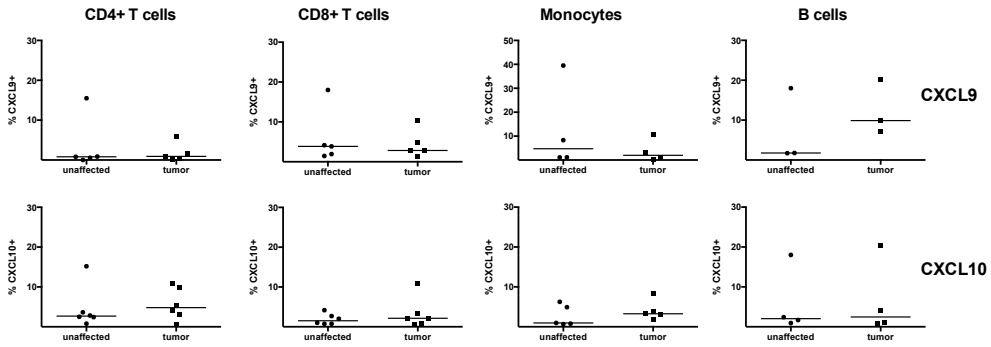

Figure 13. Human cells expressing CXCR3 ligands in colon tumors and unaffected tissues.

An increase of CXCR3 ligands has been observed in several cancer types such as basal cell carcinoma[237], ovarian cancer[238], B-cell lymphoma[239] and melanoma[240], and has been postulated to contribute to tumor growth. Human colon cancer cells express the CXCR3-B isoform[241], which has growth-inhibitory properties and inhibits chemotaxis[105]. Over-production of CXCR3 ligands can lead to a down regulation of CXCR3-B, thus promoting tumor growth and metastasis[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 $CXCR3^+$ T cell frequencies have been observed in CRC compared to the unaffected mucosa[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

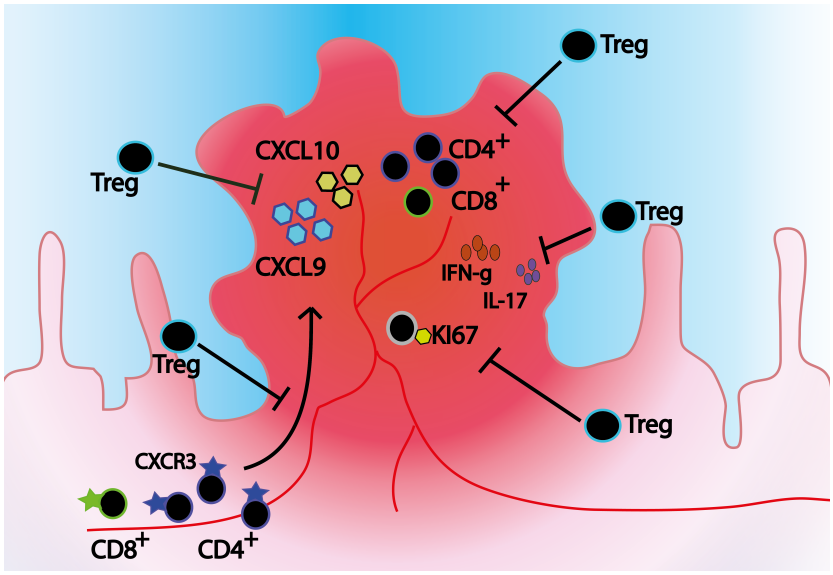

Figure 14. Treg hinders an anti-tumor response. In this thesis Treg suppressive capacity on several different anti-tumor mechanisms have been examined. Treg can both inhibit migration and proliferation of effector T cells. Furthermore, Treg decrease secretion of CXCL9 and 10 thereby hindering migration 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 $APC^{min/+}$ mouse model of intestinal tumors was used for this work since we could show that the *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-γ, $CXCR3^+$ 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[243]. Furthermore, a strong Th1 response has been correlated to improved anti-tumor response and overall patient survival[147,148,155,211,244]. 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[245-249]. 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[216,218,250-253]. In some studies tumor regression have been observed following Treg depletion[216,254]. 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[255,256], OX-40 a co-stimulatory molecule of the TNF receptor family[257] and GITR[258], 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[200], 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 $APC^{min/+}$ 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[227] thereby preventing an immune response. Peng *et al*, have shown that through blocking of PD-1, both CXCL10 and IFN-γ increased at the tumor site without decreasing Treg frequencies[227]. Several clinical trials conducted with PD-1 monoclonal antibodies to treat cancer have nevertheless had a relatively low complete response rate[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 *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 PGE2 synthesis by cyclooxygenase has been shown to reduce tumor growth through increase of intratumoral CXCR3 ligands and increased Th1 responses[261-263]. Also transfections of the IL-12 gene in a model of head and neck cancer up-regulated CXCL9 expression[264]. However, both Treg and cancer cells express CXCR3[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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REFERENCES

1. Medzhitov, R. Toll-like receptors and innate immunity. *Nat Rev* **1,** 135–145 (2001).
2. Luckheeram, R. V., Zhou, R., Verma, A. D. & Xia, B. CD4+T Cells: Differentiation and Functions. *Clin Immunol* **2012,** 1–12 (2012).
3. Wong, P. & Pamer, E. G. CD8 T cell responses to infectious pathogens. *Annu rev immunol* **21,** 29–70 (2003).
4. Landsverk, O. J. B., Bakke, O. & Gregers, T. F. MHC II and the endocytic pathway: regulation by invariant chain. *Scand J Immunol* **70,** 184–193 (2009).
5. Iwasaki, A. & Medzhitov, R. Control of adaptive immunity by the innate immune system. *Nat immunol* **16,** 343–353 (2015).
6. Ivanova, E. A. & Orekhov, A. N. T Helper Lymphocyte Subsets and Plasticity in Autoimmunity and Cancer: An Overview. *Biomed Res Int* **2015,** 1–9 (2015).
7. Pieper, K., Grimbacher, B. & Eibel, H. B-cell biology and development. *J Allergy Clin Immunol* **131,** 959–971 (2013).
8. Kanaly, S. T., Nashleanas, M., Hondowicz, B. & Scott, P. TNF receptor p55 is required for elimination of inflammatory cells following control of intracellular pathogens. *J Immunol* **163,** 3883–3889 (1999).
9. MacLeod, M. K. L., Kappler, J. W. & Marrack, P. Memory CD4 T cells: generation, reactivation and re-assignment. *Immunology* **130,** 10–15 (2010).
10. Mowat, A. M. & Agace, W. W. Regional specialization within the intestinal immune system. *Nat Rev Immunol* **14,** 667–685 (2014).
11. Cheroutre, H. & Kronenberg, M. Mucosal T lymphocytes—peacekeepers and warriors. *Springer Semin Immun* **27,** 147–165 (2005).
12. Zhang, K., Hornef, M. W. & Dupont, A. The intestinal epithelium as guardian of gut barrier integrity. *Cell. Microbiol.* **17,** 1561–1569 (2015).
13. Mowat, A. M. & Agace, W. W. Regional specialization within the intestinal immune system. *Nat Rev Immunol* **14,** 667–685 (2014).
14. Habtezion, A., Nguyen, L. P., Hadeiba, H. & Butcher, E. C. Leukocyte Trafficking to the Small Intestine and Colon. *Gastroenterology* **150,** 340–354 (2016).
15. Microbiota-Mediated Inflammation and Antimicrobial Defense in the Intestine.
16. Fodde, R., Smits, R. & Clevers, H. APC, signal transduction and genetic instability in colorectal cancer. *Nat rev Cancer* **1,** 55–67 (2001).
17. Watson, A. J. M. Claudins and barrier dysfunction in intestinal inflammation: cause or consequence? *Gut* **64,** 1501–1502 (2015).
18. Pabst, O. & Mowat, A. M. Oral tolerance to food protein. *Mucosal Immunol* **5,** 232–239 (2012).
19. Hart, A. L. *et al.* Homing of immune cells: role in homeostasis and intestinal inflammation. *Inflamm. Bowel Dis.* **16,** 1969–1977 (2010).
20. Jung, C., Hugot, J.-P. & Barreau, F. Peyer's Patches: The Immune Sensors of the Intestine. *Int J Inflam* **2010,** 823710 (2010).
21. Ohno, H. Intestinal M cells. *J. Biochem.* **159,** 151–160 (2016).
22. Andrian, von, U. H. & Mempel, T. R. Homing and cellular traffic in lymph nodes. *Nat Rev Immunol* **3,** 867–878 (2003).
23. Macpherson, A. J. & Smith, K. Mesenteric lymph nodes at the center of immune anatomy. *J Exp Med* **203,** 497–500 (2006).
24. Brucklacher-Waldert, V., Carr, E. J., Linterman, M. A. & Veldhoen, M. Cellular Plasticity of CD4+ T Cells in the Intestine. *Front. Immunol.* **5,** 696 (2014).
25. Koboziev, I., Karlsson, F. & Grisham, M. B. Gut-associated lymphoid tissue, T cell trafficking, and chronic intestinal inflammation. *Ann. N.Y. Acad. Sci* **1207,** E86–E93 (2010).
26. Preza, G. C., Yang, O. O., Elliott, J., Anton, P. A. & Ochoa, M. T. T Lymphocyte Density and Distribution in Human Colorectal Mucosa, and Inefficiency of Current Cell Isolation Protocols. *PloS one* **10,** e0122723 (2015).
27. Cheroutre, H., Lambolez, F. & Mucida, D. The light and dark sides of intestinal intraepithelial lymphocytes. *Nature Reviews Immunology* **11,** 445–456 (2011).
28. Raphael, I., Nalawade, S., Eagar, T. N. & Forsthuber, T. G. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine* **74,** 5–17 (2015).
29. Lin, J. & Weiss, A. T cell receptor signalling. *J Cell Sci* **114,** 243–244 (2001).
30. Smith-Garvin, J. E., Koretzky, G. A. & Jordan, M. S. T cell activation. *Annu rev immunol* **27,** 591–

619 (2009).
31. Corthay, A. A three-cell model for activation of naïve T helper cells. *Scand J Immunol* **64,** 93–96 (2006).
32. Corthay, A. How do regulatory T cells work? *Scand J Immunol* **70,** 326–336 (2009).
33. Zhang, N. & Bevan, M. J. CD8+ T Cells: Foot Soldiers of the Immune System. *Cell* **35,** 161–168 (2011).
34. Catalfamo, M. & Henkart, P. A. Perforin and the granule exocytosis cytotoxicity pathway. *Curr Opin immunol* **15,** 522–527 (2003).
35. Szabo, S. J., Sullivan, B. M., Peng, S. L. & Glimcher, L. H. Molecular mechanisms regulating Th1 immune responses. *Annu rev immunol* **21,** 713–758 (2003).
36. Hsieh, C. S. *et al.* Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* **260,** 547–549 (1993).
37. Wang, L., Wang, Y., Song, Z., Chu, J. & Qu, X. Deficiency of Interferon-Gamma or Its Receptor Promotes Colorectal Cancer Development. *J. Interferon Cytokine Res.* **35,** 273–280 (2015).
38. Bouker, K. B. *et al.* Interferon regulatory factor-1 (IRF-1) exhibits tumor suppressor activities in breast cancer associated with caspase activation and induction of apoptosis. *Carcinogenesis* **26,** 1527–1535 (2005).
39. Schroder, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75,** 163–189 (2004).
40. Schuhmann, D. *et al.* Interfering with interferon-γ signalling in intestinal epithelial cells: selective inhibition of apoptosis-maintained secretion of anti-inflammatory interleukin-18 binding protein. *Clin. Exp. Immunol.* **163,** 65–76 (2011).
41. Desmedt, M., Rottiers, P., Dooms, H., Fiers, W. & Grooten, J. Macrophages induce cellular immunity by activating Th1 cell responses and suppressing Th2 cell responses. *J Immunol* **160,** 5300–5308 (1998).
42. Harrington, L. E. *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat immunol* **6,** 1123–1132 (2005).
43. Mowen, K. A. & Glimcher, L. H. Signaling pathways in Th2 development. *Immunol rev* **202,** 203–222 (2004).
44. Licona-Limón, P., Kim, L. K., Palm, N. W. & Flavell, R. A. T_H2, allergy and group 2 innate lymphoid cells. *Nat immunol* **14,** 536–542 (2013).
45. Huber, S. *et al.* Th17 cells express interleukin-10 receptor and are controlled by Foxp3. *Immunity* **34,** 554–565 (2011).
46. Bettelli, E., Oukka, M. & Kuchroo, V. K. T(H)-17 cells in the circle of immunity and autoimmunity. *Nature immunology* **8,** 345–350 (2007).
47. Kimura, A. & Kishimoto, T. IL-6: regulator of Treg/Th17 balance. *Eur J immunol* **40,** 1830–1835 (2010).
48. Symons, A., Budelsky, A. L. & Towne, J. E. Are Th17 cells in the gut pathogenic or protective? *Mucosal Immunol* **5,** 4–6 (2012).
49. Benchetrit, F. *et al.* Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. *Blood* **99,** 2114–2121 (2002).
50. Xu, L., Kitani, A., Fuss, I. & Strober, W. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol* **178,** 6725–6729 (2007).
51. Esplugues, E. *et al.* Control of TH17 cells occurs in the small intestine. *Nature* **475,** 514–518 (2011).
52. Kryczek, I. *et al.* IL-17+ regulatory T cells in the microenvironments of chronic inflammation and cancer. *J Immunol* **186,** 4388–4395 (2011).
53. Hindley, J. P. *et al.* Analysis of the T-Cell Receptor Repertoires of Tumor-Infiltrating Conventional and Regulatory T Cells Reveals No Evidence for Conversion in Carcinogen-Induced Tumors. *Cancer Res.* **71,** 736–746 (2011).
54. Ohkura, N. & Sakaguchi, S. Regulatory T cells: roles of T cell receptor for their development and function. *Semin Immunopathol* **32,** 95–106 (2010).
55. Hsu, P. *et al.* IL-10 Potentiates Differentiation of Human Induced Regulatory T Cells via STAT3 and Foxo1. *J Immunol* **195,** 3665–3674 (2015).
56. Raghavan, S. & Quiding-Järbrink, M. Regulatory T cells in gastrointestinal tumors. *Expert Rev Gastroenterol Hepatol* **5,** 489–501 (2011).
57. Wing, K. & Sakaguchi, S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat immunol* **11,** 7–13 (2009).
58. Josefowicz, S. Z., Lu, L.-F. & Rudensky, A. Y. Regulatory T Cells: Mechanisms of Differentiation

and Function. *Annu rev immunol* **30,** 531–564 (2012).
59. Otsubo, K. *et al.* Identification of FOXP3-negative regulatory T-like (CD4(+)CD25(+)CD127(low)) cells in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. *Clin Immunol* **141,** 111–120 (2011).
60. Zhuo, C. *et al.* FOXP3+ Tregs: heterogeneous phenotypes and conflicting impacts on survival outcomes in patients with colorectal cancer. *Immunol rev* **61,** 338–347 (2015).
61. Baru, A. M. *et al.* Optimal isolation of functional Foxp3+ induced regulatory T cells using DEREG mice. *PloS one* **7,** e44760 (2012).
62. Lahl, K. *et al.* Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J Exp Med* **204,** 57–63 (2007).
63. Barnes, M. J. *et al.* CTLA-4 promotes Foxp3 induction and regulatory T cell accumulation in the intestinal lamina propria. *Mucosal Immunol* **6,** 324–334 (2012).
64. Ronchetti, S. *et al.* GITR, a member of the TNF receptor superfamily, is costimulatory to mouse T lymphocyte subpopulations. *Eur J immunol* **34,** 613–622 (2004).
65. Whiteside, T. L. What are regulatory T cells (Treg) regulating in cancer and why? *Semin Cancer Biol* **22,** 327–334 (2012).
66. Borsellino, G. *et al.* Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* **110,** 1225–1232 (2007).
67. Abbas, A. K. *et al.* Regulatory T cells: recommendations to simplify the nomenclature. *Nat immunol* **14,** 307–308 (2013).
68. Curiel, T. J. Regulatory T-cell development: is Foxp3 the decider? *Nat Med* **13,** 250–253 (2007).
69. Yadav, M., Stephan, S. & Bluestone, J. A. Peripherally induced tregs - role in immune homeostasis and autoimmunity. *Front. Immunol.* **4,** 232 (2013).
70. Gottschalk, R. A., Corse, E. & Allison, J. P. Expression of Helios in Peripherally Induced Foxp3+ Regulatory T Cells. *J immun* **188,** (2012).
71. Ding, Y., Xu, J. & Bromberg, J. S. Regulatory T cell migration during an immune response. *Trends Immunol* **33,** 174–180 (2012).
72. Olson, B. M., Sullivan, J. A. & Burlingham, W. J. Interleukin 35: a key mediator of suppression and the propagation of infectious tolerance. *Front. Immunol.* **4,** 315 (2013).
73. van Herk, E. H. & Velde, Te, A. A. Treg subsets in inflammatory bowel disease and colorectal carcinoma: Characteristics, role, and therapeutic targets. *J Gastroenterol Hepatol* **31,** 1393–1404 (2016).
74. Gorelik, L. & Flavell, R. A. Transforming growth factor-β in t-cell biology. *Nat Rev Immunol* **2,** 46–53 (2002).
75. Collison, L. W. *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* **450,** 566–569 (2007).
76. Long, J. *et al.* IL-35 over-expression increases apoptosis sensitivity and suppresses cell growth in human cancer cells. *Biochem Biophys Res Commun* **430,** 364–369 (2013).
77. Liu, J.-Q. *et al.* Increased Th17 and regulatory T cell responses in EBV-induced gene 3-deficient mice lead to marginally enhanced development of autoimmune encephalomyelitis. *J Immunol* **188,** 3099–3106 (2012).
78. Grant, C. R., Liberal, R., Mieli-Vergani, G., Vergani, D. & Longhi, M. S. Regulatory T-cells in autoimmune diseases: challenges, controversies and--yet--unanswered questions. *Autoimmun Rev* **14,** 105–116 (2015).
79. Vanasek, T. L., Khoruts, A., Zell, T. & Mueller, D. L. Antagonistic roles for CTLA-4 and the mammalian target of rapamycin in the regulation of clonal anergy: enhanced cell cycle progression promotes recall antigen responsiveness. *J Immunol* **167,** 5636–5644 (2001).
80. Francisco, L. M., Sage, P. T. & Sharpe, A. H. The PD-1 pathway in tolerance and autoimmunity. *Immunol rev* **236,** 219–242 (2010).
81. Park, H. J. *et al.* PD-1 upregulated on regulatory T cells during chronic virus infection enhances the suppression of CD8+ T cell immune response via the interaction with PD-L1 expressed on CD8+ T cells. *J Immunol* **194,** 5801–5811 (2015).
82. Riley, J. L. PD-1 signaling in primary T cells. *Immunol rev* **229,** 114–125 (2009).
83. Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M. J. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat immunol* **8,** 1353–1362 (2007).
84. Enarsson, K., Brisslert, M., Backert, S. & Quiding-Järbrink, M. Helicobacter pylori induces transendothelial migration of activated memory T cells. *Infect Immun* **73,** 761–769 (2005).
85. Sundström, P. *et al.* Regulatory T Cells from Colon Cancer Patients Inhibit Effector T-cell Migration through an Adenosine-Dependent Mechanism. *Cancer Immunol Res* **4,** 183–193 (2016).

86. Ernst, P. B., Garrison, J. C. & Thompson, L. F. Much Ado about Adenosine: Adenosine Synthesis and Function in Regulatory T Cell Biology. *J Immunol* **185,** 1993–1998 (2010).
87. Harty, J. T., Tvinnereim, A. R. & White, D. W. CD8+ T cell effector mechanisms in resistance to infection. *Annu rev immunol* **18,** 275–308 (2000).
88. Lieberman, J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* **3,** 361–370 (2003).
89. Seder, R. A. & Ahmed, R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat immunol* **4,** 835–842 (2003).
90. Johnsson-Leger, C., Aurrand-Lions, M. & Imhof, B. A. The parting of the endothelium: miracle, or simply a junctional affair? *J Cell Sci* **113,** 921–933 (2000).
91. Salmi, M. & Jalkanen, S. Lymphocyte homing to the gut: attraction, adhesion, and commitment. *Immunol rev* **206,** 100–113 (2005).
92. Enarsson, K., Lundin, B. S., Johnsson, E., Brezicka, T. & Quiding-Järbrink, M. CD4+CD25high regulatory T cells reduce T cell transendothelial migration in cancer patients. *Eur J immunol* **37,** 282–291 (2007).
93. Nandi, B. *et al.* CCR6, the Sole Receptor for the Chemokine CCL20, Promotes Spontaneous Intestinal Tumorigenesis. *PloS one* **9,** e97566 (2014).
94. Verbeke, H., Struyf, S., Laureys, G. & Van Damme, J. The expression and role of CXC chemokines in colorectal cancer. *Cytokine Growth Factor Rev.* **22,** 345–358 (2011).
95. Luster, A. D. Chemokines--chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* **338,** 436–445 (1998).
96. Ondondo, B. *et al.* A distinct chemokine axis does not account for enrichment of Foxp3 + CD4 +T cells in carcinogen-induced fibrosarcomas. *Immunology* **145,** 94–104 (2015).
97. Xie, J. H. *et al.* Antibody-mediated blockade of the CXCR3 chemokine receptor results in diminished recruitment of T helper 1 cells into sites of inflammation. *J Leukoc Biol* **73,** 771–780 (2003).
98. Koch, M. A. *et al.* The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat immunol* **10,** 595–602 (2009).
99. Thomas, S. Y. *et al.* CD1d-restricted NKT cells express a chemokine receptor profile indicative of Th1-type inflammatory homing cells. *J Immunol* **171,** 2571–2580 (2003).
100. Cella, M. *et al.* Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med* **5,** 919–923 (1999).
101. Nanki, T. *et al.* Chemokine receptor expression and functional effects of chemokines on B cells: implication in the pathogenesis of rheumatoid arthritis. *Arthritis Res. Ther.* **11,** R149 (2009).
102. Groom, J. R. & Luster, A. D. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* **89,** 207–215 (2011).
103. Sierro, F. *et al.* Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *PNAS* **104,** 14759–14764 (2007).
104. Cerny, C. *et al.* The Role of CXCR3/Ligand Axis in Cancer. *Int Trend Immun* **3,** 46–52 (2015).
105. Liu, M., Guo, S. & Stiles, J. K. The emerging role of CXCL10 in cancer (Review). *Oncol Lett* **2,** 583–589 (2011).
106. Billottet, C., Quemener, C. & Bikfalvi, A. CXCR3, a double-edged sword in tumor progression and angiogenesis. *Biochim Biophys Acta* **1836,** 287–295 (2013).
107. Kleinewietfeld, M. *et al.* CCR6 expression defines regulatory effector/memory-like cells within the CD25(+)CD4+ T-cell subset. *Blood* **105,** 2877–2886 (2005).
108. Wurbel, M.-A., McIntire, M. G., Dwyer, P. & Fiebiger, E. CCL25/CCR9 Interactions Regulate Large Intestinal Inflammation in a Murine Model of Acute Colitis. *PloS one* **6,** e16442 (2011).
109. Chen, H. J. *et al.* Chemokine 25–induced signaling suppresses colon cancer invasion and metastasis. *J. Clin. Invest.* **122,** 3184–3196 (2012).
110. McClellan, J. L. *et al.* Intestinal inflammatory cytokine response in relation to tumorigenesis in the ApcMin/+ mouse. *Cytokine* **57,** 113–119 (2012).
111. Al-haidari, A. A., Syk, I., Jirström, K. & Thorlacius, H. CCR4 mediates CCL17 (TARC)-induced migration of human colon cancer cells via RhoA/Rho-kinase signaling. *Int J Colorectal Dis* (2013).
112. Janakiram, N. B. *et al.* Adoptive transfer of regulatory T cells promotes intestinal tumorigenesis and is associated with decreased NK cells and IL-22 binding protein. *Mol. Cancer* **54,** 986–998 (2015).
113. Ghadjar, P. Chemokine Receptor CCR6 Expression Level and Liver Metastases in Colorectal Cancer. *J Clin Oncol.* **24,** 1910–1916 (2006).
114. Zipin-Roitman, A. *et al.* CXCL10 Promotes Invasion-Related Properties in Human Colorectal Carcinoma Cells. *Cancer Res.* **67,** 3396–3405 (2007).

115. Terzić, J., Grivennikov, S., Karin, E. & Karin, M. Inflammation and colon cancer. *Gastroenterology* **138,** 2101–2114 (2010).
116. Cui, G., Yuan, A., Goll, R. & Florholmen, J. IL-17A in the tumor microenvironment of the human colorectal adenoma–carcinoma sequence . *Scand J Gastroenterol* **47,** 1304–1312 (2012).
117. Wang, J. *et al.* The changes of Th17 cells and the relatedcytokines in the progression of humancolorectal cancers. *BMC Cancer* **12,** 418 (2012).
118. Vogelstein, B. *et al.* Cancer genome landscapes. *Science* **339,** 1546–1558 (2013).
119. Keerthivasan, S. *et al.* Wnt/β-catenin signaling in T-cells drives epigenetic imprinting of pro-inflammatory properties and promotes colitis and colon cancer. *Sci Transl Med* **6,** 225ra28 (2014).
120. Gaspar, C. *et al.* Cross-Species Comparison of Human and Mouse Intestinal Polyps Reveals Conserved Mechanisms in Adenomatous Polyposis Coli (APC)-Driven Tumorigenesis. *Am J Pathol* **172,** 1363–1380 (2008).
121. Grivennikov, S. I. *et al.* Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* **491,** 254–258 (2012).
122. Davies, R. J., Miller, R. & Coleman, N. Colorectal cancer screening: prospects for molecular stool analysis. *Nature Reviews Cancer* **5,** 199–209 (2005).
123. Davis, C. *et al.* Hematopoietic derived cell infiltration of the intestinal tumor microenvironment in Apc Min/+ mice. *Microsc Microanal.* **17,** 528–539 (2011).
124. Weitz, J. *et al.* Colorectal cancer. *Lancet* **365,** 153–165 (2005).
125. Oelkrug, C. & Ramage, J. M. Enhancement of T cell recruitment and infiltration into tumours. *Clin. Exp. Immunol.* **178,** 1–8 (2014).
126. Swann, J. B. & Smyth, M. J. Immune surveillance of tumors. *J. Clin. Invest.* **117,** 1137–1146 (2007).
127. Dunn, G. P., Old, L. J. & Schreiber, R. D. The three Es of cancer immunoediting. *Annu rev immunol* **22,** 329–360 (2004).
128. Koebel, C. M. *et al.* Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* **450,** 903–907 (2007).
129. Dougan, M. & Dranoff, G. The immune response to tumors. *Curr prot immunol* **20,** 20.11.1–4 (2009).
130. Cui, G. *et al.* Reduced expression of microenvironmental Th1 cytokines accompanies adenomas–carcinomas sequence of colorectum. *Cancer Immunol Immunother* **56,** 985–995 (2007).
131. Cui, G., Yuan, A., Vonen, B. & Florholmen, J. Progressive cellular response in the lamina propria of the colorectal adenoma-carcinoma sequence. *Histopathology* **54,** 550–560 (2009).
132. Fuchs, E. J. & Matzinger, P. Is cancer dangerous to the immune system? *Semin Immunol* **8,** 271–280 (1996).
133. Kurts, C. *et al.* Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med* **184,** 923–930 (1996).
134. Smyth, M. J., Godfrey, D. I. & Trapani, J. A. A fresh look at tumor immunosurveillance and immunotherapy. *Nat immunol* **2,** 293–299 (2001).
135. Curiel, T. J. Tregs and rethinking cancer immunotherapy. *J. Clin. Invest.* **117,** 1167–1174 (2007).
136. Lawrence, M. S. *et al.* Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* **505,** 495–501 (2014).
137. Garrido, F. *et al.* Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol. Today* **18,** 89–95 (1997).
138. Elgert, K. D., Alleva, D. G. & Mullins, D. W. Tumor-induced immune dysfunction: the macrophage connection. *J Leukoc Biol* **64,** 275–290 (1998).
139. Walker, P. R., Saas, P. & Dietrich, P. Y. Tumor expression of Fas ligand (CD95L) and the consequences. *Curr Opin immunol* **10,** 564–572 (1998).
140. Topalian, S. L. *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* **366,** 2443–2454 (2012).
141. Hino, R. *et al.* Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer* **116,** 1757–1766 (2010).
142. Karim, R. *et al.* Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. *Clin. Cancer Res.* **15,** 6341–6347 (2009).
143. Nomi, T. *et al.* Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clin. Cancer Res.* **13,** 2151–2157 (2007).
144. Thompson, R. H. *et al.* Costimulatory molecule B7-H1 in primary and metastatic clear cell renal cell carcinoma. *Cancer* **104,** 2084–2091 (2005).
145. Hua, W. *et al.* Accumulation of FoxP3+ T regulatory cells in the tumor microenvironment of human colorectal adenomas. *Pathology* **212,** 106–112 (2015).

146. Frey, D. M. *et al.* High frequency of tumor-infiltrating FOXP3 +regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. *Int. J. Cancer* **126,** 2635–2643 (2010).
147. Menon, A. G. *et al.* Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. *Lab Invest* **84,** 493–501 (2004).
148. Tosolini, M. *et al.* Clinical Impact of Different Classes of Infiltrating T Cytotoxic and Helper Cells (Th1, Th2, Treg, Th17) in Patients with Colorectal Cancer. *Cancer Res.* **71,** 1263–1271 (2011).
149. Yoon, H. H. *et al.* Prognostic impact of FoxP3+ regulatory T cells in relation to CD8+ T lymphocyte density in human colon carcinomas. *PloS one* **7,** e42274 (2012).
150. Thompson, E. D., Enriquez, H. L., Fu, Y.-X. & Engelhard, V. H. Tumor masses support naive T cell infiltration, activation, and differentiation into effectors. *J Exp Med* **207,** 1791–1804 (2010).
151. Hung, K. *et al.* The central role of CD4(+) T cells in the antitumor immune response. *J Exp Med* **188,** 2357–2368 (1998).
152. Kennedy, R. & Celis, E. Multiple roles for CD4+ T cells in anti-tumor immune responses. *Immunol rev* **222,** 129–144 (2008).
153. Thomas, W. D. & Hersey, P. TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. *J Immunol* **161,** 2195–2200 (1998).
154. Schattner, E. J. *et al.* CD4+ T-cell induction of Fas-mediated apoptosis in Burkitt's lymphoma B cells. *Blood* **88,** 1375–1382 (1996).
155. Haabeth, O. A. W., Bogen, B. & Corthay, A. A model for cancer-suppressive inflammation. *Oncoimmunology* **1,** 1146–1155 (2012).
156. Shanker, A. *et al.* CD8 T cell help for innate antitumor immunity. *J Immunol* **179,** 6651–6662 (2007).
157. Propper, D. J. *et al.* Low-dose IFN-gamma induces tumor MHC expression in metastatic malignant melanoma. *Clin. Cancer Res.* **9,** 84–92 (2003).
158. Lin, Y.-C. *et al.* Activated but not resting regulatory T cells accumulated in tumor microenvironment and correlated with tumor progression in patients with colorectal cancer. *Int. J. Cancer* **132,** 1341–1350 (2012).
159. Salama, P. *et al.* Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol.* **27,** 186–192 (2009).
160. Michel, S. *et al.* High density of FOXP3-positive T cells infiltrating colorectal cancers with microsatellite instability. *Br J Cancer* **99,** 1867–1873 (2008).
161. Correale, P. *et al.* Regulatory (FoxP3+) T-cell Tumor Infiltration Is a Favorable Prognostic Factor in Advanced Colon Cancer Patients Undergoing Chemo or Chemoimmunotherapy. *J Immunother* **33,** 435–441 (2010).
162. Lee, S. H. *et al.* ERK activation drives intestinal tumorigenesis in Apc(min/+) mice. *Nat Med* **16,** 665–670 (2010).
163. Yaqub, S. *et al.* Regulatory T cells in colorectal cancer patients suppress anti-tumor immune activity in a COX-2 dependent manner. *Cancer Immunol Immunother* **57,** 813–821 (2007).
164. Gounaris, E. *et al.* T-Regulatory Cells Shift from a Protective Anti-Inflammatory to a Cancer-Promoting Proinflammatory Phenotype in Polyposis. *Cancer Res.* **69,** 5490–5497 (2009).
165. Erdman, S. E. *et al.* CD4+CD25+ Regulatory Lymphocytes Require Interleukin 10 to Interrupt Colon Carcinogenesis in Mice. *Cancer Res.* **63,** (2003).
166. Blatner, N. R. *et al.* Expression of RORγt Marks a Pathogenic Regulatory T Cell Subset in Human Colon Cancer. *Sci Transl Med* **4,** 164ra159–164ra159 (2012).
167. Saito, T. *et al.* Two FOXP3(+)CD4(+) T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat Med* **22,** 679–684 (2016).
168. Ben-Baruch, A. Organ selectivity in metastasis: regulation by chemokines and their receptors. *Clin. Exp. Metastasis* **25,** 345–356 (2008).
169. Baier, P. K., Eggstein, S. & Wolff-Vorbeck, G. Chemokines in human colorectal carcinoma. *Anticancer Res.* **25,** 3581–3584 (2005).
170. Liu, C. *et al.* Chemokine receptor CXCR3 promotes growth of glioma. *Carcinogenesis* **32,** 129–137 (2011).
171. Pradelli, E. *et al.* Antagonism of chemokine receptor CXCR3 inhibits osteosarcoma metastasis to lungs. *Int. J. Cancer* **125,** 2586–2594 (2009).
172. Ma, X. *et al.* CXCR3 expression is associated with poor survival in breast cancer and promotes metastasis in a murine model. *Mol. Cancer Ther.* **8,** 490–498 (2009).
173. Itatani, Y. *et al.* The Role of Chemokines in Promoting Colorectal Cancer Invasion/Metastasis. *Int J Mol Sci* **17,** (2016).

174. Groom, J. R. *et al.* CXCR3 chemokine receptor-ligand interactions in the lymph node optimize CD4+ T helper 1 cell differentiation. *Immunity* **37,** 1091–1103 (2012).
175. Mulligan, A. M. *et al.* Tumoral lymphocytic infiltration and expression of the chemokine CXCL10 in breast cancers from the Ontario Familial Breast Cancer Registry. *Clin. Cancer Res.* **19,** 336–346 (2013).
176. Wu, Q., Dhir, R. & Wells, A. Altered CXCR3 isoform expression regulates prostate cancer cell migration and invasion. *Mol. Cancer* **11,** 3 (2012).
177. Balan, M. & Pal, S. A novel CXCR3-B chemokine receptor-induced growth-inhibitory signal in cancer cells is mediated through the regulation of Bach-1 protein and Nrf2 protein nuclear translocation. *J. Biol. Chem.* **289,** 3126–3137 (2014).
178. Harlin, H. *et al.* Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. *Cancer Res.* **69,** 3077–3085 (2009).
179. Hong, M. *et al.* Chemotherapy induces intratumoral expression of chemokines in cutaneous melanoma, favoring T-cell infiltration and tumor control. *Cancer Res.* **71,** 6997–7009 (2011).
180. Yang, X., Chu, Y., Wang, Y., Zhang, R. & Xiong, S. Targeted in vivo expression of IFN-gamma-inducible protein 10 induces specific antitumor activity. *J Leukoc Biol* **80,** 1434–1444 (2006).
181. Wang, X., Lu, X.-L., Zhao, H.-Y., Zhang, F.-C. & Jiang, X.-B. A novel recombinant protein of IP10-EGFRvIIIscFv and CD8(+) cytotoxic T lymphocytes synergistically inhibits the growth of implanted glioma in mice. *Cancer Immunol Immunother* **62,** 1261–1272 (2013).
182. Arenberg, D. A. *et al.* The role of CXC chemokines in the regulation of angiogenesis in non-small cell lung cancer. *J Leukoc Biol* **62,** 554–562 (1997).
183. Sgadari, C. *et al.* Interferon-inducible protein-10 identified as a mediator of tumor necrosis in vivo. *PNAS* **93,** 13791–13796 (1996).
184. Sgadari, C. *et al.* Mig, the monokine induced by interferon-gamma, promotes tumor necrosis in vivo. *Blood* **89,** 2635–2643 (1997).
185. Wang, L.-L. *et al.* CXC-chemokine-ligand-10 gene therapy efficiently inhibits the growth of cervical carcinoma on the basis of its anti-angiogenic and antiviral activity. *Biotechnol. Appl. Biochem.* **53,** 209–216 (2009).
186. Campanella, G. S. V., Colvin, R. A. & Luster, A. D. CXCL10 can inhibit endothelial cell proliferation independently of CXCR3. *PloS one* **5,** e12700 (2010).
187. Ishida, T. & Ueda, R. Immunopathogenesis of lymphoma: focus on CCR4. *Cancer Sci* **102,** 44–50 (2011).
188. Curiel, T. J. *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* **10,** 942–949 (2004).
189. Tu, Z. *et al.* CCR9 in cancer: oncogenic role and therapeutic targeting. *J Hematol Oncol* **9,** 10 (2016).
190. Nusse, R. Wnt signaling in disease and in development. *Cell Res.* **15,** 28–32 (2005).
191. Dennis, K. L. *et al.* Adenomatous Polyps Are Driven by Microbe-Instigated Focal Inflammation and Are Controlled by IL-10-Producing T Cells. *Cancer Res.* **73,** 5905–5913 (2013).
192. Moser, A. R., Pitot, H. C. & Dove, W. F. A Dominant Mutation That Predisposes to Multiple Intestinal Neoplasia in the Mouse. *Science* **247,** 322–324 (1990).
193. Zeineldin, M. *et al.* A knock-in mouse model reveals roles for nuclear Apc in cell proliferation, Wnt signal inhibition and tumor suppression. *Oncogene* **31,** 2423–2437 (2012).
194. Akeus, P. *et al.* Treg-cell depletion promotes chemokine production and accumulation of CXCR3(+) conventional T cells in intestinal tumors. *Eur J immunol* **45,** 1654–1666 (2015).
195. You, S. *et al.* Developmental abnormalities in multiple proliferative tissues of ApcMin/+ mice. *Int. J. Exp. Pathol.* **87,** 227–236 (2006).
196. Moser, A. R. *et al.* ApcMin, a mutation in the murine Apc gene, predisposes to mammary carcinomas and focal alveolar hyperplasias. *Proc. Natl. Acad. Sci* (1993).
197. Li, Y. *et al.* Gut microbiota accelerate tumor growth via c-jun and STAT3 phosphorylation in APCMin/+ mice. *Carcinogenesis* **33,** 1231–1238 (2012).
198. Marsh, L., Coletta, P. L., Hull, M. A., Selby, P. J. & Carding, S. R. Altered intestinal epithelium-associated lymphocyte repertoires and function in ApcMin/+ mice. *Int J oncol* **40,** 243–250 (2012).
199. Chae, W. J. *et al.* Ablation of IL-17A abrogates progression of spontaneous intestinal tumorigenesis. *PNAS* **107,** 5540–5544 (2010).
200. Lahl, K. & Sparwasser, T. In vivo depletion of FoxP3+ Tregs using the DEREG Mouse Model. *In Vitro* **707,** 157–172 (2011).
201. Manicassamy, S. *et al.* Activation of beta-Catenin in Dendritic Cells Regulates Immunity Versus Tolerance in the Intestine. *Science* **329,** 849–853 (2010).
202. Staal, F. J. T. & Sen, J. M. The canonical Wnt signaling pathway plays an important role in

lymphopoiesis and hematopoiesis. *Eur J immunol* **38,** 1788–1794 (2008).
203. Coletta, P. L. Lymphodepletion in the ApcMin/+ mouse model of intestinal tumorigenesis. *Blood* **103,** 1050–1058 (2004).
204. Chae, W.-J. & Bothwell, A. L. M. IL-17F deficiency inhibits small intestinal tumorigenesis in ApcMin/+ mice. *Biochem Biophys Res Commun* **414,** 31–36 (2011).
205. Martin, F., Apetoh, L. & Ghiringhelli, F. Controversies on the role of Th17 in cancer: a TGF-β-dependent immunosuppressive activity? *Trends Mol Med* **18,** 742–749 (2012).
206. Erdman, S. E. *et al.* CD4+CD25+ regulatory lymphocytes induce regression of intestinal tumors in ApcMin/+ mice. *Cancer Res.* **65,** 3998–4004 (2005).
207. Hadrup, S., Donia, M. & Thor Straten, P. Effector CD4 and CD8 T cells and their role in the tumor microenvironment. *Cancer Microenviron* **6,** 123–133 (2013).
208. Gooden, M. J. M., de Bock, G. H., Leffers, N., Daemen, T. & Nijman, H. W. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. *Br J Cancer* **105,** 93–103 (2011).
209. Sinicrope, F. A. *et al.* Intraepithelial Effector (CD3+)/Regulatory (FoxP3+) T-Cell Ratio Predicts a Clinical Outcome of Human Colon Carcinoma. *Gastroenterology* **137,** 1270–1279 (2009).
210. Shevach, E. M. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* **30,** 636–645 (2009).
211. Evans, C., Dalgleish, A. G. & Kumar, D. Immune suppression and colorectal cancer. *Aliment Pharmacol Ther* **24,** 1163–1177 (2006).
212. Ishida, T. & Ueda, R. CCR4 as a novel molecular target for immunotheraphy of cancer. *Cancer Sci* **97,** 1139–1146 (2006).
213. Liu, J. *et al.* Tumor-Associated Macrophages Recruit CCR6+ Regulatory T Cells and Promote the Development of Colorectal Cancer via Enhancing CCL20 Production in Mice. *PloS one* **6,** e19495 (2011).
214. Dimberg, J., Hugander, A. & Wågsäter, D. Protein expression of the chemokine, CCL28, in human colorectal cancer. *Int J oncol* **28,** 315–319 (2006).
215. Svensson, H. *et al.* Accumulation of CCR4+ CTLA-4hi FOXP3+CD25hi Regulatory T Cells in Colon Adenocarcinomas Correlate to Reduced Activation of Conventional T Cells. *PloS one* **7,** e30695 (2012).
216. Li, X., Kostareli, E., Suffner, J., Garbi, N. & Hämmerling, G. J. Efficient Treg depletion induces T-cell infiltration and rejection of large tumors. *European journal of immunology* **40,** 3325–3335 (2010).
217. Hindley, J. P. *et al.* T cell trafficking facilitated by high endothelial venules is required for tumor control after regulatory T cell depletion. *Cancer Res.* **72,** 5473–5482 (2012).
218. Teng, M. W. L. *et al.* Conditional regulatory T-cell depletion releases adaptive immunity preventing carcinogenesis and suppressing established tumor growth. *Cancer Res.* **70,** 7800–7809 (2010).
219. Matsushita, N., Pilon-Thomas, S. A., Martin, L. M. & Riker, A. I. Comparative methodologies of regulatory T cell depletion in a murine melanoma model. *J Immunol Methods* **333,** 167–179 (2008).
220. Mei, K. *et al.* Antitumor efficacy of combination of interferon-gamma-inducible protein 10 gene with gemcitabine, a study in murine model. *J Exp Clin Cancer Res* **27,** 63 (2008).
221. Mlecnik, B. *et al.* Biomolecular Network Reconstruction Identifies T-Cell Homing Factors Associated With Survival in Colorectal Cancer. *Gastroenterology* **138,** 1429–1440 (2010).
222. Chaput, N. *et al.* Tumour-infiltrating CD68+ and CD57+ cells predict patient outcome in stage II-III colorectal cancer. *Br J Cancer* **109,** 1013–1022 (2013).
223. Wu, Z. *et al.* The chemokine CXCL9 expression is associated with better prognosis for colorectal carcinoma patients. *Biomed. Pharmacother.* **78,** 8–13 (2016).
224. Ding, Q. *et al.* CXCL9: evidence and contradictions for its role in tumor progression. *Cancer Med* (2016).
225. Zhang, C. *et al.* Lack of interferon-γ receptor results in a microenvironment favorable for intestinal tumorigenesis. *Oncotarget* **7,** 1–11 (2016).
226. Hobeika, A. C., Etienne, W., Torres, B. A., Johnson, H. M. & Subramaniam, P. S. IFN-gamma induction of p21(WAF1) is required for cell cycle inhibition and suppression of apoptosis. *J. Interferon Cytokine Res.* **19,** 1351–1361 (1999).
227. Peng, W. *et al.* PD-1 Blockade Enhances T-cell Migration to Tumors by Elevating IFN-Inducible Chemokines. *Cancer Res.* **72,** 5209–5218 (2012).
228. Beatty, G. L. & Paterson, Y. IFN-γ-Dependent Inhibition of Tumor Angiogenesis by Tumor-Infiltrating CD4+ T Cells Requires Tumor Responsiveness to IFN-γ. **166,** 2276–2282 (2001).
229. Sato, N. *et al.* Actions of TNF and IFN-gamma on angiogenesis in vitro. *J. Invest. Dermatol.* **95,**

85S–89S (1990).
230. Johnson, M. *et al.* Discovery and optimization of a series of quinazolinone-derived antagonists of CXCR3. *Bioorg. Med. Chem. Lett.* **17,** 3339–3343 (2007).
231. Jenh, C.-H. *et al.* A selective and potent CXCR3 antagonist SCH 546738 attenuates the development of autoimmune diseases and delays graft rejection. *BMC Immunol.* **13,** 2 (2012).
232. Horuk, R. Chemokine receptor antagonists: overcoming developmental hurdles. *Nat Rev Drug Discov* **8,** 23–33 (2009).
233. Walser, T. C. Antagonism of CXCR3 Inhibits Lung Metastasis in a Murine Model of Metastatic Breast Cancer. *Cancer Res.* **66,** 7701–7707 (2006).
234. Cambien, B. *et al.* Organ-specific inhibition of metastatic colon carcinoma by CXCR3 antagonism. *Br J Cancer* **100,** 1755–1764 (2009).
235. Li, K. *et al.* Impact of chemokine receptor CXCR3 on tumor-infiltrating lymphocyte recruitment associated with favorable prognosis in advanced gastric cancer. *Int J Clin Exp Pathol* **8,** 14725–14732 (2015).
236. Le Gouvello, S. *et al.* High prevalence of Foxp3 and IL17 in MMR-proficient colorectal carcinomas. *Gut* **57,** 772–779 (2008).
237. Lo, B. K. K. *et al.* CXCR3/ligands are significantly involved in the tumorigenesis of basal cell carcinomas. *Am J Pathol* **176,** 2435–2446 (2010).
238. Furuya, M. *et al.* Up-regulation of CXC chemokines and their receptors: implications for proinflammatory microenvironments of ovarian carcinomas and endometriosis. *Hum. Pathol.* **38,** 1676–1687 (2007).
239. Jones, D., Benjamin, R. J., Shahsafaei, A. & Dorfman, D. M. The chemokine receptor CXCR3 is expressed in a subset of B-cell lymphomas and is a marker of B-cell chronic lymphocytic leukemia. *Blood* **95,** 627–632 (2000).
240. Monteagudo, C., Martin, J. M., Jorda, E. & Llombart-Bosch, A. CXCR3 chemokine receptor immunoreactivity in primary cutaneous malignant melanoma: correlation with clinicopathological prognostic factors. *J. Clin. Pathol.* **60,** 596–599 (2007).
241. Romagnani, P., Lasagni, L., Annunziato, F., Serio, M. & Romagnani, S. CXC chemokines: the regulatory link between inflammation and angiogenesis. *Trend Immunol* **25,** 201–209 (2004).
242. Datta, D. *et al.* Ras-induced modulation of CXCL10 and its receptor splice variant CXCR3-B in MDA-MB-435 and MCF-7 cells: relevance for the development of human breast cancer. *Cancer Res.* **66,** 9509–9518 (2006).
243. Kidd, P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern Med Rev* **8,** 223–246 (2003).
244. Ling, A. *et al.* The infiltration, and prognostic importance, of Th1 lymphocytes vary in molecular subgroups of colorectal cancer. *J Pathol Clin Res* **2,** 21–31 (2016).
245. Heriot, A. G., Marriott, J. B., Cookson, S., Kumar, D. & Dalgleish, A. G. Reduction in cytokine production in colorectal cancer patients: association with stage and reversal by resection. *Br J Cancer* **82,** 1009–1012 (2000).
246. Nakayama, H., Kitayama, J., Muto, T. & Nagawa, H. Characterization of intracellular cytokine profile of CD4(+) T cells in peripheral blood and tumor-draining lymph nodes of patients with gastrointestinal cancer. *Jpn. J. Clin. Oncol.* **30,** 301–305 (2000).
247. Kanazawa, M. *et al.* Effects of PSK on T and dendritic cells differentiation in gastric or colorectal cancer patients. *Anticancer Res.* **25,** 443–449 (2005).
248. O'Hara, R. J. *et al.* Advanced colorectal cancer is associated with impaired interleukin 12 and enhanced interleukin 10 production. *Clin. Cancer Res.* **4,** 1943–1948 (1998).
249. Shibata, M. *et al.* Decreased production of interleukin-12 and type 2 immune responses are marked in cachectic patients with colorectal and gastric cancer. *J. Clin. Gastroenterol.* **34,** 416–420 (2002).
250. Morse, M. A. *et al.* Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. *Blood* **112,** 610–618 (2008).
251. McHugh, R. S. & Shevach, E. M. Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. *J Immunol* **168,** 5979–5983 (2002).
252. Rech, A. J. & Vonderheide, R. H. Clinical use of anti-CD25 antibody daclizumab to enhance immune responses to tumor antigen vaccination by targeting regulatory T cells. *Annals of the New York Academy of Sciences* **1174,** 99–106 (2009).
253. Olsen, E. *et al.* Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **19,** 376–388 (2001).

254. Onizuka, S. *et al.* Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res.* **59,** 3128–3133 (1999).
255. Marabelle, A. *et al.* Depleting tumor-specific Tregs at a single site eradicates disseminated tumors. *J. Clin. Invest.* **123,** 2447–2463 (2013).
256. Maker, A. V., Attia, P. & Rosenberg, S. A. Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade. *The Journal of Immunology* **175,** 7746–7754 (2005).
257. Piconese, S., Valzasina, B. & Colombo, M. P. OX40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection. *Journal of Experimental Medicine* **205,** 825–839 (2008).
258. Cohen, A. D. *et al.* Agonist anti-GITR monoclonal antibody induces melanoma tumor immunity in mice by altering regulatory T cell stability and intra-tumor accumulation. *PloS one* **5,** e10436 (2010).
259. Brahmer, J. R. *et al.* Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol.* **28,** 3167–3175 (2010).
260. Berger, R. *et al.* Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies. *Clin. Cancer Res.* **14,** 3044–3051 (2008).
261. Markosyan, N. *et al.* Mammary carcinoma cell derived cyclooxygenase 2 suppresses tumor immune surveillance by enhancing intratumoral immune checkpoint activity. *Breast Cancer Res.* **15,** 17–33 (2013).
262. Bronger, H. *et al.* CXCL9 and CXCL10 predict survival and are regulated by cyclooxygenase inhibition in advanced serous ovarian cancer. *British journal of cancer* **115,** 553–563 (2016).
263. Bronger, H. *et al.* Modulation of CXCR3 ligand secretion by prostaglandin E2 and cyclooxygenase inhibitors in human breast cancer. *Breast Cancer Res.* **14,** R30 (2012).
264. Li, S., Xia, X., Zhang, X. & Suen, J. Regression of tumors by IFN-alpha electroporation gene therapy and analysis of the responsible genes by cDNA array. *Gene Ther.* **9,** 390–397 (2002).