LEUKOCYTE RECRUITMENT TO HUMAN GASTROINTESTINAL TISSUES

- STUDIES IN *H. PYLORI*-INDUCED GASTRITIS AND COLON ADENOCARCINOMAS

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ABSTRACT

Chronic inflammation is a major pathological basis for tumor development and two examples are *H. pylori* infection of the stomach mucosa and inflammatory bowel disease, which are strongly associated with gastric and colon adenocarcinoma, respectively. In this thesis we investigated *H. pylori*-induced chronic gastritis and colon adenocarcinoma in patients with no history of IBD. The overall aim was to determine how inflammation and endothelial cell function influence the composition of infiltrating cell populations in inflamed tissue as well as tumors in the gastrointestinal tract.

In the first paper we characterized the effect of *H. pylori*-induced gastritis on the expression of endothelial adhesion molecules. By *ex vivo* analyses of human stomach biopsies as well as *in vitro* stimulation of endothelial cells we concluded that endothelial E-selectin expression was induced by *H. pylori* strains expressing the Cag Pathogenicity Island, which encode several major virulence factors in *H. pylori.* The increased E-selectin expression in infected patients probably contributes to the characteristic *H. pylori*-induced chronic inflammation with a large influx of neutrophils and tissue damage.

Increased expression of chemokines is known to contribute to the progression of tumors, and we next studied the decoy chemokine receptors D6, DARC and CCX-CKR, mediating degradation of chemokines. Using real time RT-PCR on clinical samples we detected significantly decreased D6 expression in the colon tumor tissue compared to unaffected tissue accompanied by increased chemokine levels, but similar levels of DARC and CCX-CKR. Our results also indicate that D6 is expressed on lamina propria lymphocytes, mainly on infiltrating B cells. Hence the tumor environment is directed towards a more pro-inflammatory response due to the inability to inactivate chemokines and our results indicate that D6 contribute significantly to promote tumor progression.

Since we could show that the chemokine composition is altered in colon tumors we also wanted to characterize the infiltration of immune cells in tumor and unaffected colon mucosa. Lymphocyte composition and activation stage was determined by flow cytometry. We demonstrated that $CTLA4^{+}CCR4^{+}$ regulatory T cells, which may suppress tumor-specific T cell responses at the tumor site, accumulate in colon tumors. Additionally, the frequencies of activated conventional Th1 type T cells, one of the most important effector mechanisms in immunity against tumors, are decreased in the tumors. This altered lymphocyte composition in the colon tumor will probably diminish the ability of the immune system to effectively attack tumor cells.

In conclusion we have shown that chronic mucosal inflammation and tumor development change the expression of several endothelial receptors and chemokines leading to an altered immune cell composition in inflamed tissue and tumors. Our studies also indicate that the decoy chemokine receptor D6 may play an important role in tumor development.

Keywords: *Helicobacter pylori*, E-selectin, Lymphocyte homing, T lymphocytes, colon adenocarcinoma, regulatory T cells.

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ORIGINAL PAPERS:

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-III):

I. Helena Svensson[*#], Malin Hansson[#], Jan Kilhamn[#], Steffen Backert[¤] and Marianne Quiding-Järbrink[#]

Selective upregulation of endothelial E-selectin in response to *Helicobacter pylori*-induced gastritis. *Infect. Immun 77, 3109-3116. (2009).*

II. Helena Svensson[#*€], Veronica Olofsson[*€], Lars Börjesson[¤], Bengt Gustavsson[¤], Åsa Sjöling[*], Marianne Quiding-Järbrink[*] . [€]Authors contributed equally

Decoy receptor D6 is decreased in colon adenocarcinomas. *In manuscript.*

III. Helena Svensson[*#], Veronica Olofsson[*], Hanna Stenstad[*], Chakradhar Yakkala[*], Stellan Björck[¤], Lars Börjesson[¤], Bengt Gustavsson[¤] and Marianne Quiding-Järbrink[*]

Accumulation of $CCR4^{+}$ $CTLA4^{hi}$ $FOXP3^{+}CD25^{hi}$ regulatory T cells in colon adenocarcinomas correlate to reduced activation of conventional T cells. *Submitted.*

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ABBREVIATIONS

APC	Antigen Presenting Cell
BabA	Blood antigen binding Adhesin
BFA	Brefeldin A
CagA	Cytotoxin associated gene A
CagPAI	Cag Pathogenicity Island
CCX-CKR	Chemo Centryx Chemokine Receptor
CLA	Cutaneous Lymphocyte Antigen
CTLA-4	Cytotoxic T Lymphocyte associated Antigen-4
DARC	Duffy Antigen Receptor for Chemokines
DC	Dendritic cell
FAE	Follicle-Associated Epithelium
GALT	Gut-Associated Lymphoid Tissue
GlyCAM-1	Glycosylation-dependent Cell Adhesion Molecule 1
HEVs	High Endothelial Venules
HLO	Helicobacter-Like Organisms
HUVEC	Human Umbilical Vein Endothelial cells
IBD	Inflammatory Bowel Disease
IEL	Intraepithelial Lymphocytes
IL-1b	Interleukin 1b
ILF	Isolated Lymphoid Follicle
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome
LPL	Lamina Propria Lymphocytes
LPS	Lipopolysaccharide
M cells	Microfold cells
MAdCAM-1	Mucosal Addressin Cell Adhesion Molecule 1
MAPK	Mitogen Activated Protein Kinase
MFI	Mean Fluorescence Intensity
MHC	Major Histocompability Complex
Nap	Neutrophil activating protein
NK cell	Natural Killer cell
NO	Nitric Oxide
PNAd	Peripheral lymph Node Addressin
PP	Peyer's Patches
PSGL-1	P-Selectin Glycoprotein Ligand 1
RA	Retinoic Acid
ROS	Reactive Oxygen Species
SabA	Sialic acid binding Adhesin
SED	Subepithelial Dome
T4SS	Type IV Secreting System
TAM	Tumor-Associated Macrophage
TCR	T cell receptor
Th	T helper
TNF-a	Tumor Necrosis Factor alpha
Treg	regulatory T cell
VacA	Vacuolating cytotoxin A
vWF	von Willenbrand Factor
WHO	World Health Organization

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INTRODUCTION

BRIEF INTRODUCTION TO THE HUMAN IMMUNE SYSTEM

The immune system has evolved to protect our bodies from invading pathogens and chemicals that may cause infections, tissue damage and tumors. At the same time it has to allow uptake of nutrients and accept the presence of commensal bacteria in the gut, as well as be tolerant to its own tissues. To fulfill its tasks, the immune system is a very complex system built up of lymphoid organs, specialized immune cells and a lymph system that all work together to maintain homeostasis.

The immune system can be subdivided into two branches that work together to maintain homeostasis, the innate and the adaptive system. The innate system is the first line of defense and responds to an infectious agent within hours. It includes mechanical barriers such as skin and mucosal surfaces, physiological barriers such as low pH, fever response, and components of the complement system. This branch also contains a number of innate immune cells such as dendritic cells (DC), macrophages, neutrophils, mast cells, eosinophils, basophils and natural killer (NK) cells. The cells of the innate system recognize a fixed number of structures that are shared by many microbes. They also have the capability to initiate and regulate the adaptive branch by producing cytokines and chemokines that attract cells of the adaptive immune system and by presenting antigen that activates these cells.

The adaptive immune system is characterized by specificity, diversity and memory. The cells of the adaptive immune system consist of different subsets of T and B cells, each carrying a receptor with a unique specificity. The T cells migrate into lymph nodes where they use their T cell receptor (TCR) to recognize their specific antigen presented as a peptide bound to major histocompability complex (MHC) on the surface of an antigen presenting cell (APC) e.g. DCs, macrophages or B cells. When T cells encounter their antigen they proliferate and the progenitors differentiate into effector cells and memory cells, resulting in an elevated response when encountering the same pathogen once again.

T cells exhibit a wide range of functions during the immune response and they can be further subdivided into different populations according to their functions. The two main

populations of T cells are the $CD8^+$ and $CD4^+$ T cells. The $CD8^+$ cells are commonly termed cytotoxic T lymphocytes. They recognize a specific antigen and when activated they are able to destroy tumor cells or cells that are infected with an intracellular pathogen. The $CD4^+$ T cells can be divided into T helper cells (Th) and T regulatory (Treg) cells. With a certain degree of simplification, the Th cells can be said to differentiate into Th1 cells which are involved in cell-mediated immunity and secrete the cytokines IFN-γ and IL-2, Th2 cells which are involved in humoral (B cell) immunity and secrete IL-4 and IL-5, and Th17 cells which are involved in neutrophil recruitment and autoimmune disease and secrete IL-17. The regulatory T cells are a specialized type of immune cell that are able to suppress the immune response and are therefore crucial for maintaining immunological tolerance.

THE GASTROINTESTINAL IMMUNE SYSTEM

The intestinal mucosa allows the exchange of nutrients, ions and water across the epithelium, and at the same time it is challenged with massive loads of potentially harmful antigens and microbes present in the lumen. To be able to keep the balance between the host and the gut environment, the intestinal immune system is one of the largest and most complex immune systems in our body. The gut-associated lymphoid tissue (GALT) is made up of organized lymphoid structures such as Peyer's patches (PP) in the small intestine, isolated lymphoid follicles (ILF) in the large intestine and mesenteric lymph nodes (MLN). In addition to these organized structures, diffusely spread intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) are a part of the intestinal immune system [1]. The structure of the large intestine is shown in Fig 1.

The intestinal epithelium: To fulfill its digestive function, the epithelial layer of the small intestine consists of finger-like projections (villi) which are in turn covered with microvilli, expanding the intestinal surface area to $400m^2$ [2]. The cellular barrier of the gut which separates the lumen from the lamina propria consists of a single layer of epithelial cells that are sealed together with tight junctions, regulating the permeability between cells [3]. The epithelial cell layer displays several protective adaptations to prevent luminal antigens and microbes penetrating the barrier. Goblet cells secrete mucus composed of complex mucin glycoproteins, adding to the physical barrier by preventing firm adhesion of bacteria to the intestinal epithelial cells. The number of

goblet cells is higher in the large intestine than the small intestine, probably as an adaption to an increased bacterial load [2]. There are also Paneth cells in the small intestine (Fig 2). These are a specialized type of epithelial cell situated at the base of the crypt producing antimicrobial peptides such as defensins [4]. The whole epithelial surface is replaced every 2-3 days, and the antimicrobial peptides are assumed to protect the stem cells present in the crypts from invading bacteria [5]. During inflammatory conditions, Paneth cells can also be found in the large intestine, possibly to protect the damaged epithelium from invading bacteria [6].

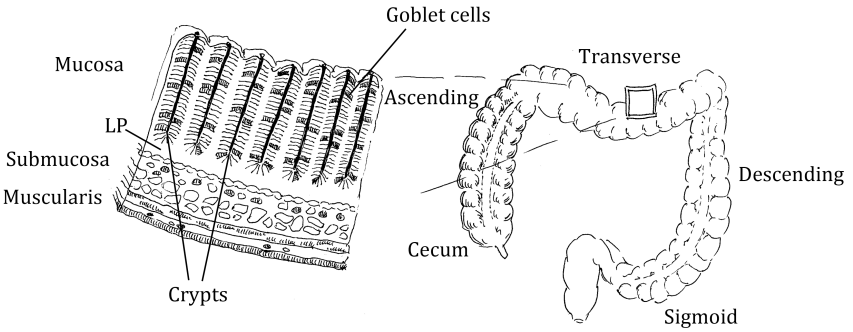


Figure 1. Anatomical and histological structure of the colon.

Immune cells of the gastrointestinal mucosa: The major inductive sites of the small intestine are the PP, which are large lymphoid aggregates covered with follicle-associated epithelium (FAE). The FAE is characteristically cuboidal and has specialized microfold (M) cells. The M cells lack surface microvilli as well as the protective mucus layer [7]. Antigens from the intestinal lumen are transported through the M cell into the underlying subepithelial dome (SED) where they are processed by DCs. From there, the stimulated DCs migrate to the T cell areas or into B cell follicles of the PP to induce T cell priming and B cell maturation [8], or they exit through the draining lymph vessels to the MLNs (Fig 2).

Activated mucosal B cells undergo isotype class switching from IgM to IgA. The main function of B cells in the local response is to collaborate with secretory epithelial cells so that IgA can be transported across the epithelium to the luminal surface. In the lumen IgA binds to microbes and toxins to neutralize them by blocking penetration through the epithelial layer, but IgA-secreting cells may also enter the blood stream and migrate to distant mucosal sites to optimize the protection against intestinal pathogens [2, 9].

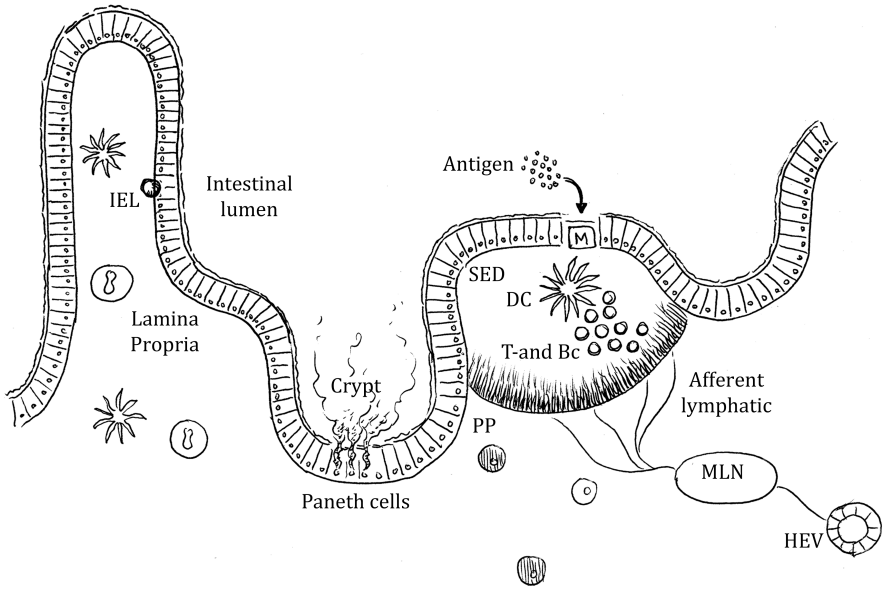


Figure 2. Organization of the intestinal immune system.

Classical T cells are activated through the TCR in response to antigenic stimulation. However, while T cells of the intestinal mucosal immune system are in close vicinity to bacteria and food antigens, they cannot be allowed to mount an inflammatory response against these antigens, therefore it is difficult to activate these cells by antigen stimuli [10]. T cells of the intestinal immune system are not only present in organized tissues, there are lymphocytes diffusely spread in the mucosa of both the small and large intestine. These cells are present both in the connective tissue of the lamina propria and within the epithelial layer. The intraepithelial lymphocytes (IEL) are a unique population of T cells consisting mainly of $CD8^+$ T cells. The majority of IEL in the human intestine use the $\alpha\beta$ TCR, and 10% (compared to 2% of the total number of T cells) use the $\gamma\delta$ TCR during healthy conditions [5]. The IELs are evolutionarily conserved, and unlike conventional T cells they have a limited diversity of their antigen receptor and are able to respond to antigenic peptides presented by conventional MHC molecules ($\alpha\beta$) or non-classical MHC molecules ($\gamma\delta$). The IEL population is very diverse however, some of the IELs do not require priming but are able to respond to an antigen instantly [11, 12]. The knowledge of IEL function in the human intestine is very limited. It has been suggested that IEL play a role in maintaining epithelial integrity by producing growth factors and secreting chemokines to recruit peripheral T cells and other inflammatory cells to the site of

inflammation [13]. Additionally, there are reports of IELs being involved in protective responses to certain pathogens, and also that they may play a role in cancer surveillance [10].

The majority of the lamina propria lymphocytes (LPL) are activated T cells, mainly $CD4^+$, but also B cells and plasma cells, and the activated phenotype of these cells suggests prior activation in PP [5, 14]. The function of LPL is not as well characterized as that of cells from systemic sites, but it has been proposed that $CD8^+$ T cells in the LP have cytotoxic functions and are believed to contribute to the control of viral infections [15, 16]. The LPLs are also able to secrete cytokines such as IFN-γ and TNF-α, and a substantial proportion of LPLs are IgA-producing plasma cells, secreting their antibodies which are translocalized to the luminal side [15, 17, 18].

Anatomy and physiology of the stomach

The main functions of the stomach are to store food, initiate digestion, and dispose material into the duodenum. It is also a barrier and eliminates potentially harmful bacteria. Histologically the stomach can be divided into three major parts; the cardia is a small area where the esophagus enters the stomach, the corpus and fundus regions which occupy the middle and upper part of the stomach, and the antrum which is the most distal part emptying its content into the duodenum [19] (Fig 3). The gastric luminal surface is covered by a columnar surface epithelium which secretes a mucus layer and bicarbonate to protect the stomach lining. Invaginations of the gastric epithelium into the underlying lamina propria form the gastric pits. The pits are lined with similar types of cells as the gastric luminal surface in order to protect the mucosa against stomach juices, and several different types of these glands can be found in different parts of the stomach. The neck region of the pit contains stem cells which can migrate upwards or downwards to replace dying cells. The parietal cells and the chief cells, which produce hydrochloric acid and pepsinogen respectively, are mainly found deeper down in the glands in the fundus and corpus regions. The antrum, on the other hand, harbors the G-cells able to produce gastrin, which in turn stimulates the secretion of gastric acid [19-21] (Fig 3).

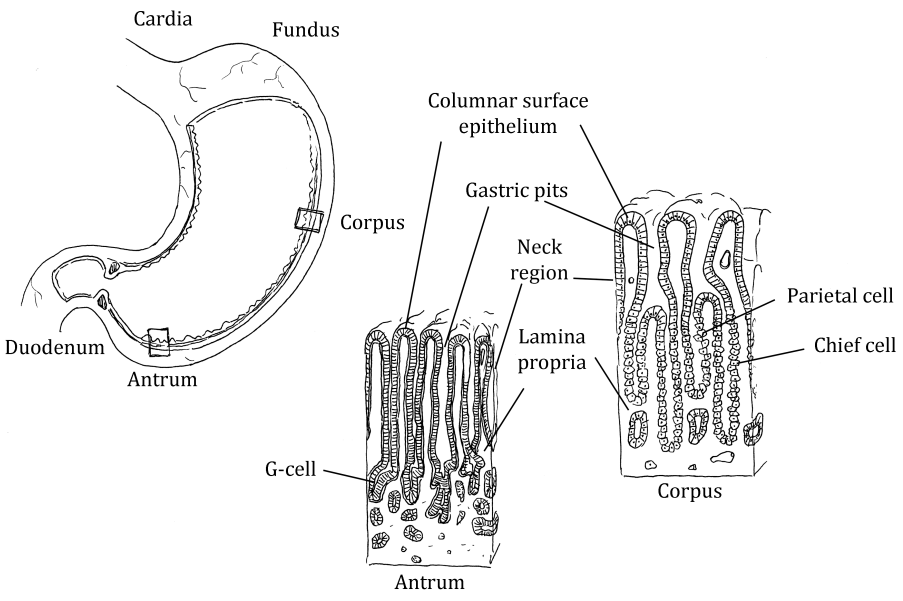


Figure 3. Regions of the stomach and their histological structure.

During normal conditions there are only few lymphocytes scattered throughout the lamina propria of the gastric mucosa [22]. Occasionally lymphocytes may also be observed in the epithelium of the normal stomach, most commonly in the surface. Plasma cells are sparse or totally absent from the stomach of healthy individuals [20], and also lymphoid follicles are believed to be absent from the normal stomach. However, there could be small aggregates of lymphocytes without germinal centers present. These aggregates are rare however, and usually located immediately above the muscularis mucosae in the distal antrum [23]. In other words, the immune system is not very active in the gastric mucosa during healthy conditions but can be activated when needed.

HELICOBACTER PYLORI

H. pylori is a gram-negative microaerophilic spiral-shaped bacterium colonizing the gastric epithelial cells in the stomach. It is also present in gastric-like metaplasia in the duodenum. *H. pylori* are generally considered to be extracellular bacteria which are free-living within the mucus lining the stomach wall, and only a fraction adhere to gastric epithelial cells [24]. However, some findings demonstrate that the bacteria is able to grow inside epithelial cells [25]. Infection is usually acquired during early childhood and without treatment, it usually persists throughout life [24, 26]. Today it is appreciated that

approximately 50% of the world's population is infected by *H. pylori*, making it one of the most common infections in the world [27]. Infection rate varies among different countries and ethnicities, and a high infection rate is correlated to a low socioeconomic status [27, 28].

Most infected individuals remain asymptomatic, but all infected individuals develop an active chronic gastritis with a characteristic histological profile involving degeneration of the surface epithelium and infiltration of acute and chronic inflammatory cells to the gastric mucosa [29, 30]. In addition to the gastritis, 10-15% of infected individuals develop peptic ulcers and 1-2% acquire gastric cancer as a consequence of *H. pylori* colonization [31]. Due to its association with cancer development, *H. pylori* was classified as a class I carcinogen by the World Health Organization (WHO) in 1994.

To enable survival and colonization in the harsh environment of the stomach *H. pylori* carry a variety of colonization and virulence factors. Variations in virulence factors present in different strains of *H. pylori* make them more or less pathogenic. However, the outcome of an *H. pylori* infection is also dependent on lifestyle and genetic factors of the infected individual. Several *H. pylori*-derived factors are important for colonization and survival of the bacteria, as seen in Fig 4.

Urease**:** To be able to survive in the acidic environment of the stomach, *H. pylori* constitutively produce large amounts of urease. Urease is present on the outer membrane but also in the cytoplasm and buffers the intracellular pH of the bacteria in the acidic environment, by converting gastric urea into ammonia and carbon dioxide [32]. Urease is important for bacterial colonization, and urease-negative *H. pylori* are unable to colonize the gastric mucosa [33].

Flagellae: Due to its spiral shape, *H. pylori* is able to use multiple flagella to move through the mucus. The bacteria is able to sense the pH gradient in the mucus layer and moves away from the acidic environment, down towards the epithelium [34, 35].

Adhesins: The bacteria express several adhesins such as bloodgroup antigen binding adhesin (BabA) and sialic acid binding adhesin (SabA), allowing them to adhere to gastric epithelial cells and thereby facilitating colonization of the gastric mucosa [36-38].

H. pylori adherance to epithelial cells also triggers the characteristic gastritis seen in most infected individuals [39].

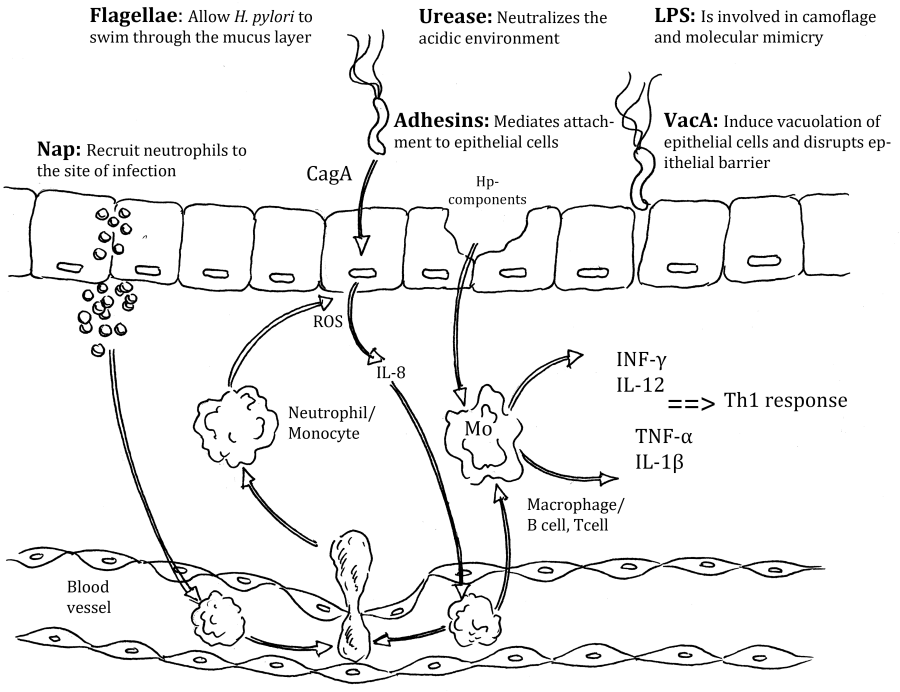


Figure 4. Overview of *H. pylori* virulence factors and pathogenesis.

Neutrophil activating protein (Nap): The *H. pylori* neutrophil activating protein (HP-NAP) has been shown to attract and activate neutrophils. It may have a role in the massive infiltration of neutrophils found in *H. pylori*-infected mucosa due to its chemotactic properties [40]. It has also been reported as being able to skew the T cell response towards a Th1 response [41].

Vacuolating cytotoxin A (VacA): The VacA protein is able to induce vacuoles in mucosal epithelial cells thereby increasing paracellular permeability [42]. It is also able to inhibit the proliferation of T cells [43]. VacA is expressed in approximately 50% of all *H. pylori* strains and is not important for bacterial colonization [44].

The Cytotoxin associated gene A (CagA) and the Cag Pathogenicity Island (CagPAI): The CagPAI locus consists of 31 genes including cagA, cagE, cagH and cagL [45] however, cagA is often used as a marker for the whole locus. Many of the genes code for a type IV

secreting system (T4SS). The T4SS is activated when the bacteria binds to a gastric epithelial cell, and the CagA protein is then inserted into the host cell through a syringe-like construction [46, 47]. Recent studies have shown that the CagL protein is targeted to the pilus surface, and its interaction with the target cell triggers the delivery of CagA [48]. Inside the host cell, the CagA protein becomes phosphorylated, interferes with the host cell signaling pathways and induces cytoskeleton rearrangements [49, 50]. Also, peptidoglycan can be translocated into epithelial cells by the T4SS, where it is recognized by the intracellular pattern recognition receptor Nod1, which activates NF-κB and induces the production of pro-inflammatory cytokines [51]. Strains carrying an intact cagPAI have been shown to be more virulent then strains lacking the cluster [52].

Lipopolysaccharide (LPS): LPS is situated in the outer cell membrane of gram-negative bacteria. It often induces strong local, as well as systemic, immune responses, but *H. pylori* LPS has much lower immunobiological activity than LPS of other bacteria due to a mutation in the lipid A component [53, 54].

The gastric mucosa during H. pylori infection.

H. pylori infection results in a characteristic active chronic inflammation of the gastric mucosa. *H. pylori*-induced inflammation is characterized by infiltration of both cells from the innate system, such as neutrophils and macrophages, and T and B cells from the adaptive system, which are specific for *H. pylori* antigens [31, 55, 56]. However, how these cells are recruited and which adhesion molecules are locally expressed is not yet fully characterized.

When *H. pylori* establishes contact with the gastric epithelial cells it induces production of the chemokine IL-8 [57], which is a strong chemotactic and activating factor for neutrophils. Infiltration of neutrophils leads to accumulation of reactive oxygen species (ROS) in the mucosa, and this together with bacterial toxins (such as VacA) damages the epithelial layer of the mucosa. The damaged epithelial layer enables *H. pylori* components to cross into the lamina propria where they activate macrophages [58, 59]. The activated macrophages in turn induce the production of pro-inflammatory cytokines such as IL-12, TNF-α and IL-1β which induce an inflammatory response that will recruit effector cells to the site. These cytokines, in particular IL-12, shift the immune response towards a Th1 response [60-62].

Lymphocytes are scattered throughout the mucosa, and during *H. Pylori* infection lymphoid follicles, consisting of a central B cell area surrounded by a thin layer of mainly $CD4^+$ T cells, are formed [63]. It is not known if these follicles are able to support local antigen presentation and B cell differentiation, but they are present in virtually all infected individuals [23]. Active chronic gastritis is also associated with an increased proportion of $CD4^+$ lymphocytes in the gastric mucosa compared to $CD8^+$ lymphocytes, due to the accumulation of $CD4^+$ T helper lymphocytes [64]. In the antrum and duodenal mucosa of *H. pylori*-infected individuals there is an increased infiltration of $CD4^+$ T helper cells as well as Tregs compared to the mucosa of uninfected individuals [65, 66]. These Tregs probably reduce the inflammation and hence promote bacterial persistance [67, 68]. Thus, it is possible that the inability to clear *H. pylori* infection is connected to the activation of Tregs, since these cells have the capacity to inhibit activation of Th1 cells, thereby limiting damage to the gastric mucosa but at the same time prolonging bacterial persistence.

There is also a strong antibody response against different *H. pylori* components. Infected individuals have elevated levels of *H. pylori*-specific antibodies in their blood as well as increased numbers of specific B cells in their stomach [56, 69]. The role for antibodies, especially IgA, in the response against *H. pylori* is far from elucidated. Moreover, patients suffering from gastric adenocarcinoma have a decreased local IgA production compared to asymptomatic *H. pylori*-infected individuals [70]. This could indicate a protective role for IgA antibodies in *H. pylori*-associated diseases.

THE LINK BETWEEN INFLAMMATION AND CANCER

In 1863, Rudolf Virchow noted leukocytes in neoplastic tissues. He suggested that these infiltrating leukocytes indicated that the cancer originated at sites of chronic inflammation and hence he was the first to propose a link between inflammation and cancer [71]. Since then, there has been accumulating evidence that there is a link between inflammation and cancer development. Inflammation is often due to an infectious agent, but an increased risk of cancer may also be imparted by chronic inflammation caused by chemical or physical agents, autoimmune inflammatory reactions, as well as by inflammation of unknown cause [71]. For instance, the risk of developing gastric cancer is tightly correlated with *H. pylori* infection. Approximately 70-90% of all gastric cancers are believed to have their origin in chronic inflammation caused by *H. pylori* infection [72].

In addition, the risk of developing colon cancer is increased in patients with the inflammatory bowel diseases Crohn's disease and ulcerative colitis [73]. Today it is estimated that 15-20% of all cancers globally develop from chronic inflammatory conditions [74].

Inflammation is a physiologic process initiated by injury due to a pathogen infection, tissue damage, chemicals or wounding. This acute inflammation initiates a cascade of cytokines and chemokines attracting mainly neutrophils to infiltrate the tissue. Acute inflammation is usually self-regulated, but if the stimulus is not removed or the resolution is disturbed, it can transform into a chronic inflammation dominated by infiltrating lymphocytes, neutrophils, and macrophages [75], all of which may contribute to cancer development.

Mechanisms contributing to tumor initiation:

TNF-α: Tumor necrosis factor alpha (TNF-α) is a major mediator of inflammation and is involved in both tissue destruction and recovery [71]. In cancer development it has a dual role; it is able to destroy blood vessels, but also to induce angiogenic factors [71]. TNF-α may initiate an inflammatory cascade with other inflammatory cytokines, chemokines and growth factors, thereby recruiting activated cells to the site. It can also initiate DNA damage and inhibit DNA repair. In addition, it may also act as a growth factor for tumor cells. The primary source of TNF-α production is activated macrophages but tumor cells may also contribute [76].

IL-6: IL-6 plays an important role in the proliferation of intestinal epithelial cells and protects normal and pre-malignant intestinal epithelial cells from apoptosis [77]. IL-6 is therefore suggested to be among the cytokines promoting tumor initiation and progression [76]. Colon cancer patients have elevated serum levels of IL-6, which is also positively correlated to tumor load, providing evidence that IL-6 is indeed important for cancer development [78].

Th17 cells: TNF-α, IL-23 and IL-6 cooperate for differentiation of the T cell subset Th17. These cells produce high levels of IL-17A and IL-17F, which promote tumor development through the enhancement of angiogenesis [79, 80].

Mechanisms contributing to tumor progression:

Macrophages: Tumor-associated macrophages (TAM) are the major infiltrating cells in most tumors [81]. Classical macrophages, or M1 macrophages, are able to kill tumor cells and ingest intracellular microorganisms. In addition to classically activated TAMs, there are alternatively activated M2 macrophages. The M2 phenotype macrophages are poor antigen-presenting cells, and are associated with immunosuppression and the promotion of tumor angiogenesis and metastasis [82, 83].

Dendritic cells: Dendritic cells classically act as messengers between the innate and adaptive immune response with a role in both activation of antigen-specific immunity and maintaining tolerance. Tumor-associated dendritic cells (TADC) have an immature phenotype and lack the ability to mount an effective T cell response against tumor antigens [84].

Additional cells: Eosinophils, mast cells and lymphocytes are also present during inflammation. These cells may secrete cytokines, reactive oxygen species and MMPs contributing to tumor growth and development [74].

Pro-inflammatory cytokines: Tumors are often rich in inflammatory cytokines, growth factors and chemokines, but lack cytokines involved in specific responses. Inflammatory cytokines and chemokines can be produced by tumor cells and also by tumor-associated leukocytes and platelets. They may promote angiogenesis, metastasis and proliferation as well as spread [71].

Chemokines: The chemokines and their receptors orchestrate the homing of leukocytes both in homeostatic conditions as well as during inflammation and cancer progression. Most tumors produce chemokines from both the CC and the CXC family. CC chemokines typically recruit multiple leukocyte subsets such as monocytes, eosinophils, dendritic cells and lymphocytes while CXC chemokines mainly recruit neutrophils and lymphocytes [80]. These recruited cells then act as a secondary source of chemokines and can affect tumor growth, cell survival, senescence and metastasis [85]. For instance, tumor cells produce chemokines such as CCL2, which recruit monocytes, that in turn supports tumor growth and spread [80].

ANTI-TUMOR IMMUNE RESPONSES

Cell-mediated immunity is believed to be the main mechanism of anti-tumor immunity of the host. $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells are among the most important cells in the destruction of tumors alongside NK cells and macrophages [86]. $CD4^+$ T helper cells are able to recognize tumor antigens via MHC class II presentation. In addition, these cells have an important anti-tumor function by producing cytokines which activate and recruit other immune cells to the tumor [87]. In particular, Th1 cells provide help to cytotoxic $CD8^+$ cells, thereby facilitating destruction of the tumor cells [86]. $CD8^+$ T cells are able to recognize tumors via MHC class I presentation of tumor antigens [88]. Type 2 $CD4^+$ cells (Th2) on the other hand do not have an anti-tumor effect since they facilitate B cell antibody production, which is not a part of the cell-mediated immune response [86]. Although tumor-reactive antibodies can have an anti-tumor effect when administered to cancer patients, most often these therapies are ineffective [89].

Macrophages can have a very diverse effect depending on their phenotype, as previously described, the M2 type is involved in the development of tumors from an inflammation background. The M1 type on the on the other hand, are able to destroy tumor cells by their production of nitric oxide (NO) as well as their production of type 1 cytokines and chemokines. M1 cells can also act as antigen presenting cells, activating cytotoxic $CD8^+$ T cells [86].

NK cells are also important in the anti-tumor response. They have a direct effect by recognition and destruction of cells that do not display MHC class I on their surface. Also, their production of cytokines play a role in the maturation of dendritic cells and $CD8^+$ T cells [86]. In contrast to the antitumor effects, there are also subsets of NK cells that are cytotoxic for, and hence regulate, activated DC and $CD8^+$ T cells [90, 91]. NK cells as well as T cells produce IFN-γ, which has also been demonstrated to be important in anti-tumor responses. IFN-γ has anti-tumor effects in inhibiting cell proliferation and angiogenesis as well as in regulating the generation and activation of Treg [92, 93].

TUMOR ESCAPE MECHANISMS

As described above, there are multiple ways in which our immune system can fight tumor cells. However, tumor cells have evolved different strategies to enable them to

avoid recognition by the different immune effector mechanisms. In this overview, which is far from complete, the main focus is on mechanisms initiated by the tumor.

As discussed earlier, cytotoxic $CD8^+$ T cells recognize tumor cells via MHC class I expression. Many malignant cells have a down-regulated or lost expression of MHC class I resulting in an inability of CTL to destroy their tumor target cells [94, 95]. An alteration in the MHC class I molecule may also prevent the NK cell-induced lysis of tumor cells [96]. Another mechanism for tumor escape is down-regulation of the antigen processing machinery in response to cytokines produced by tumor cells [97]. In addition to recognition of its antigen in an MHC complex, the T cell requires a co-stimulatory signal triggered by the interaction between co-stimulatory molecules of the B7 family (B7.1 and B7.2) on the APC and CD28 on the T cell. If this signal is absent the T cell may become anergic (unresponsive). In the tumor environment there are also co-inhibitory molecules of the B7 family (B7-H1 and B7-H4), which may induce T cell apoptosis and hence promote tumor escape [98, 99]. Also, human tumors are genetically unstable and are therefore constantly changing their antigenic profile. Tumor-specific CTLs present at the tumor site eliminate tumor cells expressing a specific antigen and leave antigen-negative tumor cells. With time, this results in the selection of tumor cells with antigen loss (also called epitope loss) in the tumor [100].

The tumor microenvironment also contains soluble factors that potentially inhibit the anti-tumor T cell functions. IL-10 and TGF-β are considered to be two of the most important cytokines in tumor escape and they can be produced by the tumor itself, but also by non-tumor stromal cells [101]. IL-10 has been shown to modulate the balance of B7 molecules towards immune suppression and also inhibit DC-mediated priming of CTLs [99, 102]. TGF-β has a dual role in tumor progression and is able to act both as a suppressor and a promoter. TGF-β suppresses tumor initiation and early development by induction of apoptosis and suppression of growth factor and cytokine expression. TGF-β regulates both the clonal expansion and the cytotoxicity of $CD8^+$ T cells and is thereby able to inhibit these functions. Another important function is the induction of FOXP3 in T cells, generating induced Tregs. [103]. The immune suppressive effect of Tregs in the tumor environment will be discussed in the section concerning Tregs.

Another tumor escape mechanism is tumor counterattack. Expression of the death receptor FasL on tumor cells may induce apoptosis in activated T cells [104], whereas some tumor cells exhibit dysfunctional apoptotic pathways enabling them to resist apoptosis [105].

COLON CANCER

Colon cancer is one of the most common malignancies word-wide, both regarding incidence and mortality together with breast, lung and prostate cancer. Cancers of the colon and the rectum are the third most common types of cancer world-wide and the fourth most common cause of cancer mortality [106, 107]. The prevalence of colon cancer is higher in industrialized countries compared to developing countries [106]. In Europe, the survival for adults is approximately 72% in the first year and 54% within 5 years [108].

Table 1. TNM classification **[106].**

T – Primary Tumor		**N – Regional Lymph Nodes**	
TX	Primary tumor cannot be assessed	**NX**	Regional lymph nodes cannot be assessed
T0	No evidence of primary tumor	**N0**	No regional lymph node metastasis
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria	**N1**	Metastasis in 1 to 3 regional lymph nodes
T1	Tumor invades the submucosa	**N2**	Metastasis in 4 or more regional lymph nodes
T2	Tumor invades the muscularis propria		
T3	Tumor penetrates through the muscularis propria intothe subserosa or into non-peritonealised pericolic or perorectal tissue	**M – Distant Metastases**	
		MX	Distant metastasis cannot be assessed
T4	Tumor directly invades other organs or structures and/or perforates the visceral peritoneum	**M0**	No distant metastasis
		M1	Distant metastasis

Most often colon cancer occurs sporadically, due to accumulation of mutations and only 5% of the cases are considered to be inherited [109]. The most important exogenous factor identified so far is diet. It has been proposed that red meat and substantial alcohol consumption contribute to colon cancer, whereas foods containing dietary fibers, garlic and milk seem to have a protective effect. Other risk factors for the development of

colon cancer are smoking and presence of inflammatory bowel disease (IBD) [106, 110], whereas long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) seems to have a protective effect [111, 112].

Today the primary treatment for colon cancer is surgery, and in some cases adjuvant therapy, which can be different kinds of treatments including chemotherapy or radiation treatment, which are given after the primary treatment to reduce the risk of relapse and death [106]. The treatment strategy is partially dependent on the tumor stage, according to the TNM classification system[106] (Table 2). Although radiotherapy and chemotherapy is improving, there is still a need for an efficient immunotherapy against colon tumors [113, 114]. However, for the immunotherapy to be efficient, the tumor-specific cells have to be able to migrate to the tumor site where they can exert their function.

MECHANISMS FOR TRANSENDOTHELIAL MIGRATION

The immune systems ability to recognize and respond to foreign antigen is dependent on cell-to-cell contact and therefore, a proper distribution of lymphocytes to various lymphoid tissues and effector sites throughout the body is fundamental for proper immune functions [115]. Most mature lymphocytes continuously circulate from the blood to the tissues and back to the blood again as much as one to two times per day [14, 115]. However, this recirculation is in no way random, but tightly regulated by several different lymphocyte-endothelial interactions dependent on the interplay between lymphocyte surface molecules and molecules expressed on the endothelium, but also tissue specific chemokines and their corresponding receptors [115].

The adhesion molecule families of most interest in the process are the selectins, the integrins and the Ig superfamily. The selectins (L-, P- and E-selectin) are a family of transmembrane carbohydrate-binding molecules. E- and P-selectins are expressed on activated endothelium and initiate the recruitment process by binding passing leukocytes [116]. Integrins are a family of transmembrane heterodimeric glycoproteins consisting of one α- and one β-subunit that are covalently linked together [117]. The integrins mediate cell adhesion and transmit signals within the cell that lead to cell spreading, retraction, migration and proliferation [118]. When the leukocyte becomes stimulated through its chemokine receptors, a conformational change in the integrins is initiated, enabling a firm adhesion to the endothelium [119]. The Ig-superfamily comprises

of proteins with shared structural features in the so-called Ig-domain. Some of the members are important in adhesion as being counter-receptors for integrins, such as ICAM-1 and 2, VCAM-1 and MAdCAM-1 [120]. To leave the blood stream, lymphocytes need to undergo four different adhesion steps: rolling, activation, firm adhesion and transmigration (Fig 5).

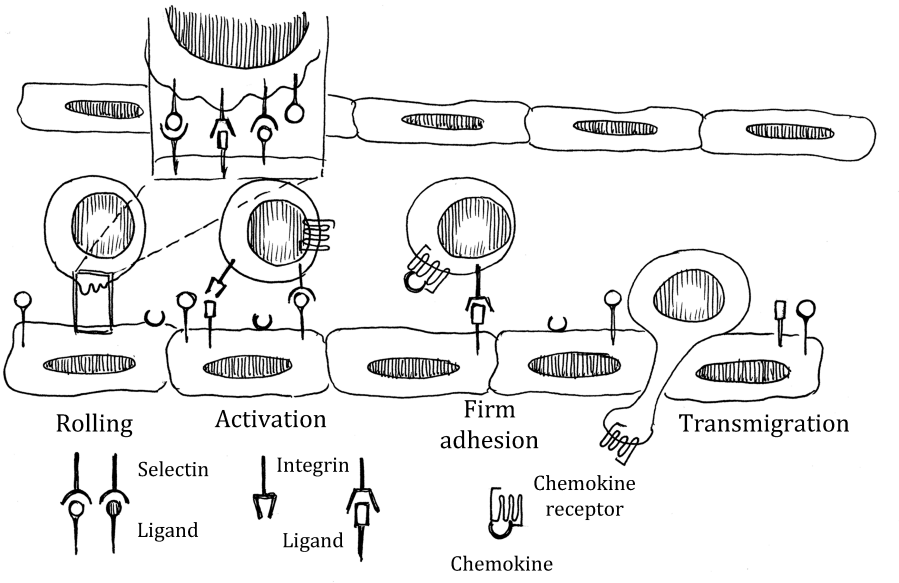


Figure 5. Overview of transendothelial migration.

L-selectin (CD62L) is expressed on the surface of most lymphocytes and binds to peripheral lymph node addressin (PNAd), mucosal addressin CAM-1 (MAdCAM-1) or glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), situated on the endothelial cells in the blood vessel wall [121, 122]. This binding is necessary for homing since it initiates rolling of the lymphocyte, but is too weak to promote extravasation. Rolling slows down the lymphocyte, causing it to roll on the endothelial surface, allowing it to effectively sample the endothelial surface and thereby enhance the chance of encountering chemokine ligands present. Rolling also allows G-protein-coupled chemokine receptors (such as CCR7) on the lymphocyte to come into contact with immobilized chemokines (such as CCL19) on the endothelial cell surface. Binding of the chemokine receptor to its ligand mediates a conformational change of the lymphocyte integrin that enables the integrins to bind with high affinity to their ligands on the endothelium, leading to cell arrest. This firm adhesion is mediated by the binding of

integrins on the lymphocyte surface, such as $\alpha_4\beta_7$ (gut-homing integrin), $\alpha_4\beta_1$, (VLA-4) and $\alpha L\beta_2$ (LFA-1), to their respective receptors, MAdCAM-1, VCAM-1 and ICAM-1 [123, 124]. Once the lymphocyte arrests there is a rearrangement of the cytoskeleton, making the cell flatten and finally cross through the epithelium [125].

Homing to specific tissues

During homeostatic conditions, cells of both the innate (neutrophils, monocytes, NK cells, eosinophils and basophils) and adaptive (T and B cells) immune system circulate in the blood. Monocytes and granulocytes are unable to re-circulate between blood and tissue, whereas mature lymphocytes constantly re-circulate to find their antigen. Naïve lymphocytes are programmed to re-circulate through basically any secondary lymphoid tissues until they encounter their antigen. Lymphocytes pass from the blood to the lymphoid tissues through specialized venules called high endothelial venules (HEVs). This process is mediated by CCL19 produced by the HEV and CCL21 produced in the lymphoid tissue. These chemokines bind to CCR7 expressed on the surface of the naïve lymphocyte [126].

T cells that have encountered their antigen, on the other hand, preferentially migrate to a certain tissue or organ. Activated T cells display a different set of integrins on their surface depending on where in the body they are heading. The best characterized homing pathways for T cells are those leading to the gut and to the skin. Gut homing lymphocytes express integrin $\alpha_4\beta_7$, which binds to MAdCAM-1 expressed on gut-associated endothelium [127-129]. The majority of T cells in the small intestine, but few in the large intestine, express CCR9, which is the receptor for CCL25 (TECK). CCL25 is produced and secreted by the intestinal epithelium in the small intestine and becomes tethered to the endothelium to facilitate T cell entry [130-132]. Also $\alpha_E\beta_7$ binding to E-cadherin is important for the location of cells to the small intestine [132]. Additionally, in the lamina propria the majority of T cells also express CXCR3 and CCR5 [133]. There is also emerging evidence that retinoic acid (RA) generated in DC present in PP or MLN is able to imprint intestinal homing properties on activated T and B cells. The presence of RA induces expression of $\alpha_4\beta_7$ and CCR9 on both T and B cells, enabling them to home to the intestinal mucosa [134]. Overall, the small intestinal homing mechanisms are relatively well-known, whereas the mechanisms for homing to the colonic mucosa are less well defined.

Leukocyte recruitment during inflammation

The number and composition of intestinal lymphocytes is altered in an inflammatory setting. Recruitment mechanisms depend on the cell subset recruited, but also on the type of inflammation [132]. Endothelial cells may increase or decrease their expression of homing receptors, and they are also able to express new ones. Cytokines produced in response to inflammation have also been shown to influence homing by altering the expression of addressins (ligands of homing receptors) on the endothelium. For example, the proinflammatory cytokines IL-1β and TNF-α up-regulate the expression of ICAM-1 on the vessel wall [135, 136]. Inflammatory cytokines primarily lead to attraction of neutrophils, monocytes and other cells of the innate immune system, resulting in a large increase in the number of innate cells in the inflamed tissue.

Since different pathogens mount different effector responses, the homing profile for Th1 and Th2 cells is somewhat different. Th1 cells preferentially express CCR5 and CXCR3, which bind to inflammatory chemokines. They also express P-selectin glycoprotein ligand 1 (PSGL-1) on their surface and mainly utilize P- and E-selectin, which are expressed by inflamed endothelial cells. On the other hand, Th2 cells mainly express the eotaxin receptor CCR3, which is also expressed on eosinophils, basophils and mast cells, but also CCR4, CCR8 and CXCR4 [125, 137]. However, studies have shown that the majority of the CCR4-expressing memory type T cells also co-express CXCR3 and CCR5, indicating that there is no straightforward way to distinguish between Th1 and Th2 cells based on chemokine receptor expression [138]. Many of these cells do however display an activated ($CD69^+$) phenotype [139].

Th17 cells migrating to the intestine and associated lymphoid tissues predominantly express CCR6 on their surface. This chemokine receptor guides them into Peyer's patches where the CCR6 ligand CCL20 is expressed [140]. The signals that direct $CD8^+$ T cells are less elucidated but are believed to be similar to those of $CD4^+$ T cells, and when stimulated by an antigen they secrete inflammatory cytokines recruiting neutrophils, monocytes and Th1 cells to the site [137].

CHEMOKINES

Chemokines regulate not only leukocyte entry into the circulation, but also emigration from the blood, positioning in the tissue, and departure from the tissue [141]. They are a

family of approximately 50 small (8-10 kDa), structurally-related and mainly secreted molecules, characterized by the variations of a conserved cysteine motif in the mature protein. The chemokine family is usually divided into four different subclasses based on the arrangement of the two conserved N-terminal cysteine residues, CC, CXC, XC and CX3C. In the CXC chemokines the first two cysteines are separated by a single amino acid, whereas in CC chemokines they are adjacent [142, 143].

Based on their functional properties in the immune system, chemokines are also divided into homeostatic and inflammatory chemokines. Inflammatory chemokines such as CCL2, CCL5 and CCL22, are not expressed normally but rather induced as a response to injury and inflammatory stimuli in order to recruit effector cells to the site. Homeostatic chemokines such as CCL22, CCL25 and CXCL12, on the other hand, are constitutively expressed at specific sites to regulate lymphocyte localization during normal conditions [144, 145].

It was for a long time believed that chemokines formed a soluble gradient across the blood vessels in order to direct leukocytes. This is however not possible since the flow of plasma in the blood vessel would instantly wash the chemokines away [146, 147]. Instead the hypothesis is that the chemokines induce adhesion and transmigration of leukocytes while immobilized on the endothelial cell surface [146-148]. Charged sulfated sugars of the glycosaminoglycan family on endothelial cells can bind chemokines and present them to leukocytes [148, 149].

Chemokine receptors

There are receptors with affinity for CC or CXC chemokines, and usually the receptor is restricted to binding chemokines from only one subgroup. Some chemokines interact with only one receptor while others are able to bind several different receptors. Many of the receptors are able to bind several different ligands from the same group, whereas some only bind one chemokine [143, 150]. See Table 2 for examples of chemokine receptors and their ligands.

With the exception of a few, recently identified receptors, chemokine receptors belong to the seven transmembrane spanning G-protein-coupled family. These receptors all have a highly conserved DRYLAIV sequence in the second intracellular loop and engage the same intracellular pathways [141, 151]. Ligand binding to the receptor triggers an

intracellular signaling cascade, activating different cell functions. What type of function is triggered is dependent on cell type and receptor expression. Chemotaxis, angiogenesis, up-regulation of integrins and proliferation are some examples of cell activation as a response to chemokine binding [143, 152].

Table 2. Selected **c**hemokine receptors and their ligands.

RECEPTOR NAME	LIGANDS	EXPRESSED ON
CXCR3	CXCL9, CXCL10, CXCL11	Mainly Th1 cells, Activated T- and NK-cells.
CXCR4	CXCL12	T cells.
CCR4	CCL2, CCL3, CCL5, CCL17, CCL22, etc	Mainly Th2 cells. T-, B- and NK- cells. Neutrophils, endothelial cells.
CCR5	CCL3, CCL4, CCL5	T-and B-cells, macrophages, monocytes and lymphoid organs.
CCR7	CCL19	Activated T-and B-cells.
CCR8	CCL1	Monocytes, Th2 cells and thymus.
CCR9	CCL25	T cells in thymus, small intestine and colon.
CCR10	CCL2	T cells and endothelial cells.

Decoy chemokine receptors

In addition to the classical signaling receptors inducing certain functions in the responding cell, there is a group of "silent" receptors, also known as interceptors (internalizing receptors), with distinct specificity and tissue distribution, consisting of the chemokine receptors D6, DARC and CCX-CKR. Common for these receptors is that the DRY motif in the second intracellular loop, which is involved in G-protein coupling, is not conserved, making them unable to signal in the classical way or to mediate chemotaxis [153-159].

D6

The chemokine receptor D6 binds with high affinity to at least 12 inflammatory CC chemokines, but none of the constitutively expressed ones [153, 158, 160, 161]. It is expressed mainly on lymphatic endothelial cells at barrier tissues such as lung, skin and gut [162], and is also strongly expressed by trophoblasts in the placenta [161]. Our study (paper II) as well as McKimmie *et al* have also found low levels of D6 on circulating leukocytes [163].

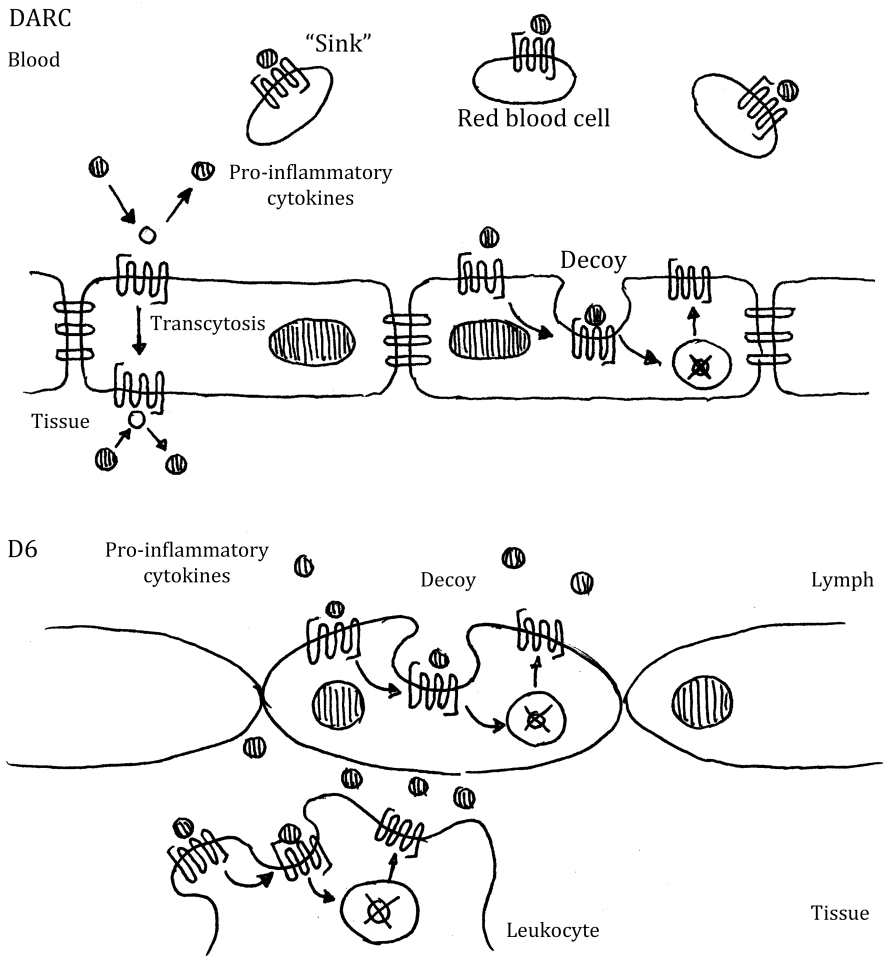


Figure 6. Proposed functions for DARC and D6.

In several aspects D6 is a unique receptor, it constitutively re-circulates to and from the cell surface to internalize and degrade chemokines in a process that is not dependent on ligand binding [153, 158, 160, 164]. When the ligand is internalized by the receptor, it is retained and targeted for degradation while D6 returns to the cell surface available for binding new pro-inflammatory CC chemokines [164] (Fig 6). In contrast to many other chemokine receptors, D6 expression is not down-regulated upon binding [164]. The proposed role for D6 is therefore, to be a scavanger for inflammatory chemokines. Furthermore, D6 expressing cells show an increased scavenging rate when exposed to increasing chemokine concentrations, probably due to translocation of intracellular D6,

situated in vesicles, to the cell surface [164, 165]. D6 has therefore been postulated to act in a dose-dependent manner.

As mentioned above, D6 is strongly expressed in lymphatic vessels and its position enables it to function as a gatekeeper for inflammatory CC chemokines, preventing diffusion into afferent lymphatics and transport to the lymph nodes [153]. It thereby reduces the bioavailability of inflammatory CC chemokines *in vivo*, which is important to resolve an inflammatory response, avoiding development of chronic inflammation [154, 156].

D6 $^{-/-}$ mice have been generated and the experiments conducted are consistent with the role of D6 as a scavenger *in vivo*; unable to clear chemokines from inflamed sites. For example, D6 $^{-/-}$ mice have an exacerbated inflammatory response following application of phorbol ester to the skin, resulting in an aggressive cutaneous inflammatory pathology similar to that of psoriasis [154]. D6 has also been shown to be an early regulator of tumor formation in mice, most likely in tumors in which inflammation is an important mechanism [166]. Recently it was reported that D6$^{-/-}$ mice are more susceptible to dextran sulfate sodium (DSS)-induced inflammation and subsequent tumor formation [167].

In human breast tumors, D6 expression is positively correlated with increased disease-free survival [168]. It was also recently reported that D6 expression is elevated in IBD-associated colon cancer, suggesting that D6 plays a critical role in the development of inflammation-associated colon cancer [169]. To our knowledge, D6 has not yet been characterized in colon cancer patients lacking a history of IBD, and it would therefore be interesting to examine the expression of D6 in this type of tumor.

DARC

DARC (Duffy antigen receptor for chemokines) was identified as the erythrocyte receptor for IL-8, in addition to binding malaria parasites [170, 171]. It binds both CC and CXC chemokine, and is thereby the only known mammalian chemokine receptor able to bind chemokines from more than one subfamily. Notably, all chemokines binding DARC are inflammatory [172, 173]. DARC is expressed on erythrocytes, but also by endothelial cells of the venules and veins in different organs and has therefore, been suggested to act as a sink for the excess of circulating chemokines [174-176] (Fig 6). This theory has recently been

argued as Pruenester *et al* demonstrated that there is no increase in cellular degradation of cytokines that can be associated with DARC expression. Instead, the hypothesis postulated is that DARC acts as a reservoir for chemokines and enables a consistent level of chemokines to be maintained over a longer period of time [177]. In addition to this, DARC also participates in a process known as transcytosis. In this process chemokines are carried across the endothelial cell from the basal to the apical face, exposing the chemokines to leukocytes passing in the bloodstream, thereby promoting adhesion and endothelial migration [148, 150, 176, 177].

In vitro studies have shown that tumors derived from DARC-expressing cells are significantly larger in size than tumors originating from cells lacking a DARC expression. The larger tumors also displayed more necrosis, decreased vascular density and a reduced metastatic potential [178]. In both a mouse study of melanoma and one of prostate tumor cells, it has been shown that DARC expression on endothelial cells leads to a decrease in metastatic tumor survival [179]. Few studies have been conducted with clinical samples, but in human breast cancer a low expression of DARC seems to be associated with increased lymph node metastasis [180]. To our knowledge, DARC expression has not been studied in colon cancer.

CCX-CKR

CCX-CKR (Chemo Centryx chemokine receptor) binds to the homeostatic chemokines CCL19, CCL21 and CCL25 with high affinity. Once bound to the receptor, the chemokines are internalized and targeted for destruction [159, 181]. In humans, CCX-CKR mRNA expression has been found in DC, T cells, spleen and lymph nodes, as well as in several non-hematopoietic organs [159]. So far, little is known about CCX-CKR, but it has been proposed to be involved in both homeostatic trafficking and the regulation of DC and lymphocytes trafficking during inflammatory and autoimmune conditions [182]. Also, a recent study by Feng *et al* showed that an over expression of CCX-CKR in breast cancer cells inhibits the proliferation and migration of those cells *in vitro* and to our knowledge, the expression of CCX-CKR has not yet been studied in human colon cancer. In human breast cancer, on the other hand, there seems to be a negative correlation between CCX-CKR expression and lymph node metastasis, and a low expression is correlated with reduced survival [183].

Although the decoy receptors are all important on their own, a recent study shows that they may have a combined effect. Zeng *et al* studied the expression of DARC, D6 and CCX-CKR in human breast cancer and found the combination of receptors to be a prognostic marker for relapse-free survival as well as for overall survival. In contrast, each receptor by itself was only favorable for a relapse-free survival, but not associated with overall survival. It was also shown that co-expression of these decoy receptors is much lower in invasive, compared to non-invasive, breast cancer [184].

REGULATORY T CELLS

T cells with the ability to suppress, rather than augment, the immune response were discovered in the 1970s [185]. It was however, difficult to prove the existence of these suppressor T cells and the T cell subset was never totally accepted. In 1995 a unique population of $CD4^+CD25^+$ T cells essential for the prevention of autoimmunity reactions was discovered. These cells were referred to as regulatory T cells (Tregs) [186]. Murine studies have shown that depletion of Treg results in various autoimmune diseases such as inflammatory bowel disease, gastritis and type 1 diabetes but also enhances the immune response against non-self antigens such as commensal bacteria [186-188].

Markers of Treg

FOXP3: The forkhead family transcription factor FOXP3 was discovered as a critical regulator of Treg development, function and homeostasis in 2003 [189, 190]. The importance of FOXP3 is shown in patients with *FOXP3*-mutations which develop a severe, fatal systemic autoimmune disorder called Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome. It is an early onset disorder only present in boys and causes enlargement of the secondary lymphoid organs, type 1 diabetes, infections and food allergies [191, 192]. At present, FOXP3 is the most specific marker for distinguishing between Tregs and non-Tregs. The drawback however, is that FOXP3 is an intracellular marker, making us unable to sort living cells based on its expression, and also conventional T cells are able to transiently express FOXP3 upon stimulation [193, 194].

CD25: The most extensively used marker for characterization and isolation of Tregs is the IL-2 receptor α-chain, CD25. The expression of CD25 on human peripheral blood $CD4^+$ T cells is heterogenous. Approximately 3% of $CD4^+$ T cells in the blood express high

levels of CD25 ($CD25^{high}$), and this population harbors the Tregs. Low to intermediate levels of CD25 are expressed on activated effector and memory cells, making up 20% of the total $CD4^+$ T cells in blood. Additionally there is a group of cells lacking CD25 expression which are mainly naïve cells, but probably also resting memory cells [195].

CTLA-4: Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is constitutively expressed on Tregs, but also on activated T cells [196]. It is the structural homologue of co-stimulatory molecule CD28 and is an important negative regulator of both T cell and APC activation. Both CTLA-4 and CD28 binds to CD80/86 on antigen presenting cells (APC). However, binding of CD28 transmits a stimulatory signal to the cells, whereas CTLA-4 delivers an inhibitory signal to APC and T cells [196, 197]. Therefore, CTLA-4 is necessary for Treg function. The drawback with CTLA-4 as a Treg marker is that it is not exclusively expressed by Tregs, and also it is an intracellular marker therefore, it is not possible to sort living cells with based on its expression.

CD127: The IL-7 receptor (CD127) is down-regulated on $FOXP3^+$ cells in peripheral blood, and also on $FOXP3^+$ cells expressing low levels or no CD25. In combination with CD4 and CD25, it is an efficient way to identify Treg by surface markers and provides the ability to sort living cells [198, 199].

Suppressive mechanisms of Treg

Treg have a suppressive effect on $CD4^+$ and $CD8^+$ T cells and in addition, can also suppress DC, NK cells, NKT cells, mast cells and B cells [200, 201]. The suppressive mechanisms of Treg in all these different settings remains to be determined, but several mechanisms have been proposed. Possibly, Treg are able to use different effector mechanisms in different settings. Alternatively, different, as yet uncharacterized, subpopulations of Treg utilizing different effector mechanisms may exist.

Immunosuppressive cytokines: The most important inhibitory cytokines secreted by Treg are IL-10 and TGF-β [202]. In our previous human *in vitro* studies it was indicated that the ability of natural Tregs to suppress immune cells is independent of IL-10 and TGF-β [203]. In contrast, there are also studies showing that IL-10 and TGF-β is indeed involved in the suppressive mechanism. While these contradictory results may be due to the study design, this contradiction is also seen in mouse studies. Interestingly, some studies show

that these cytokines are important in models of inflammatory bowel disease but not for suppression of gastritis [204-206].

Targeting DCs: One way for Tregs to indirectly suppress effector T cells is to reduce the capacity of DC to activate T cells. Cells are in direct contact with each other, and seem to be able to break up the stable contact between APC and the naïve $CD4^+$ T cell [207]. Tregs down modulate CD80 and CD86 on the APC in a CTLA-4-dependent manner [208], making the APC unable to activate naïve T cells.

Cytolysis: It has been suggested that Tregs also control immune responses through cytolysis. *In vitro* it has been shown that human Tregs express granzyme A and small amounts of granzyme B, and that these cells, in a perforin-dependent way, are able to kill autologous activated $CD4^+$ and $CD8^+$ T cells, DCs and monocytes [209]. This mechanism seems to be of little importance in the gastric mucosa since a study by Enarsson *et al* showed that Tregs from both tumor and unaffected gastric mucosa do not express granzyme A, granzyme B or perforin [210].

Tregs in cancer

Tumors have developed numerous strategies with which they can avoid an anti-tumor response, and one is to suppress the immune system by increasing the amount of Tregs. However, the mechanisms by which Tregs home to tumors are poorly characterized. In the tumor, Treg are capable of suppressing a wide range of anti-tumor responses, such as $CD4^+$ helper T cells, $CD8^+$ T cells, NK cells and NKT cells [211]. Several studies show increased numbers of circulating Tregs in gastric cancer patients compared to healthy individuals [212, 213]. In addition, a recent study indicates that proliferation of Tregs is promoted inside gastric tumors (Kindlund *et al*, unpublished). It has previously been shown in our group that patients with *H. pylori*-induced gastric cancer not only have a higher frequency of Tregs [65, 203], but also that these patients display a reduced transendothelial migration of conventional T cells. The effect was largely dependent on cell-cell contact and migration was restored after Treg depletion [203].

Increased Treg infiltration into tumors has been shown to correlate with poor clinical outcome for several epithelial tumors, including breast cancer and ovarian cancer [214, 215]. It seems though, that Tregs may be beneficial for the individual in some cases. Studies in colon cancer patients show increased numbers of Tregs in the tumor compared to the

surrounding tissue, but also that a high Treg frequency correlates to a better prognosis [216-218], also in combination with chemotheraphy or chemoimmunotherapy [219].

Treg trafficking

To be able to directly suppress T cells, Tregs need to migrate to the same areas as the effector T cells. It is still a bit of a mystery as to how Tregs migrate relative to conventional T cells during both normal conditions and immune responses. Tregs isolated from the thymus express high levels of CCR8, and this receptor has been proposed to be involved in Treg differentiation or migration [220]. In peripheral blood, Tregs express CCR4 and CCR8, making them able to migrate to dendritic cells and sites of antigen presentation in inflamed tissue and secondary lymphoid tissue [221]. Upon T cell priming, CXCR5 is induced on Tregs and the cells can be found in the T-B cell border as well as in germinal centers, possibly suppressing B cells and antibody secretion [222]. Surprisingly most Tregs are negative for the gut homing receptor $\alpha 4\beta 7$ but express cutaneous lymphocyte antigen (CLA) [223]. However, Tregs manage to somehow migrate to the intestinal mucosa despite lacking this receptor. In murine studies, RA treatment induces $CD4^+$ T cells to express FOXP3 and also to acquire expression of $\alpha_4\beta_7$ as well as CCR9 [224]. Additionally, co-culture of naïve T cells with splenic DC in the presence of TGF-β and RA generates gut-homing Tregs [225]. However, it is not yet known if the same mechanisms are utilized in humans.

SPECIFIC AIMS

The overall aim of this thesis was to investigate the mechanisms for recruitment of leukocytes to *H. pylori*-infected as well as tumor-associated mucosa.

The specific aims were:

- To investigate the effect of *H. pylori* infection on endothelial adhesion molecule expression and to identify the potential *H. pylori*-derived factors involved.
- To characterize the immune cells infiltrating colon tumors, and the mechanisms for their recruitment.
- To determine the expression of decoy chemokine receptors in colon tumors.

VOLUNTEERS AND BRIEF METHODS

HUMAN VOLUNTEERS AND COLLECTION OF MATERIALS

The regional board of ethics in medical research in west Sweden approved the studies in this thesis and informed consent was obtained from all participating individuals.

PAPER I

H. pylori positive and negative participants in the first study were recruited among blood donors at Sahlgrenska University hospital. Infection with *H. pylori* was initially determined by serological analysis and later on confirmed or excluded by culture on Scirrow agar plates and pathology reports. From each individual ten antrum biopsies were collected by endoscopy. Three of those were snap frozen and stored in -70°C for subsequent protein extraction, three were embedded in optimal cutting temperature, snap-frozen and stored in -70°C for immunohistochemical analysis and three were put in RNA Later (Ambion) over night and then stored in -70°C for subsequent RNA isolation. The last biopsy was formalin fixed, paraffin embedded and analyzed by an experienced histopathologist for the grade of gastritis and presence of *Helicobacter*-like organisms (HLO) using the updated Sydney system [226]. Two weeks after the endoscopy the uninfected individuals were treated with ASA (1.5 mg/day) for three days, and biopsies were collected following the same procedure as previously described.

In the second part of this study patients who had undergone colectomies due to ulcerative colitis five to ten years earlier were recruited from the follow-up program at the Department of Surgery at Sahlgrenska University Hospital. The patients had undergone a continence surgery by the construction of a pelvic pouch with an ileal anastomisis. Subclinical pouchitis results in a neutrophil-mediated inflammation, which do not need to be treated. None of the patients had any ulcerative colitis or acute pouchitis during three years prior to sampling and they were all in good general health. Five biopsies were collected from the ileal pouch and twelve pinch biopsies from the duodenum of each patient [227].

In addition, plasma samples from *H. pylori*-infected and uninfected individuals were collected among blood doners at Sahlgrenska University Hospital and stored at -20°C until further use.

PAPER II AND III

Patients undergoing partial colectomy at Sahlgrenska University Hospital due to colon adenocarcinomas were included in this study. None of the patients had undergone radiotherapy or chemotherapy for at least three years prior to the operation, and patients with autoimmune diseases were excluded from the study. Information about tumor stage, differentiation grade and metastasis was retrieved from the routine pathology report. We also followed the patients for 22 to 54 months and recurrence of cancer or cancer-related death was recorded. During or immediately after colectomy a strip of the tumor was collected along with a strip of unaffected mucosa. We defined unaffected mucosa, as being collected at least five centimeters away from the tumor. Small pieces of the tissue were snap frozen for protein extraction, pieces were also embedded in OCT for subsequent IHC, and some were collected in RNAlater for isolation of RNA. The remaining tissue was transported in cold PBS and used for isolation of LPL.

H. PYLORI STRAINS AND BACTERIA PREPARATION

All the *H. pylori* strains used were a kind gift from Dr. Steffen Backert at University Collage, Dublin. The following isogenic mutants of the *cag*PAI-positive strains P1 and P12 were produced by the insertion of a kanamycin or chloramphenicol resistance gene cassette: P1Δ*virD4,* P1Δ*virB10,* P1Δ*cagA,* P1Δ*napA,* P1Δ*vacA,* P1Δ*ureA and* P12Δ*cagL* [48, 228].

All bacteria were cultured on Colombia ISO-A plates, P1 isogenic mutants with an addition of chloramphenicol and P12 isogenic mutants with an addition of kanamycin to ensure that no other strains of *H. pylori* grow on the plates. Before use in stimulation assays, bacteria were scraped off, resuspended in PBS and diluted to OD_{600}=1.

SUB CULTURING OF ENDOTHELIAL CELLS AND STIMULATION WITH *H. PYLORI* BACTERIA

Primary Human Umbilical Vein Endothelial cells (HUVECs) were purchased and grown in M200 medium supplemented with the LSGS kit. To examine the cell-surface expression of E-selectin, HUVEC were incubated in medium containing *H. pylori*, TNF-α (positive control) or PBS (negative control) for 6 h, which has been determined to be a suitable incubation time for this experiment.

REAL TIME RT-PCR

A relative quantification real time RT-PCR was used to detect the mRNA levels of E-selectin in *H. pylori*-infected and uninfected gastric mucosa. The presence of the *cag*PAI in *H. pylori* strains on the other hand was detected by an absolute quantification real time RT-PCR. Relative quantification was also used in the colon cancer study to detect differences in mRNA levels of D6, DARC and CCX-CKR in unaffected and tumor colon mucosa. The RT-PCR is based on the same principles as a regular PCR reaction, but with the advantage that it detects the amount of DNA product after every cycle, and not only the plateau phase as in regular PCR.

RNA is extracted from the cells or the tissues of interest using the RNeasy Mini protocol which is based on binding of mRNA to a silica membrane while other components in the cell lysates are rinsed off during several centrifugation steps. The samples are then treated with DNase to remove genomic DNA, thereby avoiding interference with the product of interest. Thereafter, the RNA integrity is checked by running the samples on an agarose gel, and concentration measured on nanodrop. Before running the RT-PCR the extracted RNA has to be converted to complementary DNA (cDNA), since the DNA polymerase can act only on DNA templates. The cDNA reaction is performed using a kit containing omniscript reverse transcriptase, an enzyme that is reversely transcribing the RNA into cDNA.

To amplify only the product (mRNA) of interest, specific primers are designed. Since cDNA contains only the coding sequence of the gene the cDNA, in contrast to genomic DNA, contains no introns and this is utilized when designing the primers. To avoid amplifying the genomic DNA and thereby get erroneous results, the primers usually are designed to span over a region of the gene that includes and exon-exon boundary making the primers able to only bind to the cDNA. The first step in PCR is denaturation of the DNA to separate the two cDNA strands, thereafter the temperature is lowered to the annealing temperature which allow the primers to bind to their corresponding complementary regions in the gene, finally elongation is performed at a temperature optimal for the DNA polymeras. For each PCR cycle the material is duplicated leading to an exponential amplification, and due to a fluorescent reporter molecule the amplification process can visualized.

ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to determine serum levels of E-selectin in blood plasma from *H. pylori*-infected and uninfected individuals, and to detect the concentrations of CXCL11 (I-TAC), CCL17 (TARC), and CCL22 (MDC) in saponin extracted biopsies.

ELISA is a method that can be used to detect the concentration of a specific antigen in a sample with the help of a standard curve of known concentrations. In this thesis, duoELISA kits were used. First, a 96-well plate is incubated with an antibody specific for the antigen of interest. This will coat the surface of the well. Non-specific binding sites will thereafter be blocked. The standards and the samples are added to the well and the antigen binds to the immobilized antibody, any unbound antigen is washed away. An antibody conjugated to the enzyme HRP is added to detect the antigen, and the excess washed away. When the substrate (TMB) solution is added to the wells, HRP catalyzes the oxidation of TMB by using hydrogen peroxide as the oxidizing agent, developing a blue color proportional to the amount of antigen present in the sample. To be able to read the absorbance the development is stopped by sulfuric acid (H_2SO_4) and the shift in pH makes the color changes into yellow. The absorbance is measured at 450nm, and the concentration in each well can be calculated from the standard curve.

CYTOMETRIC BEAD ARRAY (CBA)

Cytometric bead array (CBA) was used to determine the levels of different chemokines in tumor and unaffected tissue. The CBA technique uses a series of spectrally discrete particles that can capture and quantify soluble antigens, enabling us to measure the concentrations of several chemokines in each sample simultaneously. We used BD-CBA kits in which the capture beads for each chemokine has a known size and fluorescence, making it able to detect and separate them using flow cytometry. Each capture bead has been conjugated with a specific antibody, and is first incubated with the sample or standard and is then detected by an antibody coupled to a fluorescent dye. This results in complexes, which can be measured using flow cytometry which records the fluorescence characteristics of both the bead and the detector. The detector reagent provides a fluorescent signal that is in proportion to the amount of antigen bound to the beads. The concentrations of the unknown samples are then calculated from a known standard curve included in each experiment.

IMMUNOHISTOCHEMISTRY

Immunohistochemical methods are used to locate a target molecule in single cells or in a tissue. In the present studies we used 8μm thick frozen tissue sections to detect the distribution of different T cells in the tissues, as well as the presence of endothelial adhesion molecules. Antibodies against proteins recognize the native, folded structure of the protein and therefore this structure needs to be preserved by a chemical fixation, in this case acetone. In this thesis two different methods have been used, the avidin-biotin complex method for light microscopy and an immunofluorescence method.

The avidin-biotin complex method: The high affinity of avidin for biotin is utilized to amplify the signal from a specific antigen, to facilitate detection. Three different reagents are necessary; a primary antibody (sometimes biotinylated) to detect the target antigen, a secondary biotinylated (or HRP-conjugated) antibody, and an avidin-biotin complex to detect the biotinylated antibody. Since this method involves an enzymatic reaction, it is important not only to block endogenous avidin and biotin, but also endogenous peroxidase. Avidin is pre-incubated with biotinylated horseradish peroxidase to form complexes, and due to the high affinity of avidin for biotin macromolecular complexes may form, also, the ratio is such that there is always an available biotin-binding site available for binding of the biotinylated secondary antibody (Fig 7). To visualize the peroxidase and thereby the antigen, a chromogen solution is required, in this thesis DAB was used. When oxidized by peroxidase DAB generates a permanent brown precipitate where the antigen is situated, which can be visualized in a light microscope. The slides were counterstained with Meyer's hematoxyline to be able to identify where in the tissue our antigen is positioned. Once the slides are mounted they can be archived for future reference.

Immunofluorescence: Immunofluorescence on tissue sections use the same basically principles as the avidin-biotin complex method. Since no enzyme reaction is used there is no need for blocking endogenous peroxidase or incubate with a substrate solution. The primary or secondary antibody is labeled with a fluorescent dye that is excited by light of a specific wavelength in the visible spectrum (Fig 8). Sections stained with fluorescent antibodies were cover slipped with mounting medium containing DAPI, which stains the nucleus and facilitates the orientation in the tissue section.

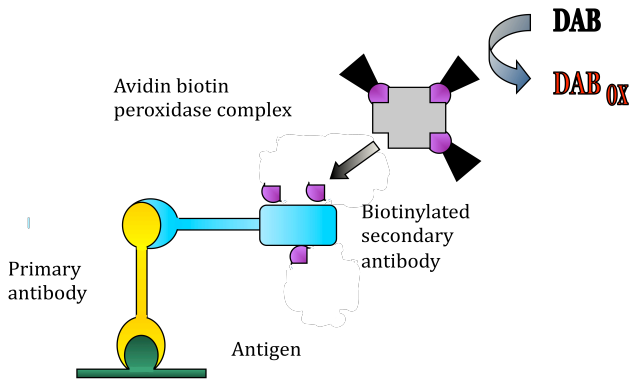


Figure 7. The avidin-biotin complex method.

In all immunohistological methods it is important to use a negative control to confirm the specificity of the primary antibody. The negative control should be obtained from the same species as the primary antibody, but should also be of the same class and subclass and incubated at the same protein concentration as the primary antibody.

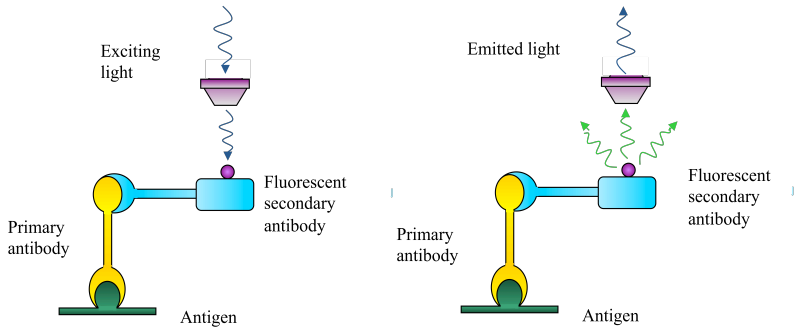


Figure 8. Immunfluorescence method.

ISOLATION OF LPL AND PBMC

To analyze the composition of lymphocytes in the lamina propria from tumor and unaffected mucosa we isolated the cells from these tissues. The fat and muscle layers were removed by dissection and the remaining tissue cut into small pieces. These were incubated in HBSS containing EDTA and DTT to remove the epithelium and intraepithelial lymphocytes (IEL). The epithelial integrity is disrupted by the reducing agent DTT and chelating agent EDTA, the latter is a binding to calcium and prevent joining of cadherins between cells thereby detaching adherent cells. Subsequent incubation in collgense/DNAse solution makes the LPLs come into solution. The use of collagenase detaches the cells from each other by breaking down the extracellular matrix. Since DNA from lyzed cells in the medium can cause cells to clump, DNAse is

added to break down any free DNA and thereby prevent clumping. As a last step the suspension is filtered through a mesh to obtain a more pure LPL population. The cells are then washed and counted under a light microscope.

PBMC were isolated from blood using a density gradient medium (Ficoll-paque). After centrifugation, the density gradient will create different fractions of the blood such as plasma, red blood cells and a lymphocyte band that will be collected, washed and counted under a light microscope. These cells were also characterized by flow cytometry and represent the systemic immune system, rather than a local response.

FLOW CYTOMETRY

Flow cytometry was used to analyze surface and intracellular molecules on lymphocytes and endothelial cells. The cells are first labeled with fluorescent antibodies specific for the characteristics of interest. The cells are required to be in solution and are then recorded in a flow cytometer. A laser beam is directed onto a stream of fluid, and a number of detectors are aimed at the point where the laser hits the stream. One records the forward scatter (FSC) and one the side scatter (SSC) which enables us to select (gate) the lymphocyte or endothelial cell population depending on their size (FSC) and granularity (SSC). The flurochromes bound to the antibodies used for detection of specific antigens (different cell populations or homing phenotypes) are excited to a higher energy when hit by the beam. This energy is released as a photon, unique for every fluorochrome. The cytometer records which fluorochromes are present on the cells as well as the intensity of those, but it also record cells with no flouorochromes. In this way you may gate your cells of interest and analyze their homing phenotype, activation state or expression of adhesion molecules for instance.

CHEMOTAXIS ASSAY

In our studies we analyzed the migration of isolated lamina propria lymphocytes towards a chemoattractant on the other side of a membrane. In short, the upper chamber is loaded with cell suspension containing a specific number of cells diluted into cell medium. The lower chamber is loaded with cell medium containing the chemokine of interest. After incubation the cells that have migrated into the lower compartment are collected, marked with antibodies and TrueCount beads (BD) added. Using flow cytometry this able us not only analyze which cells that migrate towards the

chemoattractant but also to quantify them. This method is very useful to determine if chemokine receptors expressed on the cell surface are actually migrating towards its ligands or not. However, it is a very simplified system and *in vivo* there may be several additional factors involved in the chemotaxis of these cells (Fig 9).

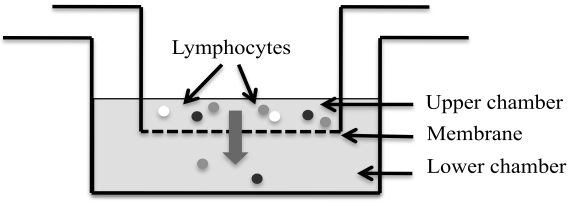


Figure 9. Schematic picture of chemotaxis assay.

FOXP3 METHYLATION ANALYSIS

The transcription factor FOXP3 has been linked to the suppressive activity of Tregs but may also be transiently expressed by human conventional T cells upon stimulation. To analyze if the $FOXP3^+$ cells in the tumor and unaffected mucosa carries the suppressive phenotype we conducted a methylation analysis. This method aims to detect methylation of one of the dinucleotide CpG in the FOXP3 promotor region. Methylated CpG-islands sterically hinders the RNA-polymerase from binding to the promotor region and the FOXP3 gene is not transcribed. In the case of a demetylated promoter region there are no obstacles for the RNA-polymerase and the FOXP3 gene may be transcribed and the Treg is able to exert its suppressive function. We began by sorting $CD4^+$ cells into a $CD4^+FOXP3^+$ population and a $CD4^+FOXP3^-$ population using a FACSAria cell sorter based on the same principles as flow cytometry previously described. Thereafter genomic DNA from these cells was isolated and bisulphite converted. The bisulphite treatment converts all non-methylated cytosines (C) to uraciles (U) whereas the cytosines binding a methyl group are protected from conversion. The coding strand is then amplified by PCR, and in this process all U's are interpreted as T's (thymidine). The PCR product is then subjected to MS-SNUPE (Methylation-Sensitive Single Nucleotide Primer-Extension). This utilizes a primer that bind to the sequence upstream and terminates immediately 5´to the CpG being investigated. When the primer has annealed to its target sequence a single-nucleotide extension reaction is performed in the presence of DNA-polymerase and labeled dNTP. In this way it is possible to determine the ratio of cystein to thymine by capillary electrophoresis by analyzing their corresponding peaks. This method enables us to determine if the $FOXP3^+$ cells in the

tissue have a stable expression, i. e. demetylated promotor site, or if they are recently induced to express FOXP3. It is not possible, though, to draw any conclusions about their suppressive activity *in vivo.*

STATISTICAL METHODS

In the present study we have used the nonparametric tests Mann-Whitney and Wilcoxon Signed rank test. We chose non-parametric test since we cannot assume that our data has a normal distribution. Also, when working with tumors some values could be extremely high or low, and a non-parametric test does not give this value too much impact on the test outcome since it uses ranks instead of absolute values. The Mann-Whitney test is the non-parametric alternative for the *t* test for comparing data from two independent groups. All values are ranked as if they were from a single sample, and then the sum of the ranks from each group is calculated and the P value extracted from a table. The Wilcoxon Signed rank test is the non-parametric equivalent of the paired *t* test and in contrast to the Mann-Whitney test we treat the difference between the paired values as the sample for calculating the ranks. The sum of the ranks is converted into a P-value according to a table for the Wilcoxon test.

RESULTS AND DISCUSSION

ADHESION MOLECULE EXPRESSION IN THE GASTROINTESTINAL MUCOSA DURING INFLAMMATION

H. pylori-induced gastritis alters local adhesion molecule expression

Endothelial cells have a key function in the recruitment of leukocytes to inflamed tissues. To study the influence of *H. pylori*-induced gastritis on adhesion molecule expression in the human gastric mucosa, we collected biopsies from *H. pylori*-infected as well as healthy individuals. Biopsies from uninfected subjects were histologically normal without inflammation or infiltration HLO. In biopsies from *H. pylori*-infected individuals on the other hand, active chronic inflammation and HLO were observed in all biopsies. The up-dated Sydney system was used to calculate a mean chronic inflammation score of 1.88 ± 0.46 (mean ± SD) and the mean active inflammation score was 1.0 ± 0.93. The mean HLO score was 1.75 ± 0.46 and there was no sign of atrophy or intestinal metaplasia in any of the subjects.

We first examined the expression profiles of adhesion molecules E-selectin, ICAM-1, VCAM-1 and VAP-1 by immunohistochemistry and compared the mucosal expression of each adhesion molecule between individuals with *H. pylori*-induced gastritis and uninfected individuals. We found that the endothelial expression of E-selectin was low in the mucosa of most uninfected volunteers, whereas *H. pylori*-infected individuals had a significantly higher expression of E-selectin (Fig 10 and Fig 1, Paper I). On the other hand, adhesion-molecules ICAM-1, VCAM-1 and VAP-1 had a considerable variation within the two groups, and no significant differences were found (Fig. 2, Paper I). This was a bit surprising since a previous study by Hatz *et al* [229] reports an increased expression of both ICAM-1 and VCAM-1, but not E-selectin, in the gastric mucosa of *H. pylori*-infected an uninfected individuals. The contradictory results may be due to the different strategies used in recruitment of volunteers. In our study, all participants were recruited among blood donors at Sahlgrenska University Hospital and they were all asymptomatic. In the study of Hatz *et al*, the participants were instead recruited among patients suffering from dyspepsia and seeking medical care. An additional explanation is that a difference in *H. pylori* strain may influence the adhesion-molecule profile. Previous *in vitro* studies from our lab show that different clinical isolates of *H. pylori*

influence the expression of adhesion-molecules differently, even though they all express the major virulence factors, such as *cag*PAI and vacA, to a similar extent [210, 230].

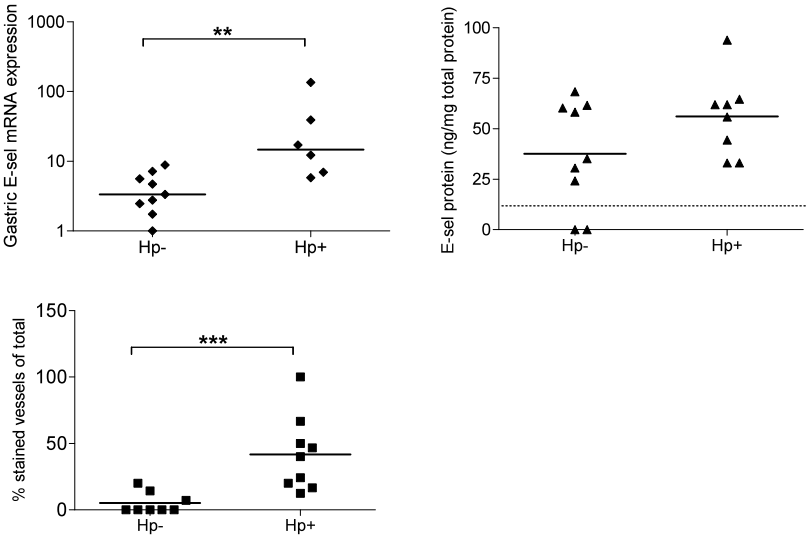


Figure 10. Expression of E-selectin in *H. pylori*-infected and –uninfected gastric mucosa. Symbols represent individual values and the line represents the median. **A.** mRNA levels of E-selectin, measured by relative-quantification real-time RT-PCR. The E-selectin mRNA expression is presented as the change standardized to HPRT and normalized to the lowest mRNA value. **, P< 0.005. **B.** E-selectin in saponin extracted tissues was measured by ELISA and adjusted to total protein levels in the extract. Dotted line represent the detection limit. **C.** E-selectin expression in sections was determined by immunohistochemistry and presented as the proportion positive vessels out of all vessels, as determined by von Willebrand factor. ***, P< 0.0001.

Next we investigated if the increased endothelial expression of E-selectin is present exclusively in *H. pylori*-induced gastric inflammation or if it is rather a phenomenon common for all inflammations in the gastrointestinal tract. We chose to use tissue from colectomized patients with jejunal pouchitis to compare to healthy duodenum from the same patient. This chronic inflammation has a similar inflammatory profile to *H. pylori*-induced gastritis, with an accumulation of neutrophils. Despite this, there was no difference between the inflamed and healthy duodenal expression of E-selectin or any of the other adhesion molecules investigated (ICAM-1, VCAM-1, VAP-1 and CLEVER-1) (Fig 3A, Paper I). We also studied E-selectin expression in chemically induced gastritis to see if the increased E-selectin expression was mimicked in aspirin-induced gastritis. In this experiment the normal biopsies used as internal controls were taken from the same individuals before treatment with aspirin. Aspirin-induced gastritis is relatively mild and characterized by a large production of pro-inflammatory cytokines and recruitment

of neutrophils [231], a profile similar to *H. pylori*-induced gastritis. However, results show that E-selectin expression is similar in the vessel endothelium of the gastric mucosa before and after treatment with aspirin (Fig 3B, Paper I).

This led us to draw the conclusion that *H. pylori*-induced gastritis exclusively up-regulates the expression of E-selectin in vessel endothelium of the gastric muocsa. The specific up-regulation of E-selectin and not other adhesion molecules probably contributes to the large influx of neutrophils and macrophages seen in *H. pylori*-induced gastritis. Selective up-regulation of E-selectin, and not adhesion molecules ICAM-1 and VCAM-1 that are classically up-regulated during inflammation, may be a mechanism used by the bacteria to influence the immune cell population entering the tissue. If the bacteria are able to modulate the type of immune cells entering the tissue this may contribute to the persistence of the bacterium at the epithelial surface.

Induction of E-selectin expression is a local response due to increased mRNA levels

To address the question of whether the increased E-selectin expression is a local endothelial response to the presence of bacteria or rather a general up-regulation in a systemic response to inflammation, we examined gastric and serum levels of E-selectin using ELISA. Saponin-extracted gastric biopsies from both *H. pylori*-infected and asymptomatic, uninfected individuals were low in E-selectin expression. Even though there was no statistically significant difference, the data was skewed towards an increased E-selectin concentration in the infected individuals compared to uninfected (median 56.3 and 39.7 ng/mg total protein, respectively) (Fig 10). In serum from *H. pylori*-infected and uninfected individuals E-selectin had an almost identical median serum concentration (18.8, range 11.5-31.5 and 18.9, range 9.4-49.3, respectively) (Fig 10). Therefore, it seems that the increased expression of E-selectin is restricted to the endothelium in the gastric mucosa in response to *H. pylori* infection, rather than being part of a systemic response to infection.

There are many ways in which the expression of a gene can be regulated. In our study we examined whether the selective up-regulation of E-selectin expression seen on gastric endothelial blood vessel walls was caused by an increase in E-selectin mRNA. RNA was extracted from gastric biopsies from *H. pylori*-infected and uninfected individuals. The levels of E-selectin mRNA were then analyzed using relative

quantification real time RT-PCR. To be able to compare different samples, the mean fold change was standardized to an endogenous reference gene, HPRT. Expression of the housekeeping gene HPRT was not influenced by *H. pylori* infection making it an appropriate choice. To obtain results that were easily comparable, E-selectin expression was also normalized to the lowest expressed value (Fig 10). We found that there was a significant increase in E-selectin mRNA levels in *H. pylori*-infected individuals (p=0.0048) compared to uninfected individuals. These results confirmed that the increased E-selectin expression was indeed due to an increased transcription of the E-selectin gene rather than being regulated post-transcriptionally (Fig 5, Paper I).

H. pylori induce E-selectin expression in a cagPAI-dependent manner

Since *H. pylori* is able to specifically induce expression of E-selectin on endothelial cells *in vivo*, we wanted to identify the bacterial factors involved. To study this *in vitro*, HUVEC were cultured with the *cag*PAI positive *H. pylori* strains P1 and P12 and, the cells were analyzed by flow cytometry. Incubation time for the experiment was optimized to 6 h and the optimal concentration of the positive control TNF-α was measured to be 100 ng/ml. *H. pylori* strains P1 and P12, as well as TNF-α, always induced an increased expression of E-selectin on the HUVECs compared to the unstimulated control (PBS) (Fig 6A, Paper I), showing that the *in vitro* system was functional. Next we used isogenic mutants of the P1 and P12 strains in parallel with the wild-type strain (wt) to be able to identify bacterial phenotypes involved in the induction of E-selectin expression. Stimulation with the isogenic mutant P1Δ*vacA* induced E-selectin expression to the same extent as the wt P1, indicating that the VacA cytotoxin is not involved in induction of E-selectin expression on HUVEC (Fig 6B, Paper I). Furthermore, P1Δ*cagA*, P1Δ*napA* and P1Δ*ureaseA* were all able to induce significant expression of E-selectin, but to a lower extent than the wt. Furthermore, the isogenic mutants P1Δ*virD4* and P1Δ*virB10*, which carry mutations in two of the structural proteins of T4SS in the *cag*PAI, totally abolished E-selectin expression on the endothelial cells. This was also the case for the P12Δ*cagL* mutant which was unable to induce E-selectin expression on the HUVEC cells, in contrast to the parental P12 wt strain which induced significant E-selectin expression (Fig 6A and B, Paper I). These results would imply the T4SS encoded by the *cag*PAI is the most important E-selectin inducing *H. pylori* component. Previously, our group has found that Δ*virD4* and Δ*virB7* mutants have a reduced ability to activate endothelial cells

to support T cell transendothelial migration [210], which is in agreement with the results of this study.

Since the CagA protein was not necessary for induction of E-selectin expression, another mechanism other than CagA phosphorylation must be important for signal transduction. A possible candidate is the CagL protein since it is the only product encoded by the *cag*PAI that contains an arginine-glycine-aspartate (RGD) motif [48], thereby enabling it to serve as a site of recognition for integrins [232]. CagL is targeted to the pilus surface where it interacts with integrin β1 and leads to the activation of Src and FAK, which in turn activates JUN N-terminal kinase (JNK). Since JNK is necessary for endothelial expression of E-selectin, it seems likely that this signal pathway is participating in the induction of E-selectin by *H. pylori* [233-235]. Nod1 reacts with peptidoglycan introduced by the T4SS, and part of the *cag*PAI-dependent signaling may also be mediated by Nod1 [51]. Activation of Nod1 results in the activation of NF-κB, but also of mitogen activated protein kinase (MAPK) and JUN N-terminal kinase (JNK) [236], all of which are involved in endothelial expression of E-selectin [233].

Since *H. pylori* requires a functional *cag*PAI to induce E-selectin expression *in vitro*, we studied whether the *H. pylori*-strains from our clinical specimens carry the *cag*PAI. Absolute quantification real-time RT-PCR was used to analyze the presence or absence of the CagA gene, which is commonly used as a marker for the entire *cag*PAI, in five clinical isolates and four antrum specimens (no isolates available). From all of the five clinical isolates and three of the biopsy specimens we were able to retrieve *H. pylori*-DNA in accordance with our positive control *hpaA*, which is carried by virtually all *H. pylori* strains. In fact, all the clinical strains of *H. pylori* tested were positive for CagA, and thereby *cag*PAI-positive. Our results further indicate that the increased E-selectin expression seen *in vivo* may indeed be a result of the expression of a functional *cag*PAI.

THE COLON MUCOSA OF CANCER PATIENTS

Characterization of LPL in tumor and unaffected colon mucosa

Lymphocyte subsets are similar in tumor and unaffected mucosa

LPL isolated from tumor and unaffected mucosa of patients undergoing colectomy was analyzed with regard to surface molecule expression using flow cytometry. We found that $CD4^+$ and $CD8^+$ T cell subsets were present in similar frequencies in both tissue types. Also, the $CD56^+CD3^-$ NK cells were present in similar, but low, frequencies in both tumor and unaffected mucosa. In contrast, $CD19^+$ B cells were significantly decreased in the tumor compared to the unaffected colonic mucosa (Fig 1, Paper III). This result is similar to a recent finding in our group that B cells and IgA antibody-secreting cells are decreased in gastric tumors [70]. T cell-mediated immunity is however, believed to be more important for anti-tumor responses than B cells [237], and we therefore continued characterizing the T cells to see whether there were differences in the specific T cell subsets.

The frequency of regulatory T cells is significantly increased in colon tumors

Since Treg are able to suppress the immune response and may be of benefit for the tumor, we examined the presence of putative Treg among the LPL. Treg were identified as being $CD4^+CD25^{high}$ cells, and those cells were also invariably $Foxp3^+$ and $CD127^{low}$, as stated in the literature (Fig 2, Paper III) [198]. We found that the frequencies of Treg were significantly higher ($P< 0.0001$) in tumor colon mucosa compared to unaffected colonic mucosa from the same patients. Since FOXP3 can be transiently expressed by human conventional T cells following stimulation, we analyzed if colonic $FOXP3^+$ cells were stably expressing FOXP3 by assessing the methylation status of the *FOXP3* promotor region using Ms-SNUPE. We found that there was an almost complete demethylation in the analyzed CpG site of the *FOXP3* promotor in $CD4^+Foxp3^+$ cells from both the tumor and unaffected tissue (100% demethylation in the tumors and 97-100% in unaffected tissue), indicating that they stably express FOXP3 as part of an established Treg phenotype, rather than being due to a recent activation of conventional T cells. When comparing these results to $CD4^+FOXP3^-$ cells from the colonic tissue, we found these cells to have a lower demethylation (range 51-100% and 53-74% in the tumor and unaffected tissue, respectively) (Fig 11). So indeed, the Tregs found in tumor tissue seem to stably express FOXP3 as part of an established Treg phenotype able to suppress the

effector cells trying to fight the tumor cells. However, it is not known how and why Tregs accumulate in the tumor, but their presence seems to be in favor of the tumor rather than the patient.

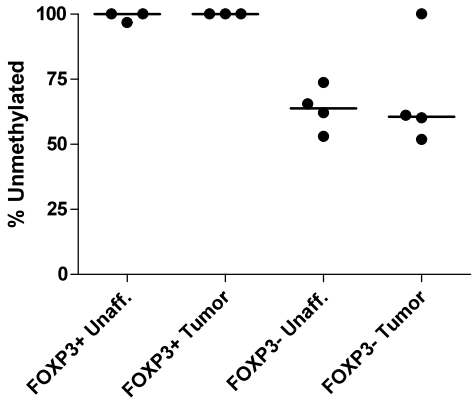


Figure 11. FOXP3 demethylation in FOXP3$^+$ and FOXP3$^-$ LPL from unaffected and tumor tissue. Symbols represent individual values and the line represents the median.

Treg in vivo are in close proximity with CD4$^+$ and CD8$^+$ T cells

With the knowledge that the frequency of Treg is increased in the tumor mucosa compared to unaffected colon mucosa, we wanted to see if these cells interact with other T cells to exercise their suppressive function using immunofluorescence (Fig 3, Paper III). In the unaffected mucosa, the submucosa was clear of T cells, and CD4$^+$ cells was scattered throughout the lamina propria; this was also true for Tregs. The number of CD8$^+$ cells was also low and present in the lamina propria but also in between epithelial cells (Fig 3C and D, Paper III). In the tumor, the CD4$^+$, CD8$^+$ and Tregs were only rarely scattered between tumor cells. Instead, the cells were located in the lamina propria-like tissue surrounding islands of tumor cells. The three different cell types were co-localized in the lamina propria-like parts, and some Tregs were found to be in close vicinity with CD4$^+$ or CD8$^+$ cells (Fig 3G, Paper III), maybe executing their regulatory effect.

Conventional T cells from the tumor display lower frequencies of activated cells

For the immune cells to exert their effector functions they need not only to be at the right place at the right time, but also they have to be activated. To see whether the cells

situated in the tumor and unaffected mucosa of colon cancer patients were activated, we analyzed recently activated CD69$^+$ T cells by flow cytometry. We found that CD69$^+$ T cells were frequent in the unaffected lamina propria, but present in significantly lower numbers in the tumor (Fig 4A, Paper III). Also, there were higher frequencies of CD4$^+$CD25^{int} activated cells expressing the IL-2R in the unaffected compared to the tumor-associated mucosa (Fig 2A, Paper III), suggesting that T cells present in the tumor mucosa have impaired activity therefore, making it difficult to mount a proper response against the tumor.

When looking at the distribution of naïve CD45RA$^+$, CD4$^+$ and CD8$^+$ cells, this was similar in the tumor and the surrounding mucosa. However, we found higher frequencies of CD8$^+$ Granzyme B$^+$ putative CTLs in the tumor than in the unaffected mucosa, which could indicate an ongoing anti-tumor response (Fig 4B, Paper III). Granzyme B has previously been described as an effector molecule used by a fraction of the Tregs in murine systems, where it appears to be involved in Treg-mediated suppression [238]. However, in our study we found the conventional CD4$^+$ lymphocytes to express low frequencies of Granzyme B, whereas the Tregs completely lacked expression of Granzyme B.

CTLA-4 has been identified as an effector molecule important for mediating the suppressive effects of Treg [239], and virtually all Tregs in the tumors did express CTLA-4. Conventional CD4$^+$ T cells had a lower and more varied expression of CTLA-4. In an effort to measure the amount of CTLA-4 expressed by the cells, we compared the mean fluorescence intensity (MFI) between the two tissue types. This showed that Treg had more CTLA-4 per cell than conventional CD4$^+$ T cells, both in the tumor and in the unaffected tissue (Fig 4D, Paper III). On conventional T cells, CTLA-4 is increased in the later stages of T cell activation, and down-regulates the activity of the T cell expressing it [240]. We found CTLA-4 expression to be higher in conventional CD4$^+$ cells from the tumor compared to CD4$^+$ cells from unaffected mucosa (Fig 4C, Paper III). These cells are susceptible to inhibition of their effector functions through ligation of CTLA-4, once again dampening the anti-tumor response. The higher frequency of CTLA-4$^+$ cells in the tumor is in accordance with the lower frequency of CD69$^+$ CD4$^+$ cells, resulting in fewer activated cells and a higher rate of effector suppression. Indeed, several pro- and anti-

tumor responses seem to exist side by side in the tumor, and the fine balance between them probably decides the final outcome.

HOMING TO THE TUMOR AND UNAFFECTED MUCOSA

The lymph vessel density is increased in tumor colon mucosa

Since angiogenesis is a common feature in colon tumors, we examined the possibility that our results were reflecting changes in lymph or blood vessel density. An altered vessel density could influence not only the expression of chemokine receptors situated in the vessel walls, but also there would be more paths leading to the tumor, possibly resulting in an increased homing of immune cells to the tumor tissue. We used immunohistochemistry to examine vessel density in the colon mucosa and found that the blood vessel density visualized with the blood endothelial marker von Willenbrand Factor (VWF) was similar in the tumor and unaffected mucosa (Fig 2B, Paper II). This suggests that the expression of blood endothelial markers (discussed later) as well as lymphocyte homing is not dependent on differences in blood vessel density. In contrast, the lymph vessel density, as detected by the lymph endothelial marker LYVE-1, was increased in 9 out of 10 tumor samples investigated ($p<0.05$) (Fig 2A, Paper II). The importance of lymph and blood vessel densities will be discussed more extensively in the chemokine receptor section.

T cells from tumor colon mucosa have an altered expression of mucosal homing receptors

Even though the homing mechanisms used by lymphocytes to enter healthy intestinal mucosa are quite well known, little is known about lymphocyte homing to tumors in the gastrointestinal tract. To better understand the mechanisms of T-cell migration to colon tumors, we compared homing receptor expression on conventional $CD4^+$ and $CD8^+$ LPL and Tregs from tumor and unaffected mucosa. These studies showed a significantly decreased ($p<0.05$) expression of the mucosal homing receptor $\alpha4\beta7$ on $CD4^+$ cells in tumor tissue compared to unaffected tissue (Fig 5A, Paper III). The endothelial ligand for $\alpha4\beta7$, MAdCAM-1, is obviously also important for the homing of $\alpha4\beta7^+$ cells into the mucosa and was measured using real time RT-PCR. We found that MAdCAM-1 expression was significantly reduced in the tumor compared to the unaffected tissue and these results were also confirmed by immunohistochemistry, showing a lower density of MAdCAM-1^+ vessels in the tumor-associated mucosa (Fig 6A and B, Paper III). Previously, studies from our group have shown that gastric cancer patients have a

decreased expression of MAdCAM-1 on tumor blood vessels and this was, as in the present study, accompanied by fewer $\alpha4\beta7^+$ LPL in the tumor mucosa compared to unaffected mucosa [241].

Integrin $\alpha E\beta7$ serves to attach lymphocytes to E-cadherin on epithelial cells, but has also been implicated in Treg function [242, 243]. It was found to be expressed by more $CD4^+$ tumor-infiltrating T cells than $CD4^+$ T cells infiltrating the unaffected mucosa in the majority of the patients. On $CD8^+$ T cells, the expression of $\alpha E\beta7$ was much higher than on $CD4^+$ T cells, probably reflecting the eventual intraepithelial position of many $CD8^+$ T cells, as indicated by immunofluorescence stainings (discussed above). Interestingly, there was also a significant increase in $\alpha E\beta7$ expression on tumor-infiltrating Treg compared to Treg in unaffected tissue (Fig 5B, Paper III), even though we were not able to locate any Treg in the epithelium by immunofluorescent stainings. However, a recent study shows that $\alpha E\beta7^+$ Tregs are antigen experienced effector/memory cells and are able to develop in the periphery [243]. We therefore speculate that $\alpha E\beta7^+$ Treg detected in the tumor may arise locally and could be effective in mediating down-regulatory signals.

The homing receptor for secondary lymphoid organs, L-selectin, has previously been shown to be up-regulated on T cells migrating to gastric tumors [241]. In colon cancer patients however, there was no consistent difference between the frequencies of L-selectin$^+$ conventional T cells and Tregs in the tumor and in the surrounding tissue. The L-selectin ligand, PNAd, on the other hand, could not be detected at all in the colon lamina propria, in the tumor or in the unaffected mucosa. This observation would then suggest that L-selectin$^+$ T cells present in the colonic lamina propria have used mechanisms other than PNAd to migrate into the tissue.

CHEMOKINES AND CHEMOKINE RECEPTORS IN TUMOR AND UNAFFECTED COLON MUCOSA

Chemokine receptor expression is altered on LPL in tumor colon mucosa

The expression of chemokine receptors on the lymphocyte surface and presence of its specific chemokine is crucial for specific lymphocyte homing, and may explain the altered homing of T cells to colon tumors. To further evaluate mechanisms of lymphocyte homing to colon tumors, we analyzed the expression of selected chemokine receptors and their respective chemokine ligands.

Chemokine receptor CXCR3 is mainly expressed on activated Th1-type lymphocytes and CTL and is abundant on lymphocytes in the mucosa of the normal intestine [244]. Flow cytometry analysis showed that CXCR3 was expressed on significantly fewer $CD4^+$ and $CD8^+$ LPL ($p<0.01$) in the tumor mucosa compared to the unaffected mucosa (Fig 7A, Paper III). In the Tregs there was also a trend towards a decreased CXCR3 expression on cells from the tumor mucosa, but this reduction was not significant. $CD4^+$ Th1 lymphocytes expressing CXCR3 have been shown to be important for anti-tumor responses [245, 246], and the reduction seen in $CXCR3^+$ cells most likely mirrors a reduction of Th1 cells and $CD8^+$ CTL in the tumor area [247, 248]. Since chemokines are also crucial in the recruitment of lymphocytes, we measured the concentrations of CXCR3 binding chemokines CXCL9, CXCL10 and CXCL11 by ELISA. We found that CXCL9 was present in similar concentrations in tumor and unaffected mucosa. Surprisingly, CXCL10 and CXCL11 were both present at significantly higher concentrations in the tumor mucosa, even though both $CD4^+$ and $CD8^+$ T cells expressing CXCR3 were significantly lower in the tumor compared to the unaffected mucosa. This led us to hypothesize that tumor evasion from $CXCR3^+$ cells is probably not dependent on chemokine production by the tumor microenvironment but rather some yet unidentified mechanism.

The chemokine receptor CCR4 is associated with Th2 and Treg responses and is primarily expressed on Th2-type $CD4^+$ T cells and Tregs [67, 221, 249]. The expression of CCR4 was similar on $CD8^+$ T cells in tumor and unaffected mucosa (Fig 12). In contrast, the expression of CXCR3 on conventional $CD4^+$ T cells and Treg was higher in the tumor compared to the unaffected mucosa (Fig 12 and Fig 7B, Paper III). The CCR4 ligand CCL17 was present in similar concentrations, while the second ligand CCL22 was significantly increased in the tumor. This finding could explain the increased number of $CCR4^+$ T cells accumulating in the tumor mucosa. Our previous study also showed increased expression of CCR4 on gastric tumor-associated Tregs accompanied by increased concentrations of CCR4 ligands. [67]. This makes us hypothesize that increased tissue concentrations of CCR4 ligands probably contribute to the recruitment of $CCR4^+$ cells to the tumor mucosa.

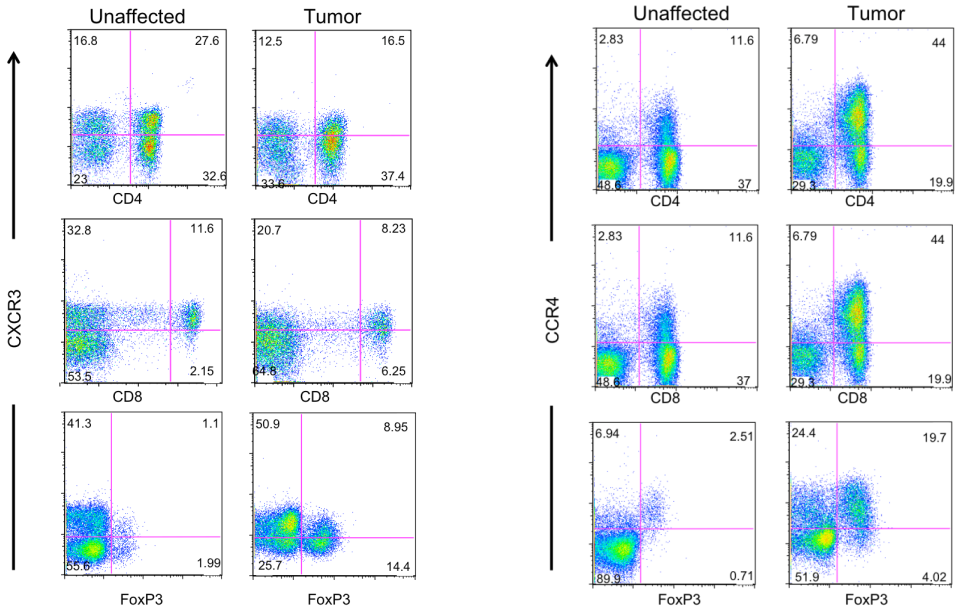


Figure 12. Flow cytometry was used to investigate the expression of CXCR3 and CCR4 on $CD4^+$ and $CD8^+$ T cells from unaffected and tumor mucosa from the same individual. Picture show flow cytometric analysis of one representative experiment.

Chemokine receptor CCR5 is usually co-expressed with CXCR3 on Th1 cells however, this receptor was expressed in similar frequencies in tumor and unaffected mucosa. This was also the case for chemokine receptors CXCR4, CCR7, CCR9 and CCR10, indicating that these chemokine receptors may be of less importance in the response against colon tumors. Together these data suggest a selective exclusion of $CXCR3^+$ conventional T cells from the tumor-associated mucosa, with a concomitant recruitment of $CCR4^+$ Treg and conventional $CD4^+$ T cells.

Transendothelial migration of lymphocytes is regulated by mulitiple mechansisms

Lymphocytes isolated from tumor and unaffected mucosa were allowed to migrate towards CCR4-binding chemokines CCL17 and CCL22, CXCR3-binding chemokine CXCL11, as well as the positive control SDF-1α in transwell assays. The positive control (SDF-1α) induced migration of all cell populations during the different experiments. Migration towards all of the tested chemokines was also induced, but the amount of cells migrating varied considerably between the different experiments (Fig 13) and the different populations of lymphocytes showed no difference in their ability to migrate towards the different chemokines. The different responses may be due to the stage and type of the tumor, and also to the tumor microenvironment. Furthermore, in the

negative control, where only medium was added to the lower well, we could detect spontaneous migration. This is a phenomenon not seen when using PBMC, suggesting that LPL have a phenotype ready to migrate. It seems though that the migration of LPL is more complicated than just following a single chemokine gradient, and that the *in vivo* situation is regulated by multiple factors contributing to the guidance of cells to a specific location.

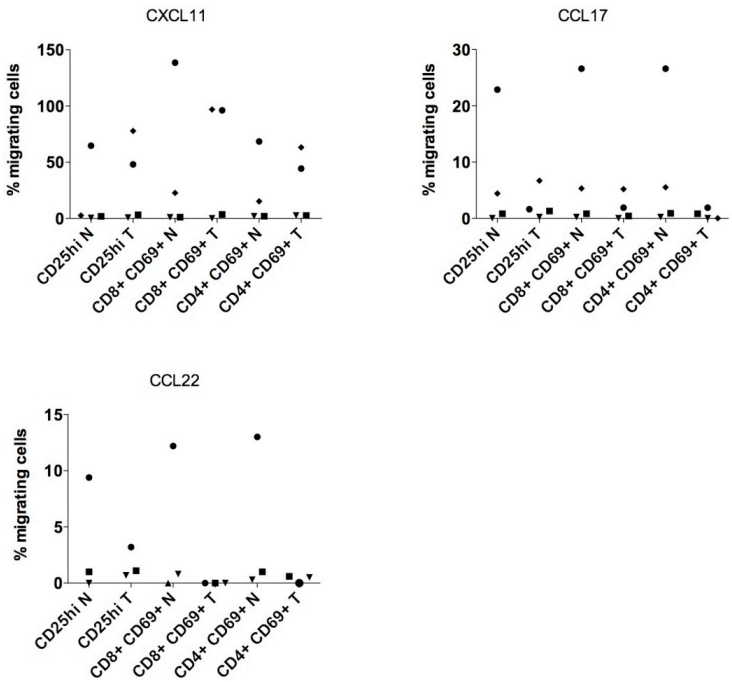


Figure 13. Lamina propria $CD25^{hi}$ (putuative Tregs), and recently activated ($CD69^{+}$) $CD4^{+}$ and $CD8^{+}$ T cells were allowed to migrate towards CXCL11, CCL17 or CCL22. Graph represents percent migrating cells of the input population when spontaneous migration been subtracted. N represents cells from unaffected tissue and T cells from the tumor tissue. The same type of symbol represent the same patient in the experiments.

Expression of decoy receptor D6 is decreased in tumor mucosa

During the last years several non-signaling chemokine receptors have been discovered. Since then they have been studied at various sites and under various conditions, but little is known about their presence and functions in tumors. We selected the three most well known non-signaling or decoy receptors, D6, DARC and CCX-CKR, to determine the expression of these receptors in the colon tumor mucosa. The receptor mRNA expression was measured using real time relative quantification PCR and related to 18S

rRNA. We could detect a substantial and significant ($p<0.005$) decrease, generally about 10-fold, of D6 mRNA expression in tumor compared to unaffected mucosa (Fig 1A, Paper II). This could not be explained by an accompanying decrease in lymph vessel density since it is actually increased in the tumor, as mentioned earlier. Interestingly, if lymph endothelial cells are the major source of D6, the decreased expression is even more remarkable. Thus, the observed decrease of D6 expression in colon tumors is not simply a reflection of lymph vessel density, but instead this receptor appears to be actively regulated.

We also measured the concentrations of selected D6-binding chemokines CCL2, CCL5, CCL8 and CCL22 by ELISA. The concentrations of CCL2, CCL5, and CCL8 were not altered when comparing tumor and unaffected mucosa, CCL22 was however, present at significantly higher concentrations in the tumor mucosa as already discussed (Table 3). As seen in Table 3, the expression of all pro-inflammatory cytokines measured, except for TNF-α, was increased in the tumor tissue whereas the T cell-associated cytokines were present at similar levels in unaffected and tumor tissue. The elevated level of CCL22 may be due to the lower expression of decoy receptor D6, which is an important scavenger for this chemokine. A very recent study found elevated levels of D6 in IBD-induced colon tumors and somewhat surprisingly, this was not accompanied by a reduction in chemokine levels [169]. These results are contradictory to the present study and may be explained by the fact that our patients do not have a history of IBD, making it difficult to compare these two types of cancer. *In vitro* studies using D6 over-expressing human breast cancer cells show that these cells have impaired proliferation and invasion compared to control cells. Also, an *in vivo* xenograft model indicated that these D6 over-expressing cells grew slower, and the incidence of metastasis in the lungs was decreased [168]. Studies using D6-deficient mice demonstrate that these mice are more susceptible to chemically-induced skin tumors [166], further indicating the importance of D6 *in vivo.* A possible consequence of decreased D6 in the tissue would be elevated levels of the chemokines that bind to D6, as previously described [154]. Here we show that there are increased levels of CCL22 in tumor mucosa, approximately three times higher compared to unaffected tissue. Simultaneous secretion of CCL22 and suppression of D6 expression by colon tumors would be a sophisticated strategy to recruit immunosuppressive Tregs in order to escape anti-tumor immune responses.

Table 3. Cytokine concentration in pg/mg total protein (mean ± standard deviation).

	UNAFFECTED TISSUE	TUMOR
CCL2	61±48	148±232
CCL5	119±72	131±165
CCL8	218±457	132±146
CCL22	124±111**	304±320
CXCL8	7.0±6.3***	190±354
CXCL9	109±205	248±254
IL-1β	13.1±6.3**	92.4±131
IL-2	1.5±1.4	1.3±1.6
IL-4	4.1±2	3.3±2.2
IL-5	1.6±1.2	1.6±1.2
IL-6	2.3±1 *	52±117
IL-10	3.7±2	2.5±1.5
IL-12p70	5.3±2.7	3.7±2.4
IFN-γ	2.1±1.6	2.9±1.6
TNF-α	3.3±1.5	2.73±1.7

* $p<0.05$ when comparing tumor and unaffected tissue
** $p<0.01$ when comparing tumor and unaffected tissue
*** $p<0.001$ when comparing tumor and unaffected tissue

We also measured the levels of DARC and CCX-CKR mRNA which were not significantly different in tumor and unaffected mucosa (Fig 1B and C, Paper II). Since DARC is expressed on blood vessel walls we, as described earlier, measured the blood vessel density and found it to be similar in both tumor and unaffected mucosa. However, to be certain that the DARC mRNA does not originate from a different source to the blood vessel walls, we used double immunofluorescent staining with DARC and the blood endothelial marker von Willerbrand Factor (vWF). When related to vWF, expression of DARC protein was not altered in tumor compared to non-tumor colon mucosa, confirming the real time PCR results (Fig 2A and B, Paper II). The corresponding analysis could not be performed for D6 and CCX-CKR, since antibodies for immunofluorescence are not available. The DARC-binding chemokine CXCL9 was present in approximately the same concentration in both tumor and unaffected mucosa, whereas CXCL8 had a significantly higher concentration in the tumor mucosa (Table 3). DARC does not seem to regulate chemokine levels, but rather makes chemokines accessible to circulating leukocytes [177]. It has also been described to have suppressive

effects on tumor metastasis in malignancy by reducing angiogenesis and preventing tumor spread [179, 180]. In this study, DARC was expressed at similar levels in tumor and unaffected colon, both at mRNA and protein level. It is however possible that investigation of distant metastases would give different results. Another possibility is that different tumor types utilize different mechanisms for progression and spread.

Decoy receptor D6 is expressed on human lymphocytes

Since D6 transcription was significantly lower in the tumor colon mucosa compared to the non-tumor mucosa, we wanted to investigate the cellular source of D6. Previously, D6 has been detected in both lymph endothelial cells and mononuclear leukocytes in human colon tissue [169]. Recent studies report that D6 is expressed by circulating B cells, macrophages and dendritic cells [250], and that $CD45^+$ mononuclear cells in human colon express D6 [169]. Flow cytometry was used to determine surface expression of D6 on different populations of LPL isolated from tumor and unaffected colon mucosa. We found that approximately 15% of the $CD19^+$ B cells in unaffected LP expressed D6 on their surface and 5% in the tumor (Fig 4, Paper II). To identify which subset of B cells that express D6, we further characterized these cells. Preliminary data from a limited number of patients indicate that the subset of B cells in the lamina propria expressing D6 are mainly naïve IgM^+ B cells, and that this is the case in both tumor-associated and unaffected mucosa. Lamina propria B cells from unaffected mucosa expressed higher levels of D6 compared to lamina propria B cells from the tumor, and circulating B cells from the same patient had even higher frequencies of D6-expressing B cells. At this point we are not able to speculate if the decrease in D6 on tumor-associated B cells is due to local down-regulation of D6 on B cells in the tumor microenvironment, or to impaired homing of $CD19^+$ B cells to tumor mucosa. However, we see decreased frequencies of $CD19^+$ B cells in colon tumor mucosa compared to unaffected mucosa, but it is hard to imagine that this 25% decrease in recruitment of B cells to the tumor mucosa would explain the large reduction in D6 expression detected in this study. It is more likely that active modulation of D6 contributes to reduced expression in the tumor tissue, maybe in combination with reduced B-cell recruitment. Although B cells are generally not considered to be key effector cells in anti-tumor responses, our results suggest that they are indirectly involved by shaping the repertoire of chemokines in the local tumor environment. This issue needs to be further investigated in future experiments.

CORRELATION OF TUMOR PARAMETERS WITH DECOY RECEPTOR EXPRESSION

Expression of decoy receptor D6 is altered depending on colon localization

The distribution of D6, DARC and CCX-CKR has not yet been fully characterized, and it is not known if their expression can be correlated to age, sex or type and location of the tumor. In this study we found that decoy receptor expression is not dependent on age or sex of the patient either in the tumor or unaffected mucosa. D6 expression in unaffected mucosa varies considerably between individuals, but appears to be higher in the more distal parts of the colon (Fig 14). To compensate for the individual variation, we used the individual difference in D6 expression between unaffected and tumor mucosa when relating D6 expression to different tumor parameters. Using this approach, we were not able to find any correlation between D6 expression and lymph node spread or the presence of distant metastasis in this rather limited material. Interestingly, D6 expression was significantly lower in tumors from the sigmoid part of the colon compared to the cecum, when comparing to unaffected tissue (Fig 5, Paper II). Even though mentioned earlier, it is important to emphasize that, despite the change in D6 expression seen in the unaffected mucosa within the colon, the tumor mucosa always has a lower expression of D6. The correlation found for D6 was not noted for DARC or CCX-CKR, but exclusively for D6. This observation is not easily explained and a larger patient material is needed to make definitive conclusions. Our possible reason for the different D6 expression in the proximal and distal large intestine may be their different embryonic origin, with the distal part originating from the hindgut and the proximal part from the foregut.

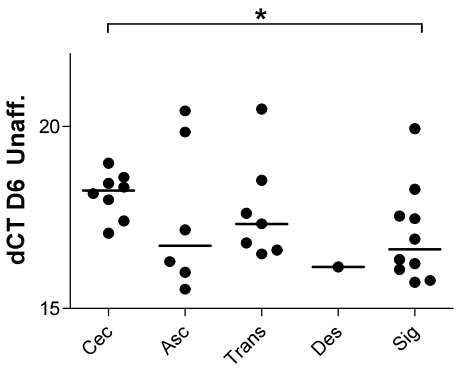


Figure 14. Correlation of D6 expression with location in colon. Please note that a low cycle corresponds to a higher expression of D6.

Survival rate and Treg frequencies are not correlated in colon tumors

Several recent studies have been able to correlate the frequencies of Tregs in tumors with either poor prognosis or better survival [214, 218, 251, 252]. However, in this limited material, there was no significant correlation between Treg frequencies and relapse-free survival of the patients. A recent study has shown that there is actually a positive correlation between higher Treg frequencies in colorectal tumors and increased survival [218]. This finding is somewhat surprising since our observations indicate that Tregs in colon tumors are active immunosuppressive cells. Also, several other studies have shown that Treg infiltration in other types of solid tumors correlates with poor prognosis in ovarian, pancreatic and hepatocellular carcinomas[215, 251, 252]. In ovarian cancer, it has been shown that Tregs in the tumor suppress tumor-specific immunity, contribute to tumor growth and have an inverse correlation with patient survival. It is suggested that the tumor Tregs primarily exert their suppressive function by inhibiting effector cell function, and that suppression of naïve T cells in lymph nodes is of less significance in the suppression of anti-tumor responses [215]. The patient material in this study was relatively small and the overall survival good, making it difficult to correlate between Treg frequencies and survival.

CONCLUDING REMARKS

The recruitment of immune cells from blood into inflamed and/or tumor tissues is crucial for the ability to clear inflammation, as well as to fight tumor cells and prevent tumor growth and spread. Multiple factors influence the subsets of cells that are recruited, and these factors are altered depending on, for instance, local inflammatory signals, environmental factors and pathogen-induced changes.

During infection, endothelial cells have a key function in promoting leukocyte entry into inflamed areas. The type of cells recruited from the blood stream are, to a large part, dependent on the adhesion molecules expressed on the endothelial cell surface. In this thesis we studied the effect of *H. Pylori* infection on adhesion molecule expression on endothelial cells. We found that E-selectin was significantly and specifically up-regulated in gastric tissue from *H. pylori*-infected individuals, and we were also able to induce E-selectin expression on HUVEC *in vitro*. Further, we identified a functional type IV secretion system, encoded by the CagPAI, to be crucial for E-selectin expression *in vitro.* Also, it seems that that CagL protein, targeted to the pilus surface and mediating binding to $\alpha 5\beta 1$ integrins, is important in this process. The increased expression of E-selectin on blood vessel walls probably contributes to the large influx of neutrophils seen in *H. pylori*-infected gastric mucosa, and this could be one possible explanation as to why CagPAI$^-$ strains are less pathogenic than CagPAI$^+$ strains. Activated neutrophils are a main source of reactive oxygen species (ROS) and reactive nitrogen species (RNS). As mentioned previously, this results in tissue damage, but also it contributes to oxidative/nitrosative DNA damage. In gastric cancer, mutations in the tumor-suppressive gene *p53* are frequently observed [253] and it is therefore possible that the influx of neutrophils, due to increased E-selectin expression in the gastric mucosa, contributes to the development of gastric cancer. Even though there are additional sources of ROS/RNS in the *H. pylori*-infected stomach, such as vascular endothelial cells and the bacterium itself, neutrophils are, as previously mentioned, believed to be the main source. One may speculate that the risk of developing gastric cancer would be dramatically reduced if oxidative stress in the mucosa was reduced. This could be achieved by reducing the entry of neutrophils into the gastric tissue by blocking endothelial expression of E-selectin with antibodies. On the other hand, this could

possibly reduce the ability of the immune response to limit bacterial numbers, since neutrophils phagocytose and kill the bacteria, and also have the capacity to migrate through the epithelium to the gastric lumen.

Our group previously found that fewer activated cells, and more naïve and central memory cells, accumulate in *H. pylori*-induced gastric adenocarcinomas and that this was probably due to a shift in adhesion molecule expression on tumor-associated endothelial cells. To determine if the same mechanisms are utilized in tumors throughout the gastrointestinal tract, we chose to study tumors originating in the colon. We found that in colon tumors there is also a decreased frequency of activated cells and a large increase in Tregs in the tumor-associated mucosa compared to unaffected mucosa. Treg infiltration of the tumor has been shown to be beneficial for the patient in a colon cancer study by Salama *et al*. [218]. This finding is a bit surprising since a majority of the studies correlate Treg infiltration in the tumor with poor clinical outcome and reduced overall survival. More research is needed, but it seems that in most cases it would probably be beneficial to reduce Treg function in cancer patients. A reduction of Treg function would, in theory, reduce the immune suppression and boost anti-tumor immunity, enhancing the chance of tumor eradication.

There are several approaches that may be used to reduce the number of Tregs in the tumors, such as reducing Treg trafficking, function or differentiation. However, the current lack of Treg-specific surface markers is a problem. Recognition of CD25 and blockage of CTLA-4 are two of the most recently developed targets. Unfortunately they lack specificity since these target molecules are also expressed by activated effector T cells [254]. The most specific marker for Tregs known at the moment is FOXP3, and since FOXP3 is an intracellular marker it cannot be targeted with antibodies. However, vaccination of mice with Foxp3 mRNA-transfected DCs stimulated Foxp3-specific CTLs and resulted in reduced numbers of Tregs in the tumor, but not in the periphery, and also enhanced anti-tumor immunity [255]. If possible to transfer to human conditions, this method would be preferable, since depleting all FOXP3+ cells most likely would result in an autoimmune disease.

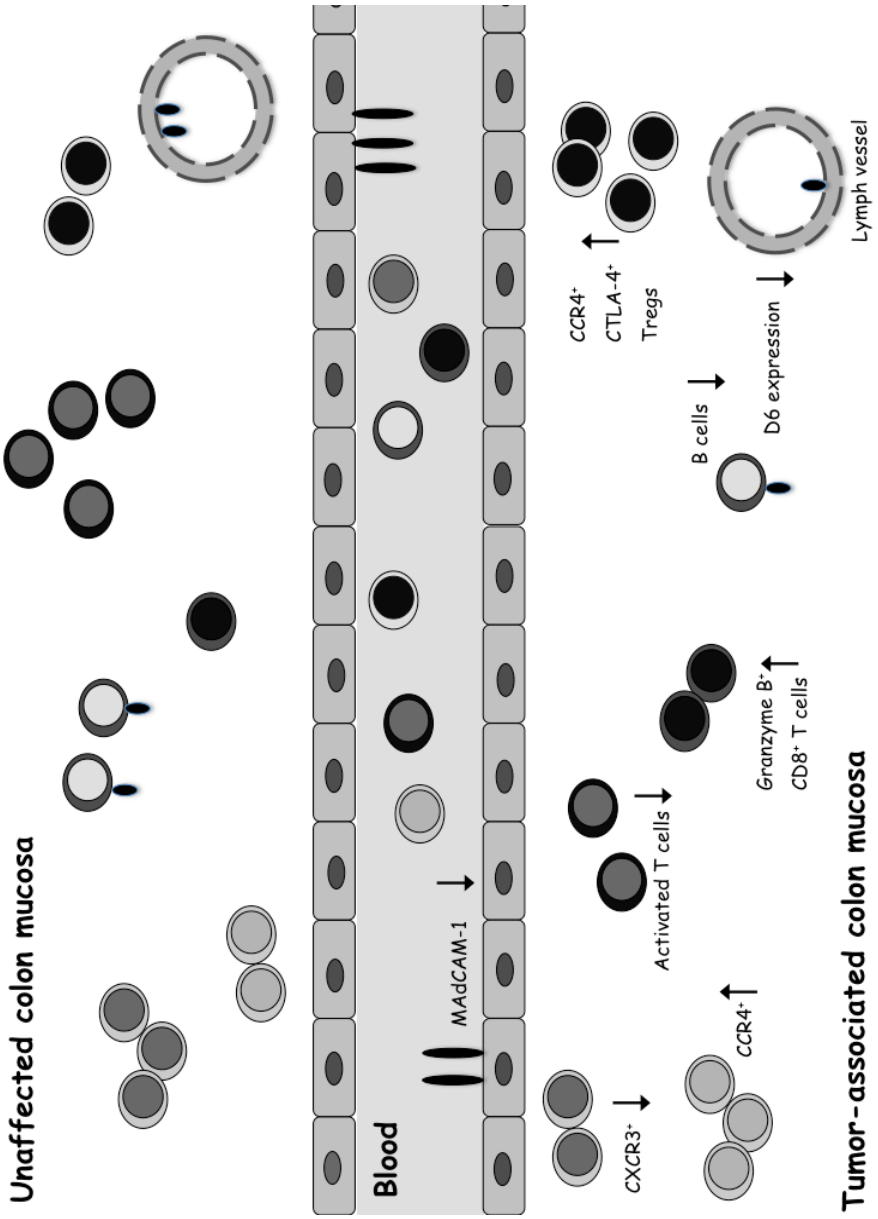
To avoid autoimmune disease it may be preferable to avoid the physical elimination of Tregs and instead inactivate them or block their traffic into the tumor site. In this thesis

we found that Tregs in the tumor display higher levels of CTLA-4 and also more frequently express CCR4 on their surface. At the same time, the CCR4 ligand CCL22 is present in higher concentrations in the tumor tissue. One possible explanation for the increased levels of CCL22 in the tumor is that the expression of D6, acting as a scavenger for this chemokine, is significantly decreased in the tumor-associated mucosa. These findings may be utilized in blocking Treg entry into the tumor and, although the mechanisms by which Treg home to tumors in the gastrointestinal tract are not well defined, we suggest that the increased concentration of CCL22 contributes to the recruitment of $CCR4^+$ cells to the tumor mucosa. Another anti-tumor approach would therefore be to selectively knock-down the production of CCL22, or to over-express D6 to increase the scavenging of CCL22. In a study by Wu *et al.* [168], D6 was over-expressed in a human breast cancer cell line and the results show that both the proliferation and invasion of these cells is inhibited *in vitro* and *in vivo*. These studies further strengthen our beliefs that chemokines and chemokine receptors in the tumor micro-environment could play multifaceted roles in tumor development and progression. We therefore hypothesize that D6 plays an important role by scavenging tumor-supportive chemokines and inhibiting chemokine-induced angiogenesis. Further, we believe that D6 is a promising molecule in anti-tumor immunity and that its potential in anti-tumor treatment should be evaluated. However, one has to bear in mind that each tumor is different and the treatment always has to be adjusted according to type, stage and individual differences.

As previously mentioned, D6 expression is decreased in the tumor-associated mucosa. Interestingly, in the unaffected mucosa, D6 expression was lower in the sigmoid part of the colon compared to the cecum. This pattern of expression was, on the other hand, not seen in the tumor-associated mucosa, even though the tumors were localized at different sites in the colon. We did however, see a tendency towards more advanced tumors expressing less D6, making us hypothesize that D6 expression may be used as a prognostic marker. Studies including more patients with different stage tumors and also possibly adenomas need to be performed to draw any conclusions about the importance of D6 as a prognostic marker for colon cancer.

In conclusion in this thesis we have shown that chronic mucosal inflammation and tumor development change the expression of several endothelial receptors and

chemokines leading to an altered immune cell composition in inflamed tissue and tumors. In additon, our studies also indicate that the decoy chemokine receptor D6 may play an important role in tumor development.



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