Regulatory $CD4^+FOXP3^+$ T cells in *Helicobacter pylori*-induced disease

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”…Oroa Dig inte innan Du vet, och när Du vet

är det ofta försent, -så varför oroa sig?...”

handledarens ord till doktoranden

ABSTRACT

Helicobacter pylori colonize the gastric or duodenal mucosa of approximately half of the worlds' population. Although most individuals are asymptomatic, *H. pylori* infection cause peptic ulcers or gastric cancer in 10-15 % and 1-2 %, respectively, of the infected individuals. It has previously been suggested by us and others that the life-long infection caused by *H. pylori* may be due to dysregulation of the immune response and that the host often fails to eradicate the infection due to the presence of regulatory T cells that down-regulate the proper immune response. In this thesis we have further analyzed the suppressive capacity, frequency, location and proliferation of regulatory T cells both locally at the site of infection and systemically in the blood in individuals with duodenal ulcer and gastric cancer. We could show by immunohistochemistry that FOXP3-expressing $CD4^+$ T-cell numbers increase in areas of duodenal gastric metaplasia compared to normal duodenal mucosa and are associated to $CD4^+$ T-cell aggregates in the duodenum of *Helicobacter pylori*-infected duodenal ulcer patients. The increase of FOXP3-expressing T cells in the antrum of infected individuals was dependent on the presence of *Helicobacter pylori*, since eradication therapy resulted in 4-fold lower levels of FOXP3 and IL-10 mRNA in the antrum. Higher numbers of $CD4^+FOXP3^+$ T cells were found in duodenal ulcer patients than in asymptomatic *H. pylori* infected individuals. These results show that $CD4^+FOXP3^+$ expressing T cells are increased at the site of infection and decrease when the bacteria are eradicated.

When analysing patients with gastric cancer we found increased numbers of $CD4^+FOXP3^+$ T cells in the tumor compared to tumor-free gastric mucosa. We could determine that gastric $CD4^+CD25^{high}$ T cells expressed FOXP3 and were able to suppress *H. pylori*-induced T-cell proliferation and IFN-γ production. These regulatory T cells (Treg) expressed increased levels of the homing receptor L-selectin and CCR4, which indicated that the increased levels of $CD4^+FOXP3^+$ Tregs in tumors are due to active recruitment to the tumor mucosa.

Suppressive activites of Tregs have been proposed to be exerted by the anti-inflammatory cytokines IL-10 or TGF-β. To evaluate the immune response in individuals that developed gastric cancer compared to asymptomatic individuals we stimulated T cells from both peripheral blood and gastric mucosa of *H. pylori*-infected gastric cancer patients with *H. pylori* antigens. All T cells from gastric cancer patients produced high amounts of IL-10, while the IL-10 production from blood T cells of *H. pylori*-infected asymptomatic subjects was low. Furthermore, the mRNA levels of IL-10 were increased in the gastric mucosa of GC patients and the frequency of activated $CD8^+$ T cells was markedly reduced compared to asymptomatic individuals.

Finally, we analysed $CD4^+FOXP3^+$ T cells from gastric cancer patients by flow cytometry. We found that $CD4^+FOXP3^+$ T cells from gastric cancer patients have significantly higher levels of proliferation than $CD4^+FOXP3^-$ T cells within the tumor, and that $CD4^+FOXP3^+$ cells within tumors proliferate significantly more than $CD4^+FOXP3^+$ cells in tumor free mucosa and in blood. When $CD4^+$ T cells were isolated directly from the tumor and tumor free mucosa and sorted into $CD25^{high}$ and $CD25^{low/-}$ populations we found that $CD4^+CD25^{low/-}$ T cells express higher transcription levels of both IFN-γ and TGF-β compared to $CD4^+CD25^{high}$ T cells, but $CD4^+CD25^{high}$ have a higher IL-10 / IFN-γ ratio, which indicates a suppressive function. Furthermore, the tumor mucosa had a higher expression of the pro-inflammatory cytokines IFN-γ and IL-8 compared to tumor-free mucosa.

In conclusion, we show increased numbers of $CD4^+FOXP3^+$ T cells both in the mucosa of duodenal ulcer patients and in gastric tumor tissue. This increase is associated to the presence of *H. pylori*, local inflammation or cancer, and is mediated by both increased recruitment into the mucosa and increased proliferation of resident $FOXP3^+$ cells. Furthermore, the local $CD4^+FOXP3^+$ T cells are associated to IL-10 expression but lack of IFN-γ, while TGF-β is produced to a larger extent by other T cells. We believe that these findings contribute to increased understanding of the immunoregulatory processes related to *H. pylori*-induced diseases.

Keywords: Regulatory T cells, *Helicobacter pylori*, duodenal ulcer, gastric cancer, FOXP3

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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-IV):

I. Bert Kindlund, Åsa Sjöling, Malin Hansson, Anders Edebo, Lars-Erik Hansson, Henrik Sjövall, Ann-Mari Svennerholm and B. Samuel Lundin

FOXP3-expressing $CD4^+$ T-cell numbers increase in areas of duodenal gastric metaplasia and are associated to $CD4^+$ T-cell aggregates in the duodenum of *Helicobacter pylori*-infected duodenal ulcer patients. *Helicobacter, In press*

II. Karin Enarsson, Anna Lundgren, Bert Kindlund, Mikael Hermansson, Giovanna Roncador, Alison H Banham, B. Samuel Lundin and Marianne Quiding-Järbrink

Function and recruitment of mucosal regulatory T cells in human chronic *Helicobacter pylori* infection and gastric adenocarcinoma. *Clin Immunol* 2006 Dec; 121 (3): 358-68

III. B. Samuel Lundin, Karin Enarsson, Bert Kindlund, Anna Lundgren, Erik Johnsson, Marianne Quiding-Järbrink and Ann-Mari Svennerholm

The local and systemic T-cell response to *Helicobacter pylori* in gastric cancer patients is characterised by production of interleukin-10. *Clin Immunol* 2007 Nov; 125 (2) 205-13

IV. Bert Kindlund, Åsa Sjöling, Jenni Adamsson, Anders Janzon, Lars-Erik Hansson, Mikael Hermansson and B. Samuel Lundin

Increased proliferation of $CD4^+FOXP3^+$ T cells in gastric cancer mucosa contribute to higher local numbers of regulatory T cells. *Submitted*

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PAPER I-IV

ABBREVIATIONS

AS	Asymptomatic individuals
CD	Cluster of Differentiation
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic Cell
DGM	Duodenal Gastric Metaplasia
DU	Duodenal Ulcer
FCM	Flow cytometry
FOXP3	Forkhead box P3
GC	Gastric Cancer
H.p.	*Helicobacter pylori*
HPRT1	Hypoxanthine-guanine phosphoribosyl transferase
IDO	Indoleamine 2,3-dioxygenase
IL-8	Interleukin-8
IL-10	Interleukin-10
IFN-γ	Interferon- γ
LPL	Lamina propria lymphocytes
PBMC	Peripheral blood mononuclear cells
Treg	Regulatory T cells
TGF-β	Transforming growth factor β
TIL	Tumor Infiltrating Lymphocytes
TREC	T-cell receptor excision circles

INTRODUCTION

Helicobacter pylori

Helicobacter pylori is a gram-negative, microaerophilic, curved and rod-shaped bacterium with multiple polar flagellae. It is one of the most common pathogenic bacteria that infect the human population and approximately half of the world's population is estimated to be or to have been infected (Kuipers, et al. 1995; Rothenbacher and Brenner 2003). *H. pylori* specifically colonises humans and its only niche is the human stomach mucosa or the gastric-like metaplasia in the duodenum (Steer 1984; Wyatt, et al. 1987). It expresses a number of virulence factors, including, BabA2, SabA, HpaA and UreA that mediate the attachment to the gastric mucosa and help the bacterium to survive in the acidic environment of the stomach, as well as a toxin (VacA) and other host-affecting proteins such as CagA, which is injected into epithelial cells through a bacterial type-IV secretion system. *H. pylori* infection is usually acquired in childhood and is often life-long in absence of antibiotic therapy (Everhart 2000). Infection by *H. pylori* infection is therefore regarded as a chronic disease.

***H. pylori* infections cause peptic ulcers or gastric cancers**

All infected subjects will develop an inflammation (chronic gastritis) of the stomach mucosa in response to the infection, but the majority do not experience any problems caused by the infection, and can unknowingly carry the infection for years as asymptomatic carriers. It has been estimated that approximately 85% of infected individuals are asymptomatic. However, approximately 10-15 % of all infected individuals are at risk of developing gastric or duodenal ulcer disease and another 1% will develop gastric adenocarcinoma as a consequence of *H. pylori* infection(Peek and Blaser 2002; Suerbaum and Michetti 2002) The diseases risk seems to vary with age, geographical location and ethnicity, so host susceptibility as well as environmental and bacterial factors influence the outcome of infection (Graham, et al. 1994).

Duodenal ulcer

One reason for the different outcomes of *H. pylori* infection is that in some subjects the infection induces an antral predominant non-atrophic pattern of gastritis, and this result in increased release of the gastric hormone gastrin and thereby increased acid secretion. This phenotype is associated with an increased risk of duodenal ulcer disease but little, if any, increased risk of gastric cancer (Uemura, et al. 2001). Peptic ulcers are divided into gastric ulcers and duodenal ulcers, which develop in the stomach and duodenum, respectively. Colonization of duodenum is much more complicated for *H. pylori* compared to colonization of the stomach. Patients with an increased gastric acid secretion also have higher levels of acid in the duodenum due to leakage from the stomach. When the epithelial layer is exposed to increased acid levels in duodenum it may transform to be more of the same type as the gastric epithelial layer. Such areas in the duodenum are referred to as duodenal gastric metaplasia (DGM) and the gastric-like layer of DGM make it possible for *H. pylori* to colonize these areas (Steer 1984; Wyatt et al. 1987).

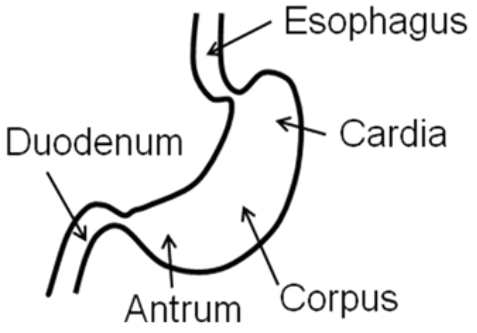

Figure 1:
The different compartments of the human gastrointestinal tract mentioned in the text.

Gastric Cancer

H. pylori-induced gastric cancer develops during several decades. After initial infection, individuals will develop a chronic gastritis, which is predominantly located either in the antrum or corpus of the stomach, or alternatively equally distributed in both locations (pan-gastritis). Those with pan-gastritis or corpus-predominant gastritis may subsequently develop atrophic gastritis – *e.g.* destruction and loss of the gastric glands. This histological picture is associated with increased gastrin release but reduced or absent acid secretion and is associated with an increased risk of developing gastric cancer (El-Omar, et al. 1997; Uemura et al. 2001). In a subset of individuals with atrophic gastritis there is also development of intestinal metaplasia, e.g. transformation of gastric epithelial cells into intestinal-like cells. The metaplastic epithelium may then undergo further genotypic and phenotypic disarrangements and thereby progress to dysplasia and malignant transformation, i.e. adenocarcinoma (Kuipers et al. 1995). In a prospective study it was shown that the risk of developing gastric cancer within 10 years is 15 and 35 times greater for individuals with pan-gastritis or corpus-predominant gastritis compared to individuals with antrum-predominant gastritis, respectively (Uemura et al. 2001).

Gastric cancer epidemiology

Gastric cancer is the fourth most common type of cancer with at least 900.000 new cases per year, but the second most common in leading to death. There is a considerable geographic variation in incidence of gastric cancer around the world, but almost two thirds of the cases of gastric cancer occur in developing countries and 42% in China alone (Parkin, et al. 2005). Areas of high-risk for development of gastric cancer are East Asia (China, Japan), Eastern Europe, and parts of Central and South America (Parkin et al. 2005). In Sweden the numbers of new cases of gastric cancer have decreased and today around 1000 new cases are reported every year, indicating a reduction of cases to a third of the cases reported in 1970. People moving from high-risk areas to low-risk areas have the same risk of developing gastric cancer as the people who stay, but the children born in low-risk areas have the same risk of developing gastric cancer as other people in their new country (Kamineni, et al. 1999). This supports that environmental factors in early life are important for cancer development.

Epidemiological, clinical, and animal studies have established a central role for *H. pylori* in gastric carcinogenesis and provided insights into the mechanisms and biologic relationships between bacterial infection, host genetics, nutrition, and environmental factors (Mbulaiteye, et al. 2009). More than 90% of gastric cancers are characterized as adenocarcinoma, i.e. derived from glandular epithelial cells (Dickson, et al. 2004). Gastric cancer is divided into cardia (the esophagogastric junction) and non-cardia (distal part of stomach) cancer depending on the location of tumor, and 65-85% of all gastric cancer cases are non-cardia cancer. It has been estimated that more than half of the world-wide non-cardia gastric cancers are caused by *H. pylori* infections (HCC-Group 2001).Non-cardia gastric cancer has a male to female ratio of approximately 2:1 (Chandanos and Lagergren 2008). There are two histological main types of gastric carcinoma, diffuse (undifferentiated) and intestinal (well-differentiated) (Lauren 1965). *H. pylori* infection increases the risk of developing non-cardia cancer but seems not to be involved in cardia cancer (HCC-Group 2001) where smoking and obesity are risk factors (Calle, et al. 2003; Gonzalez, et al. 2003). Factors related to diet and food preservation, such as high intake of salt-preserved foods and dietary nitrite or low intake of fruit and vegetables, are likely to increase the risk of stomach cancer (Brenner, et al. 2009; Jakszyn and Gonzalez 2006; Riboli and Norat 2003). Recently it has been shown that regular use of aspirin or other NSAIDs have a protective function against non-cardia cancer but not against cardia cancer (Abnet, et al. 2009).

Immune responses to *H. pylori* infection

Infection with *H. pylori* generally leads to an immune response characterised by a large infiltration of various immune cells into the infected gastric mucosa such as antibody producing B-cells, neutrophils, macrophages, $CD8^+$ and $CD4^+$ T cells. The T cells and the general immune response seem to be polarised towards production of Th1 cytokines such as IL-12 and IFN-γ in infected individuals (Bamford, et al. 1998; Lindholm, et al. 1998). However, previous studies from our group have shown that the presence of $CD4^+CD25^{high}$ regulatory T cells down-regulate the T-cell response to *H. pylori* in the blood of asymptomatic *H. pylori*-infected subjects (Lundgren, et al. 2003). Furthermore, the number of $CD4^+CD25^{high}$ T cells were also shown to be increased in the infected antral and duodenal mucosa compared to uninfected mucosae (Lundgren, et al. 2005a). A working hypothesis further evaluated in this thesis is that the regulatory T cells suppress the mucosal immune responses to minimise the tissue damage, but at the same time this contribute to the persistence of *H. pylori* infection (Lundgren et al. 2005a) and increase the risk for development of gastric cancer by reducing the anti-tumor immune responses.

Regulatory T cells

History

Already in 1970, the first publications reporting the existence of specific T cells that not only augmented but also dampened the immune response, and that these down-regulating T cells were different from helper T cells (Gershon and Kondo 1970). The new T cell population that dampened immune responses was called Suppressor T cells and was intensively studied during a number of years, but then this line of research ended quite abruptly. The reason was that the researchers did not find the I-J region in the MHC complex, which was assumed to encode the I-J molecule with the supposed suppressive function (Green, et al. 1983; Kronenberg, et al. 1983) but also that no reliable cell surface markers for distinguishing suppressor T cells from other T cells were found. However, a decade later in the 90s this line of research was re-initiated by the findings of the oral tolerance-induction by TGF-β producing T regulatory cells, called Th3 cells (Fukaura, et al. 1996; Miller, et al. 1992). Finally, in 1995, Sakaguchi et al demonstrated that mouse T cells with suppressor activity was confined to a minor subset of T cells that constitutively expressed the IL-2 receptor α subunit, CD25 (Sakaguchi, et al. 1995), the $CD4^+CD25^+$ T cells were termed regulatory T cells (Treg). In humans, the Tregs were further recognized to be T cells with high expression of CD25 i.e $CD25^{high}$ cells (Baecher-Allan, et al. 2001).

$CD4^+$ regulatory T cells are now categorized into two main classes; naturally occurring Tregs (nTregs), which develop their regulatory function in the thymus, and the regulatory T cells that up-regulate their regulatory function in the periphery or *in vitro* during various experimental conditions, *i.e.* induced Treg (iTreg) (Shevach 2006). In addition there are also Tr1 cells, characterized by high IL-10 production (Groux, et al. 1996) and Th3 cells which produce high levels of TGF-β (Fukaura et al. 1996); the definitions of these cell populations are overlapping and they are not all mutually exclusive. In humans, simulation may under certain conditions be sufficient to induce FOXP3 expression in $CD4^+CD25^-$T cells (Walker, et al. 2003).

Regulatory T cells markers

Regulatory T cells express CD25, FOXP3, GITR, CD27 and CTLA-4 but have low expression of CD127 and CD49d, independent of whether they originated from the thymus or were induced in the periphery. These markers are often used to define the Treg cell population and high expression of **CD25**, the IL-2 receptor α chain, is one of the most extensively used markers for the isolation and characterization of Treg both in mice and humans. However, one major drawback is that activated effector T cells also express this receptor (Baecher-Allan et al. 2001). Apart from $CD25^{high}$ the following markers may be used:

FOXP3, forkhead box P3, is a transcription factor and a characteristic marker, which control phenotype and function of regulatory T cells (Buckner and Ziegler 2008) and has been linked with a suppressive phenotype (Mahic, et al. 2006; Pillai, et al. 2007; Walker, et al. 2005; Walker et al. 2003). In support of the use of FOXP3 as a marker for regulatory T cells the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of the FOXP3 gene (Bennett, et al. 2001). FOXP3 mutations in IPEX patients result in dysfunction of $CD4^{+}$ $CD25^{high}$ cells and in effector T cells, which leads to a high incidence of autoimmune or inflammatory diseases including type 1 diabetes, thyroiditis, inflammatory bowel disease and allergic diseases (Bacchetta, et al. 2006). FOXP3 has emerged as a useful marker for distinguishing Tregs but one major drawback is that intracellular staining is needed, which means that living cells cannot be sorted with regard to FOXP3 expression.

CTLA-4, Cytotoxic T-Lymphocyte Antigen 4, also known as CD152. CTLA-4 is similar to the T-cell costimulatory protein CD28, and both molecules bind to CD80 and CD86 on antigen-presenting cells. CTLA 4 transmits an inhibitory signal to T cells and to antigen-presenting cells, whereas CD28 transmits a stimulatory signal (Alegre, et al. 2001; Levings, et al. 2001b; Wing, et al. 2005; Zheng, et al. 2008).

GITR, glucocorticoid-induced TNFR-related protein, interaction of GITR on Treg with its ligand (GITR-L) on APCs has the opposite effect compared to CTLA-4-CD80/86, since signaling via GITR has an inhibitory effect on Treg suppression.

CD27 together with CD25 are a useful combination for distinguish regulatory T cells from effector T cells in inflamed tissue (Ruprecht, et al. 2005). However, Duggleby claim that CD27 is most useful to identifying Treg in the cell lines obtained after expansion of $CD4^+$ $CD25^+$ cells and its expression may not reliably identify the Treg cell population in other T-cell populations such as those found in joints (Duggleby, et al. 2007).

Low levels of CD127, IL-7 receptor chain, have been shown on regulatory T cells surfaces (Liu, et al. 2006; Seddiki, et al. 2006). In gastric cancer patient it was recently shown that $CD4^+CD25^+CD127^{low/-}$ can be used as a selective marker to enrich Treg (Shen, et al. 2009).

CD49d is a new marker that is expressed on proinflammatory PBMC but is absent on immune-suppressive Treg cells. The combination of anti-CD49d with anti-CD127 makes it possible to separate $CD49d^-CD127^{low}$ cells as an "untouched" $FOXP3^+$ Treg cell population free from contaminating effector cells (Kleinewietfeld, et al. 2009).

The identification of specific molecular markers of Treg remains an important goal in human immunology and will not only aid in the elucidation of Treg but also provide novel targets for immunotherapy (Knutson, et al. 2007).

Tr1 cells

Tr1 cells are characterized by producing particularly high levels of cytokine IL-10 and are one of the main T-cell mediators of cytokine-dependent immune regulation (Groux, et al. 1997). Tr1 cells arise in the periphery when naive $CD4^+$ T cells are activated by tolerogenic antigen-presenting cells (APCs) in the presence of IL-10 (Roncarolo, et al. 2006). Regulation of the immune responses, *in vivo* and *in vitro*, is through secretion of IL-10 and transforming growth factor-β (TGF-β), that have a suppressive capacity on both naive and memory T-cell responses (Barrat, et al. 2002; Levings, et al. 2001a). The suppressive effects of Tr1 cells are not completely dependent on IL-10 and TGF-β (Groux et al. 1997), additional mechanisms such as cytotoxicity may also contribute (Grossman, et al. 2004b). Although Tr1 cells need to be activated *via* the T-cell receptor (TCR) to exert their suppressive function, once activated, they mediate bystander suppressive activity against cells that recognize other antigens. Unfortunately, specific cell-surface markers of Tr1 cells are not known, and it is therefore not possible to isolate the cells *ex vivo*.

Suppressive mechanisms used by regulatory T cells

Suppressive mechanism that Treg may use can be divided into four different groups; suppression by inhibitory cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by modulation of dendritic-cell maturation or function (Vignali, et al. 2008).

The inhibitory **cytokines** most important for Treg suppression are IL-10 and TGF-β (Nakamura, et al. 2001; Zhang, et al. 2001; Zheng, et al. 2004). The role of cytokine suppression by Treg has been thoroughly studied in several mouse models. Thus, both IL-10 and TGF- β are important for maintaining gut homeostasis and protect from inflammatory bowel disease-like conditions in mice (Asseman, et al. 1999). In human, some studies have shown that the natural Treg-suppression is independent of IL-10 and TGF-β (Enarsson, et al. 2007; Levings et al. 2001b) whereas other studies have shown that IL-10 and TGF-β are involved in the suppression (Strauss, et al. 2007). Recently in mouse experiments, IL-35 has been proposed as a novel inhibitory cytokine that may be specifically produced by Treg cells and is required for maximal suppressive activity (Collison, et al. 2007). However, human Treg cells do not express detectable amounts of IL-35 (Bardel, et al. 2008).

Suppression by **cytolysis** is suggested to be carried out by granzyme A, granzyme B and perforin. In human this has been shown on *in vitro* stimulated PBMC (Grossman, et al. 2004a). However, gastric mucosal $CD4^{+}CD25^{high}$ T cells isolated from tumor and tumor-free mucosa do not express Granzyme A, Granzyme B or Perforin (Enarsson 2005). It has also been suggested that Treg may use two other cytotoxic mechanisms: the TRAIL-DR5 (tumor-necrois-factor-related apoptosis-inducing ligand-death receptor 5) pathway (Ren, et al. 2007) and galectin-1 (Garin, et al. 2007).

Suppression by **metabolic disruption** is described as a mechanism that mediate suppression of the effector T-cell target (Vignali et al. 2008). The high expression of CD25 (IL-2Rα) empowers Treg cell to “consume” IL-2 and therefore starve actively dividing effector T cells by depleting the IL-2 they need to survive (de la Rosa, et al. 2004; Thornton and Shevach 1998) but this has been questioned by others (Fontenot, et al. 2005). The coordinated expression of CD39/CD73 on Treg which generate the inhibitory molecule adenosine can suppress effector T cells by binding to their A2A receptor (Deaglio, et al. 2007). Treg can also use cyclic AMP (cAMP) to suppress effector T cells by transfer through membrane gap junctions (Bopp, et al. 2007).

Suppression by **modulation of dendritic cells** is an indirect way for Treg to suppress effector T cells by reducing dendritic cell activation-capacity. Treg cells down-modulate B7-molecules on DCs in a CTLA-4-dependent way, thereby enhancing suppression of T-cell activity (Oderup, et al. 2006; Read, et al. 2000). Treg can also in an CTLA-4-dependent way induce up-regulation of indoleamine 2,3-dioxygenase (IDO) in DC, resulting in a suppression of effector T cells (Fallarino, et al. 2003). IDO catalyses the degradation of tryptophan, generating suppressive metabolites, which can anergize T cells or even induce the differentiation of new Tregs (Munn and Mellor 2007). Neuropilin-1 which is expressed mostly by Treg but not naïve T cells, promote prolonged interaction with immature DC (iDC). This will give Treg an advantage over naïve T cells in modulating the function of DCs (Sarris, et al. 2008).

Correlation between FOXP3 and a suppressive function in humans

The best marker for regulatory T cells, the transcription factor FOXP3, is presently questioned, the issue being if all cells that express FOXP3 have a suppressive function. FOXP3 is not only restricted to naturally occurring Tregs but can be expressed by conventional T cells upon stimulation (Allan, et al. 2007; Gavin, et al. 2006; Mantel, et al. 2006; Morgan, et al. 2005; Pillai et al. 2007; Walker et al. 2003; Wang, et al. 2007). The suppressive capacity of the induced FOXP3 expressing cells has been tested with varying results, in some cases stimulation results in a transient FOXP3 expression and non-suppressive function (Allan et al. 2007; Tran, et al. 2007). However, suppressive phenotypes have been observed in induced $CD4^+FOXP3^+$ cells in other experiments (Gavin et al. 2006; Long and Buckner 2008; Mahic, et al. 2008b; Pillai et al. 2007; Walker et al. 2005; Walker et al. 2003). Different experimental set ups have been used in different studies, suppressive function has been reported by using isolated induced $CD4^+CD25^+$ cells, whereas lack of *in vitro* suppressive function where reported when $CD25^+$ cells were not separated and a mixed population of T cells were used as suppressors (Buckner and Ziegler 2008). Furthermore, even $CD8^+CD25^-FOXP3^-$ T cells attain a transient $FOXP3^+CD25^+$ state during activation and acquire a suppressive function (Mahic, et al. 2008a; Pillai et al. 2007). Regardless whether the variation in suppressive capacity of induced FOXP3 expressing cells is due to different stimulation conditions or to donor-dependent factors, it is now becoming increasingly accepted that stable FOXP3 expression is prerequisite for a suppressive phenotype (Janson, et al. 2008; Wang et al. 2007)

Presence of regulatory T cells in cancer

Tumors has different strategies to avoid the anti-tumor immune response, one is to suppress the immune responses by increasing the number of regulatory T cells. In ovarian cancer and non-Hodgkin´s lymphoma, they have found that the tumor produce the chemokine CCL22 that serves to attract $CCR4^+$ Treg into the tumor tissue (Curiel, et al. 2004) (Yang, et al. 2006). Furthermore, tumor cells and tumor-educated tolerogenic APC seems to use the same mechanisms for inducing Tregs, both can secrete immunoregulatory cytokines such as TGF-β and IL-10, and both have been shown to express IDO (indoleamine 2,3 dioxygenase)(Munn and Mellor 2007; Zou 2006). Our group has shown that *H. pylori* induced T cell migration was dramatically reduced in gastric carcinoma patients compared to healthy individuals, but restored after Treg depletion. The effect of Treg cells was largely dependent on cell-cell contact, but not on IL-10 or TGF-β (Enarsson et al. 2007). The results of a number of studies looking at different cancer types show an increased number of Treg in PBMC in cancer patients compared to healthy individuals whilst a minority of studies did not (Betts, et al. 2006). In gastric cancer patients, at least two studies apart from the reports presented in this thesis have shown an increased number of Treg in PBMC in gastric cancer compared to healthy individuals (Ichihara, et al. 2003; Sasada, et al. 2003). In gastric cancer, the population of $CD4^+CD25^+$ T cells in the tumor infiltrating lymphocytes (TIL) in advanced disease was significantly higher than that in TILs of patients with early-stage disease (Ichihara et al. 2003). In ovarian cancer, Curiel et al have shown a correlation between frequencies of tumor infiltrating Tregs and survival (Curiel et al. 2004) whereas Sato observed a better prognosis in patients with a high $CD8^+$ T cells to $CD4^+FOXP3^+$ T cells ratio (Sato, et al. 2005). Furthermore, gastric cancer patients with high frequencies of $CD4^+CD25^{high}$ T cells in PBMC demonstrated reduced survival compared to those classified as low (Sasada et al. 2003).

Treg in infections

The role of regulatory T cells in HIV pathogenesis is not fully elucidated. Persistent antigens, such as HIV, are believed to promote the expansion and activation of antigen-specific Tregs and several reports have described both beneficial and detrimental roles for Treg in HIV pathogenesis (Seddiki and Kelleher 2008). In human cutaneous leishmaniasis, recent reports indicate that IL-10-producing natural regulatory T cells may play critical roles in the maintenance and loss of infection-induced immunity (Okwor and Uzonna 2008).

AIMS OF THE THESIS

- To determine the number and localisation of mucosal $CD4^+$ T cells expressing the transcription factor FOXP3 in duodenal ulcer patients and gastric cancer patients.

- To determine the local gene expression levels of anti- and pro-inflammatory cytokines and the expression of FOXP3 before and after eradication of *Helicobacter pylori*.

- To investigate cytokine mRNA production, expression of FOXP3 and the capacity to suppress *H. pylori*-induced T-cell response in human mucosal $CD4^+CD25^{high}$ regulatory T cells from *H. pylori*-infected gastric cancer patients.

- To study whether those individuals that develop gastric cancer have an altered immune response to *H. pylori* compared to individuals that remain asymptomatic

- To study the level of activity among the cytotoxic $CD8^+$ T cells and the relation between $CD4^+FOXP3^+$ T cells and $CD8^+$ T cells in tumor compared to tumor-free gastric mucosa.

- To study the mechanisms behind increased levels of regulatory $CD4^+FOXP3^+$ T cells in the gastric tumor mucosa.

MATERIALS AND METHODS

Patients and volunteers

All experiments in this thesis were performed using biopsies from human duodenum and/or gastric mucosa and/or blood samples collected from patients at the Sahlgrenska University hospital, Gothenburg, or healthy volunteers. In the different studies, patients with non-cardia gastric adenocarcinoma or pancreatic cancer, duodenal ulcer patients, *H. pylori* eradicated duodenal ulcer patients and healthy controls were included (Table 1). All individuals were tested for *H. pylori* infection as described below. None of the *H. pylori*-infected individuals had taken any antibiotic medication prior to sampling of biopsy specimens or any anti-secretory medication for at least 10 days prior to collection. Furthermore, none of the gastric adenocarcinoma patients had undergone radiotherapy or chemotherapy prior to surgery. The studies were approved by the Ethical review board for human research at Sahlgrenska Academy, and informed consent was obtained from each subject before participation in the study.

Table 1. Volunteers participating in the different studies

	Study I		Study II		Study III		Study IV	
	Number	Age (median)	Number	Age (median)	Number	Age (median)	Number	Age (median)
Hp- AS[a]	6	34 (25-55)						
Hp+ AS	21	54 (26-86)			7	60 (35-75)		
Hp+ DU[b]	11	50 (24-86)						
Erad DU[c]	6	64 (59-81)						
GC[d]	6	79 (63-85)	12	75 (55-82)	8	76 (49-84)	13	78 (63-85)
Hp- PC[e]					5	72 (64-80)		
Healthy							5	68 (57-75)
Total no.	**50**		**12**		**20**		**18**	

[a]AS – Asymptomatic; [b]DU - Duodenal Ulcer; [c]*Erad – H. pylori*-eradicated; [d]GC - gastric cancer, [e]PC – Pancreatic cancer

Diagnosis of *H. pylori* infection

For each patient, the *H. pylori* status was evaluated by different tests. Detection of antibodies specific for *H. pylori* in plasma was done by using a commercial plasma quick-test for anti-*H. pylori* IgG (Status *H. pylori*, LifeSign LLC, Somerset, NJ, USA), in house ELISA methods for detection of IgA and IgG antibodies against *H. pylori* membrane proteins (Mattsson, et al. 1998) as well as with an EIA-G III ELISA kit (Orion Diagnostics, Finland). Biopsies from gastric and duodenal mucosa were sonicated and cultured on Columbia-Iso agar plates, and the number of *H. pylori* DNA copies in the biopsy-mix was quantified with an *H. pylori*-specific quantitative real-time PCR method (Janzon, et al. 2009). A patient was scored as *H. pylori* positive if positive in culturing or PCR analysis or positive in two of the antibody test.

Collection of mucosal tissue and blood samples

To collect samples for comparison of normal duodenum and duodenal gastric metaplasia (DGM), areas of DGM were identified in the *H. pylori*-infected subjects by methylene blue staining during endoscopy as previously described (Stromberg, et al. 2003a). This procedure stained the normal mucosa dark blue but left areas of DGM unstained and pink, which allowed for directed biopsy sampling from the different mucosa types. To collect biopsies for measuring gene expression in the antral and duodenal mucosa, endoscopy without methylene blue staining was performed. Collection of gastric tumor and tumor-free mucosa was performed during gastrectomy operations of non-cardia gastric adenocarcinoma patients. Gastric tumor-free mucosa was defined as tissue with normal visual appearance located at least 5 cm from the nearby tumor. From the patients who underwent gastrectomy, 40 ml of blood was collected and 10-30 ml of blood was taken from endoscopy patients.

Isolation of PBMC and LPL

Peripheral blood mononuclear cells (PBMC) were isolated from blood from the volunteers by density-gradient centrifugation on Ficoll-Paque (GE Healthcare Bio-Sciences AB, Sweden). Lamina Propria Lymphocytes (LPL) were isolated using collagenase/DNAse enzymatic digestion, as previously described (Lundgren, et al. 2005b). Briefly, the tissue was first stripped of any muscle or fat layers before being cut into small pieces and incubated with Hank´s balanced salt solution (HBSS) without calcium or magnesium containing 1mM EDTA and 1mM DTT (Sigma, Sweden) to remove the epithelium and all intraepithelial lymphocytes. Thereafter, the bottles were incubated for two hours at 37˚C in a

collagenase/DNAse solution (100 U/ml collagenase, 0,1 mg/ml DNAse, Sigma, Sweden), releasing the LPL. The lymphocytes were then run through a nylon mesh to get rid of any remaining tissue. This procedure gives an optimal yield of LPL with very little epithelium remaining.

Immunofluorescence staining

The regulatory T cells in duodenal and gastric mucosa were identified by double staining with anti-FOXP3 and anti-CD4 antibodies. Biopsies from duodenal and gastric mucosa frozen in OCT compound (Tissue-Tek; Miles Inc., Elkhart, IN, USA) and stored at -70°C, were cut into sections (6 µm thin), fixed in 4 % paraformaldehyde, and rinsed in PBS containing 0.1 % saponin, followed by blocking of biotin in the tissue (Molecular Probes, Eugene, Oregon, USA). Thereafter, the slides were incubated with mouse IgG1 anti-FOXP3 (clone 236A/E7) (Roncador, et al. 2005) or with mouse IgG1 as a negative control (DAKO, Glostrup, Denmark) followed by detection using tyramide-Alexa Fluor 488 amplification (TSA kit, Molecular Probes). The samples were then incubated with mouse IgG1 anti-CD4 (DAKO) followed by goat anti-mouse IgG1 Alexa Fluor 594 (Molecular Probes). The slides were mounted using a DAPI-containing mounting medium (Molecular Probes) in order to visualise the nuclei of the cells.

Microscopy and image analysis

The numbers of cells were counted using a conventional fluorescent microscope, Zeiss Axiovert 100TV, and the area was measured using the Zeiss Axiovision software, crypts and submucosa were not included in the analysis. The numbers of $CD4^+FOXP3^+$ positive cells in the lamina propria per mm^2 of total tissue were calculated. In Paper I each reported value represents the mean of two separate sections of the same biopsy. In Paper II the tissue sections was 4-5 times bigger and each reported value represents one separate section. To receive high quality images for publication, confocal microscopy (LSM META 510, Zeiss) was used at the Centre for Cellular Imaging at University of Gothenburg.

Flow cytometry

LPL and PBMC were stained extra- and intra-cellularly with fluorescent labelled antibodies. Different combinations of antibodies were used in the papers;

Paper I	Extracellular; α-CD4-FITC, α-CD8-PerCP, α-CD25-PE
	Intracellular; α-FOXP3-APC
Paper II	Extracellular; α-CD4-FITC, α-CD8-PerCP, α-CD25-PE, α-CD69-APC, α-integrin-β7-PE, α-integrin-αE-FITC, α-L-selectin-PE, α-CXCR3-FITC, α-CCR4-PE, α-CCR5-PE, α-CCR9-PE
Paper III	Extracellular; α-CD8-FITC, α-CD25-PE
	Intracellular; α-Perforin-PE
Paper IV	Extracellular; α-CD4-FITC, α-CD8-PERCP, α-CD25-PE, α-CD27-PE
	Intracellular; α-FOXP3-APC, α-Ki67-PE, α-CTLA-4-PE

The cells were stained for cell surface expression using optimal concentrations of antibodies followed by intracellular staining with FOXP3 permeabilization buffer kit (eBioscience, San Diego, CA)(Paper I, II and IV) or Cytofix/Cytoperm solution (BD Bioscience)(Paper III) according to the protocol of the manufacturer. After staining, the cells were fixed in formaldehyde and analyzed by flow cytometry using a FACSCalibur equipped with a blue and a red laser (BD, San Jose, CA). The Flow Cytometry data were analysed by Flow Jo (Flowjo, Oregon, US).

Isolation of RNA from biopsies and sorted cells

The mucosal tissue biopsies collected from gastric cancer patients were immediately placed in RNAlater (Ambion) and stored at -70°C until extraction of RNA. Isolated LPL and PBMC were sorted into $CD4^{+}CD25^{high}$ and $CD4^{+}CD25^{low/-}$ T cells using a FACSVantage SE (BD Bioscience) as previously described (Lundgren et al. 2005a). The cells were placed in lysis-buffer with 1% β-mercaptoetanol and stored at -70˚C until extraction of RNA. Isolation of total RNA from biopsies was performed according to the description of the manufacturers using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The biopsies were disrupted by a glass mortar and pestle and filtered through a QIAshredder column (Qiagen, Hilden,

Germany) before RNA extraction to ensure proper lysis and disruption of the tissue. The extracted tissue RNA was checked for integrity on a 1% agarose gel and the concentration was measured using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The tissue RNA was kept at -70˚C until further use. Total RNA from equal numbers of sorted $CD4^{+}CD25^{high}$ and $CD4^{+}CD25^{low/-}$ T cells were extracted from each patient, using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the description of the manufacturers and kept at -70˚C until further use .

cDNA synthesis

Isolated RNA from biopsies were converted to cDNA by using the Omniscript Reverse Transcriptase kit (Qiagen) and cDNA synthesis of RNA from sorted cells were done by the Sensiscript Reverse Transcriptase kit (Qiagen). The instructions from the supplier were followed and Random hexamers (cat.no.79236, Qiagen, Hilden, Germany) were used as primers. The cDNA were stored in -20°C until use.

Real-time RT PCR

The gene expression analysis was run on 7500 Real Time PCR System (Applied Biosystems) and commercial Taqman gene expression assays (Applied Biosystems) were used. The gene expression was measured on cDNA from both gastric and duodenal mucosal biopsies and cDNA from sorted cell from LPL and PBMC. The analysed genes coded for FOXP3, CD4, IL-10, TGF-β, IFN-γ, IL-8 and HPRT1. The real time PCR reactions were run in 96 well plates using the standard amplification conditions described for the 7500 Real Time PCR System (Applied Biosystems) and each reaction contained 1 µl cDNA (30ng), 1 x Universal PCR Master Mix, 1 µl primer mix and ribonuclease-free water in a total volume of 20 µl . All reactions were run in duplicate and the average Ct value, the fractional cycle number at which the fluorescence passes the threshold, was calculated for each sample. HPRT1 was shown to be the best housekeeping gene for gastric and duodenal mucosal biopsies, as judged by analysis of the TaqMan® Human Endogenous Control Plate (part no 4309199) that contain assays for 11 different commonly used housekeeping genes. However, we found by comparing expressions in tumor and tumor-free mucosa that HPRT1 expression increased in tumor mucosa and hence, HPRT1 were used as the housekeeping gene in paper I and paper III but not in paper IV since samples from tumor mucosa were used for gene expression analyses in this paper. In paper I and III; the difference between the target gene and the HPRT1 reference gene (ΔCt) was always compared against a calibrator sample included and run on

each individual plate to allow comparisons between results obtained from different runs. The expression levels of the target genes were expressed as the ratio of target and reference gene ($2^{-\Delta Ct}$, according to the instructions from Applied Biosystems). In paper IV; the fold difference between tumor and tumor-free sample for each patient and gene was calculated by the Ct values (Bustin 2002). For analysis of gene expression in sorted cells, all reactions were run in duplicate and the average Ct value for each sample was calculated. The fold difference between $CD4^+CD25^{high}$ and $CD4^+CD25^{low/-}$ sample for each patient and gene was calculated by the Ct values (Bustin 2002).

Suppression assay

For investigation of Treg suppressive activity, monocytes were used as APC and isolated from PBMC by adherence of the remaining cell population after CD4 and CD8 cell depletion using magnetic beads (Dynal, Oslo, Norway). Sorted $CD4^+CD25^{high}$ cells, $CD4^+CD25^{low/-}$ cells and $CD4^+$ cells were added to 96-well plates and stimulated with either anti-CD3 or an *H. pylori* membrane preparation (MP), prepared as previously described (Mattsson et al. 1998). In co-culture experiments, $CD4^+CD25^{high}$ or $CD4^+CD25^{low/-}$ cells were cultured together with $CD4^+CD25^{low/-}$ cells. Replicated supernatants were then pooled after 48h and stored at -70°C until analysis of IFN-γ content with cytometric bead array (BD, Bioscience). After 5 days of cultivation, cell proliferation was measured with a thymidine incorporation assay.

T-cell stimulation

In paper III, antigen-presenting cells (APC) were obtained by adding PBMC ($CD4^+$- and $CD8^+$-T-cell-depleted) to round-bottomed, 96-well plates (NUNC, Aarhus, Denmark) at 10^5 cells/well and incubated for 2 h at 37°C. Thereafter, the non-adherent cells were washed away twice with PBS, and 10^5 cells of $CD4^+$ or $CD8^+$ T cells, isolated with Dynabeads (Lundin, et al. 2002), per well were added. The cells were stimulated with 10 µg/ml of *H. pylori* MP heat-killed *H. pylori* or *H. pylori* lysate (10^5 heat-killed or lysed *H. pylori* per well). As positive control, 5µg of phytohemagglutinin (PHA) (Murex Diagnostics Ltd., Temple Hill, United Kingdom) per ml was used and culture medium alone was used as negative control. The plates were then incubated in 5% CO_2 at 37°C for 5 days. Forty-eight hours after the initiation of culture, the supernatants from at least three wells were pooled and stored at -70°C until analysis of cytokines. Proliferation was measured in parallel culture wells by adding tritiated thymidine on day 5 followed by harvesting and analysis using a β–counter.

Statistical analysis

Statistical evaluation was performed using the GraphPad PRISM 3 and 4 software. The used tests were Wilcoxon matched pair test, Mann-Whitney Test and Student T test. P-values < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Localization and frequency of $CD4^+FOXP3^+$ T cells in peripheral tissue

It is possible that the inability of the immune system to clear *H. pylori* infections is due to a dysregulation of the immune response. In this thesis the presence and suppressive capacity of regulatory T cells was studied in order to further determine the role of these cells in *H. pylori* induced disease. Most studies in regulatory T cells have been performed on cells derived from blood since they are easier to obtain, but since it is more relevant to study the cells at the site of infection, this thesis is mainly focused on regulatory T-cells located in mucosal tissues and their responses to *H. pylori* infection. In order to identify the regulatory T cell populations, we have either used high expression of the IL-2 receptor α chain CD25 as a marker on $CD4^+$ cells or alternatively recently described antibodies against the nuclear transcription factor FOXP3, which is currently the most accepted marker for regulatory T cells (Buckner and Ziegler 2008; Roncador et al. 2005).

FOXP3 expressing $CD4^+$ T-cells increase in *H. pylori* infected gastric mucosa

Detection of regulatory T -cells in antral and duodenal mucosa has been performed previously in our group using anti-CD25 antibody staining of tissue sections (Lundgren et al. 2005a). The cells detected with this method were mostly $CD4^+CD25^{high}$ T cells (Lundgren et al. 2005a; Stromberg, et al. 2003b), as confirmed using flow cytometry, and this cell fraction was increased in *H. pylori* infected mucosal tissue (Harris, et al. 2008; Lundgren et al. 2005b). However, when the antibodies directed against FOXP3 became available it was evident that FOXP3 staining was not always associated with high expression of CD25. In initial studies when we compared the profiles of $CD25^{high}$ and $FOXP3^+$ cells in blood as well as peripheral tissue from cancer patients we could confirm that a large majority (more than 95%) of the FOXP3-expressing cells were $CD4^+$ T cells (Paper I). However, the flow cytometry analysis showed that although 90% of the FOXP3-expressing cells in peripheral blood co-express CD25, only 74% of the FOXP3-expressing cells in gastric mucosa do express CD25 (fig. 2). This is novel information, and given that there is a high correlation between FOXP3-expression and suppressive function (Gavin et al. 2006; Long and Buckner 2008; Mahic et al. 2008b; Pillai et al. 2007; Walker et al. 2005; Walker et al. 2003), these results may indicate that a substantial fraction of gastric regulatory T cells are CD25-negative.

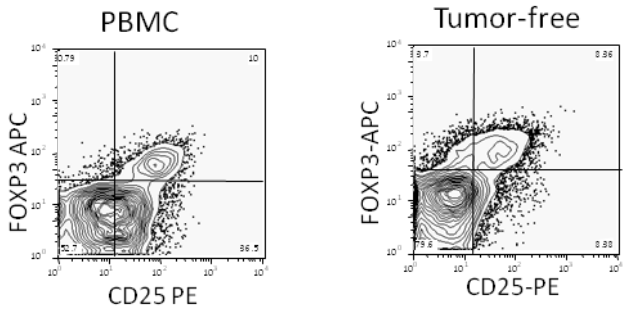

Figure 2: Expression of FOXP3 and CD25 in $CD4^+$ T cells isolated from PBMC and tumor-free gastric lamina propria of a *H.p.*+ gastric cancer patient.

Since we observed that a relatively large number of cells in the gastric mucosa were $CD25^-$ $CD4^+FOXP3^+$ T cells, the former stainings with CD25 may not have detected these cells. Therefore, we decided to investigate the localization and frequency of $CD4^+FOXP3^+$ T cells at the single-cell level in duodenal and gastric mucosa in tissue sections. To this end, we established an immunofluorescence double staining method for simultaneous detection of CD4 and FOXP3 which was then used throughout the studies (fig. 3).

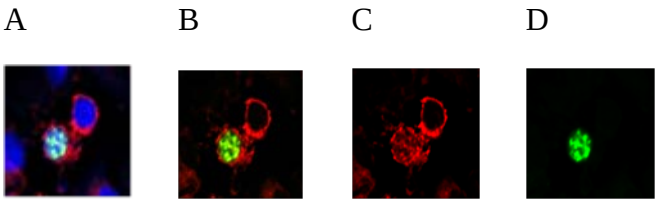

Figure 3: Immunofluorescence staining of FOXP3 and CD4 in stomach mucosa, stained as described in the methods section. CD4 staining is red, FOXP3 staining green and nuclear staining (DAPI) is blue. The images show the same area using red, green and blue filters (A), red and green filters (B), only red filter (C) and only green filter (D). Both primary antibodies are mouse IgG_1, and because of the sequential staining the red secondary antibody (AF594 goat-anti-mouse IgG_1) binds both primaries (see C). This poses no problem, due to the differential localization of CD4 and FOXP3 expression.

Using this method, we initially investigated the FOXP3- and CD4-expression in the antrum. The staining results showed that the numbers of $CD4^+FOXP3^+$ T cells were increased in the antrum of AS and DU subjects compared to uninfected subjects, demonstrating an increased $CD4^+FOXP3^+$ T cell population in the antrum of *H. pylori*-infected individuals (fig. 4) (paper I). This is agreeing well with the previous results from our group, which demonstrated increased $CD4^+CD25^{high}$ frequencies in *H. pylori*-infected stomach mucosa (Lundgren et al. 2005a).

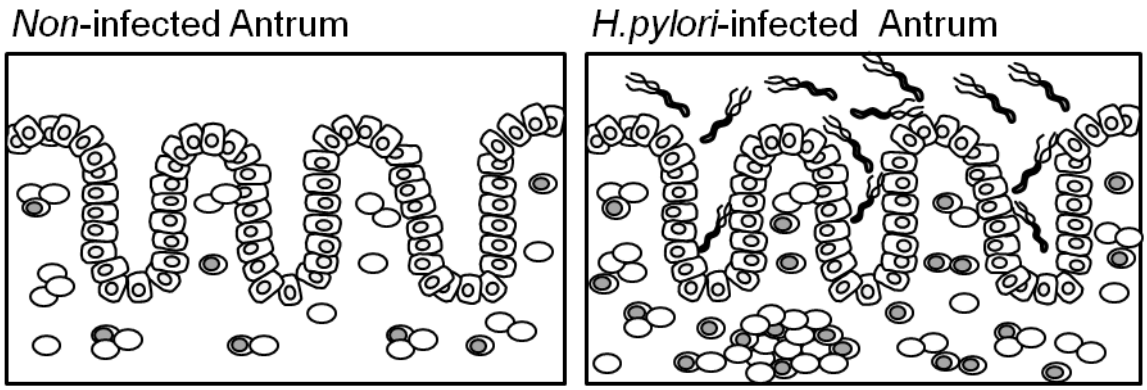

Figure 4: A schematic drawing of non-infected and *H. pylori*-infected gastric mucosa.

: $CD4^+FOXP3^+$ T cell : $CD4^+$ T cell

Increase of FOXP3-expressing $CD4^+$ T cell numbers in areas of duodenal gastric metaplasia (DGM) of DU patients.

H. pylori colonize only the areas of duodenal gastric metaplasia (DGM) in the duodenum and not the normal duodenal mucosa (Steer 1984; Wyatt et al. 1987). A way to differentiate normal duodenal epithelia from DGM is application of methylene blue during gastroscopy. The normal duodenum will then be stained blue while DGM is left unstained. The use of this method made it possible for us to pick biopsies separately from normal duodenum and DGM. In a previous study using this method of biopsy collection, the expression of CD25 and CTLA-4 was analysed with immunohistochemistry staining. This revealed an increased presence of CTLA-4 in duodenum of DU patients compared to Hp^+AS, supporting an increase of regulatory T cells in these patients (Stromberg et al. 2003b). To specifically enumerate putative Tregs in duodenum, we now analyzed FOXP3-expressing cells using our established immunofluorescence staining method, and compared areas of normal mucosa with DGM areas in the different study groups (paper I). The results showed that there was a 2-fold increase in the numbers of $CD4^+FOXP3^+$ T cells in the DGM from DU patients compared to DGM in AS individuals, and both DU and AS patients had a higher number of $CD4^+FOXP3^+$ T cells in DGM compared to normal duodenum (fig. 5) (paper I). Previously, DU patients have been shown to have 20-fold higher density of *H. pylori* in the duodenum compared to AS individuals and a 4-fold higher extent of DGM in duodenum (Hamlet, et al. 1999).Taken together, our results from both antrum and duodenum therefore indicate that increased numbers of $CD4^+FOXP3^+$ T cells are associated with presence of *H. pylori*.

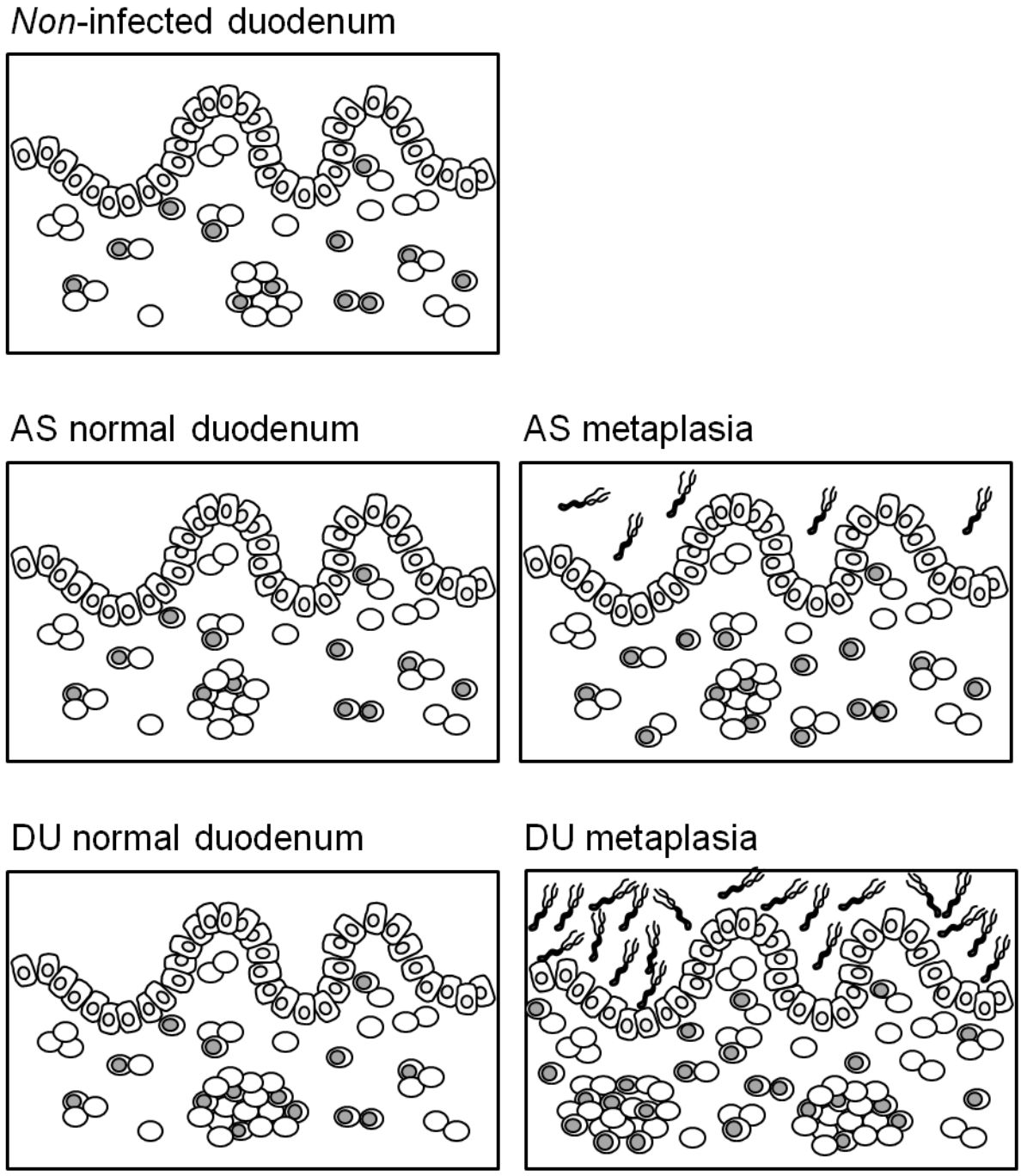

Figure 5: A schematic drawing of non-infected duodenum and normal and DGM areas of *H.pylori*-infected DU and AS individuals. In both DU and AS the number of $CD4^+FOXP3^+$ T cells are higher in DGM compared to normal duodenal mucosa. There was a 2-fold increase in the numbers of $CD4^+FOXP3^+$ T cells in the DGM from DU patients compared to DGM in AS individuals.

: $CD4^+FOXP3^+$ T cell : $CD4^+$ T cell

FOXP3 cells decrease in the antrum when *H. pylori* is eradicated

To verify that *H. pylori*-infection gives increased number of $CD4^+FOXP3^+$ T cells, we analysed the expression of FOXP3 by real-time RT-PCR in *H. pylori*-infected DU patients who had or had not been treated by antibiotics to eradicate *H. pylori* (paper I). The analysis indicated that the increased numbers of FOXP3-expressing T cells in the antrum in infected individuals were dependent on the presence of *H. pylori* bacteria, since antibiotic-treated DU patients had 4-fold decreased levels of FOXP3 mRNA in the tissue. The mRNA level for CD4 was also decreased after eradication. So, these results indicate that the numbers of $CD4^+FOXP3^+$ T cells and other T cells decline in parallel when the bacteria disappear from the gastric mucosa. This was supported by analysis of the relation between FOXP3 and CD4 mRNA, as this relation did not change markedly after eradication (paper I). In contrast to the antral mucosa, FOXP3 mRNA levels in the duodenal samples remained unchanged after *H. pylori* eradication treatment. This result was probably due to that the RNA analysis was performed on a second set of biopsies that were not collected using methylene blue staining. Since the extent of DGM is 15% in DU and only 4% in AS individuals (Hamlet et al. 1999), it is likely that the biopsies were taken from areas of normal duodenal mucosa which was not colonized with *H. pylori*. Therefore, the differences in mRNA levels of eradicated and non-eradicated DGM mucosa are expected to be greater than what was detected in the current setting. Our data support the findings that FOXP3 mRNA expression is up-regulated in antrum of *H. pylori*-infected compared to non-infected individuals (Kandulski, et al. 2008), but to our knowledge we show for the first time that the FOXP3 expression decrease in antrum after *H. pylori* eradication.

Localization of FOXP3-expressing cells

The immunofluorescence staining provided information about the localization of $CD4^+FOXP3^+$ cells. A relatively large fraction of the $CD4^+FOXP3^+$ T cells were located in aggregates of $CD4^+$ T cells, both in antral and duodenal infected mucosa. The formation of aggregates or follicles is highly associated with *H. pylori* infection and has been shown to correlate to both the severity of the gastritis and the density of bacteria (Sepulveda, et al. 2002; Zaitoun 1995). In DGM of DU patients, four out of five patients had more than 50% of their $CD4^+FOXP3^+$ T cells associated to aggregates, while none of the AS individuals reached such a high frequency of aggregate association. Among antrum samples, there was considerable variation in the association of $CD4^+FOXP3^+$ T cells to $CD4^+$ T cell aggregates, so there was no major differences observed between AS and DU individuals.

Gastric tumors showed increased levels of FOXP3 expressing cells

It has earlier been shown that $CD4^+CD25^{high}$ T-cell numbers increase in gastric tumor mucosa compared to tumor-free mucosa (Lundgren et al. 2005a). The higher expression of CD25 may reflect increased numbers of Treg in the tumor mucosa, but could alternatively also be due to increased number of activated T cells expressing high levels of CD25. For this reason, we used $CD4^+FOXP3^+$ staining to confirm an increase of regulatory T cells, as well as to determine their localization in the tumor and the surrounding tissue.

By immunofluorescence double staining of tissue sections we found that *H. pylori*-infected gastric cancer patients had significantly increased numbers of $CD4^+FOXP3^+$ cells in the tumor mucosa compared to tumor-free mucosa (paper II). By analysing cells from gastric cancer patients with flow cytometry, we confirmed an increased proportion of $CD4^+FOXP3^+$ T cells among the $CD4^+$ T cell population in the gastric tumor mucosa compared to tumor-free mucosa (fig. 6). The gastric cancer patients analyzed where both *H. pylori* positive and negative, supporting that the tumor itself was the main cause of increased numbers of $CD4^+FOXP3^+$ T cells in the tumor mucosa. This increase of $CD4^+FOXP3^+$ cells induced by the tumor is of high clinical relevance, since it has recently been shown that gastric cancer patients with increased number of $FOXP3^+$ cells in gastric tumor mucosa are associated with adverse prognosis (Perrone, et al. 2008) and that the level of $FOXP3^+$ Tregs cells decrease after tumor resection (Shen et al. 2009).

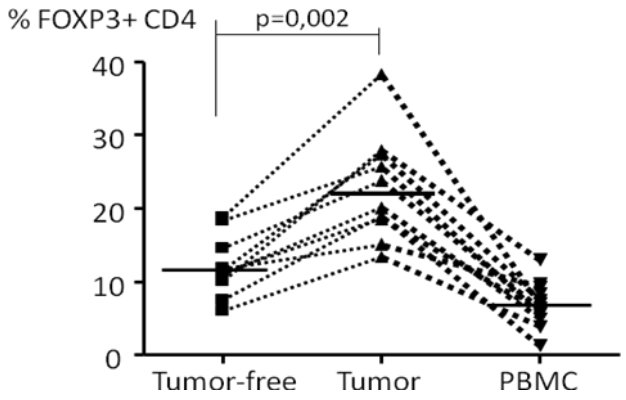

Figure 6: Flow cytometry analysis of FOXP3 frequency among $CD4^+$ T cells of tumor and tumor-free gastric mucosa and of peripheral blood.

Three different cell groups could be identified among $CD4^+FOXP3^+$ T cells with respect to CD25 expression: $CD25^{high}$, $CD25^{low}$ and $CD25^{neg}$. The $CD25^{high}$ $FOXP3^+$ population increased around two-fold in the tumor tissue compared to tumor-free mucosa. Furthermore, the levels of FOXP3 and CD25 were correlating on an individual cell basis, *i.e.* the more CD25 the more FOXP3 expression (paper IV). This agrees with recent data indicating that Treg are dependent on continuous STAT5-signaling provided by IL-2 or IL-15 (Goldstein 2009). Presumably this would occur to a higher extent in cells expressing high levels of CD25, thereby explaining the increased FOXP3-expression levels of these cells.

In gastric cancer patients, many of the $CD4^+FOXP3^+$ cells were equally scattered throughout the lamina propria and 20 % of all $CD4^+FOXP3^+$ cells were located in lymphoid follicles (paper II) which is similar to the median value for $CD4^+FOXP3^+$ cells in aggregates for AS and DU in antrum. The pattern was the same in all different groups, the $CD4^+FOXP3^+$ cells not located in aggregates/follicles were located in close contact to other $CD4^+FOXP3^+$ cells, $CD4^+FOXP3^-$ or non-$CD4^+$ cells. It is presently unknown which non-$CD4^+$ cells are in close contact with the $CD4^+FOXP3^+$ cells, and this warrants further investigation. Nevertheless, the location of $CD4^+FOXP3^+$ cells and $CD4^+FOXP3^-$ cells suggest active interactions between Treg and other T cells in the gastric mucosa in cancer as well as in tumor-free *H. pylori* infected mucosa.

It is evident that both *H. pylori* infection and the presence of a gastric tumor are factors that increase the number of $CD4^+FOXP3^+$ T cells. Presumably, both these conditions would gain an advantage of inducing a suppression of the immune response against themselves. This is supported by data showing reduced *H. pylori* numbers in mouse models were $CD25^+$ Tregs have been depleted (Rad, et al. 2006; Raghavan, et al. 2003). Furthermore, reports showing worse prognosis of cancer patients correlating to higher intratumoral numbers of Treg demonstrate a benefit for the tumor inducing increased Treg-numbers (Perrone et al. 2008). In addition, as a further complication in *H. pylori*-induced gastric cancer, a tumor developing in the presence of an *H. pylori* infection would benefit from elevated levels of regulatory T cells due to the infection.

One possible explanation for the increased number of Treg in *H. pylori*-infection and gastric tumors are the expression of cyclooxygenase II (COX-2), which increase in gastric tumors (Chen, et al. 2001) and in *H. pylori*-infected mucosa (Sawaoka, et al. 1998). COX-2 is a key enzyme in biosynthesis of PGE_2 which can upregulate IDO expression in DC (Braun, et al. 2005) and also induces FOXP3 in human $CD4^+CD25^-$ T cells (Baratelli, et al. 2005). Induced Treg upregulate the expression of COX-2 and produce PGE_2 which may both in an autocrine and a paracrine way upregulate the expression of FOXP3 (Mahic et al. 2006). This may also be linked to the increased expression of IL-8 observed both in *H. pylori*-infected mucosa and in gastric tumors (Bartchewsky, et al. 2009; Crabtree, et al. 1993; Kido, et al. 2001; Takehara, et al. 2006).

Suppressive capacity and Treg function

Suppression assays indicate a suppressive function of FOXP3$^+$ CD4$^+$CD25^{high} T cells

Based on our results we suggested that regulatory T cells increase as a response to local *H. pylori* infection of the mucosa and that the outcome of this is that the immune response against *H. pylori* is decreased (paper I). However, presence of FOXP3-expressing cells is not a proof of ongoing active suppression in the tissue. Therefore, one important question is whether the mucosal regulatory T cells in *H. pylori*-infected individuals are antigen-specific, and have the capacity to suppress the proliferation and cytokine response - *e.g.* IFN-γ production - of *H. pylori* stimulated CD4$^+$CD25$^-$ responder cells. To address this, the first step was to characterize the CD4$^+$CD25^{high} cells with regard to the expression of FOXP3 which had been directly linked to suppressive function (Walker et al. 2003). FOXP3 mRNA was readily detected in CD4$^+$CD25^{high} cells in the tumor as well in the tumor-free mucosa, while no or only weak FOXP3 expression could be detected in the CD4$^+$CD25$^{low/-}$ cells (paper II). Furthermore, analysis of the FOXP3 protein expression by flow cytometry showed that > 90% of the CD4$^+$CD25^{high} cells in both tumor and tumor-free mucosa expressed FOXP3, whereas < 20% of the CD4$^+$CD25$^{low/-}$ cells expressed this marker (paper II). Interestingly, CD4$^+$CD25^{high} cells from the tumor expressed higher FOXP3 levels compared to the tumor-free mucosa, as shown by both mRNA analysis and flow cytometry (paper II). Taken together, analyses of both FOXP3 mRNA and protein expression support that CD4$^+$CD25^{high} cells had a Treg phenotype. Based on this information, CD4$^+$CD25^{high} T cells were used as regulatory T cells in the suppression assays. Since the antibodies against FOXP3 are used for intracellular staining, the cells need to be permeabilized before staining, and they can therefore not be used in experiments that demand living cells.

Lundgren et al previously showed that *H. pylori*-infected individuals have impaired peripheral blood memory CD4$^+$ T-cell responses to *H. pylori,* which is linked to the presence of *H. pylori*-specific regulatory T cells that actively suppress the responses (Lundgren et al. 2003). Therefore, we wanted to investigate the suppressive function of gastric mucosal regulatory T cells in *H. pylori*-infected individuals on *H. pylori* stimulated T cells. To receive sufficient numbers of isolated lamina propria lymphocytes from gastric mucosa, *H. pylori*-infected gastric cancer patients that went through gastrectomy were used. As shown in paper II, depletion of CD4$^+$CD25^{high} T cells from isolated CD4$^+$ T cells from tumor-free gastric mucosa lead to an increased proliferation in seven out of eight individuals and also to increased IFN-γ

production in six of eight patients. Furthermore, co-culture experiment with $CD4^+CD25^{high}$ and $CD4^+CD25^{low/-}$ (1:1) from both tumor and tumor-free mucosa stimulated with *H. pylori* antigen showed a reduced proliferation and IFN-γ production compared to stimulation of only CD4+$CD25^{low/-}$ cells. These results demonstrated that $CD4^+CD25^{high}$ cells in *H-pylori*-infected gastric mucosa have a suppressive function on effector $CD4^+$ T cells stimulated with *H. pylori* antigen, and that expression of FOXP3 in vivo is associated to suppression. To our knowledge, this was the first study that investigated antigen-specific suppression of immune responses by local human Tregs in mucosal infection. Our results are supported by a recent study showing that $CD4^+CD25^+CD127^{low/-}$ can be used as a selective marker to enrich human Treg cells from blood and gastric mucosa and around 95% of the cells express FOXP3. The authors used co-culture assays with anti-CD3 and anti-CD28 stimulation and found that $CD4^+CD25^+CD127^{low/-}$ T cells from gastric mucosa have a suppressive function on $CD4^+CD25^-CD127^{high}$ responder cells (Shen et al. 2009).

Gene expression of $CD4^+CD25^{high}$ Regulatory T cells

Stimulation of $CD4^+CD25^{high}$ T cells from gastric tumor-free mucosa with anti-CD3 induced a very low proliferation and did not produce detectable levels of IL-10, IFN-γ or IL-2 (paper II). In comparison, $CD4^+CD25^{high}$ T cells from colon mucosa in ulcerative colitis patients have shown similar results, with the exception that IL-10 production could be detected (Holmen, et al. 2006). Analogous findings have also been made by others (Shen et al. 2009; Walker et al. 2003)

From this, the conclusion could be drawn that the $CD4^+CD25^{high}$ T cells have a low cytokine production, but we wanted to study this *in vivo*. Therefore, we sorted PBMC and LPL from gastric tumor and tumor-free mucosa into $CD4^+CD25^{high}$ and $CD4^+CD25^{low/-}$ T cells for mRNA expression analysis. RNA from sorted cells of each population was converted to cDNA and quantitative real-time RT-PCR was used to analyze the expression of IL-10, TGF-β, IFN-γ and the putative house-keeping gene HPRT1. We found that $CD4^+CD25^{high}$ T cells expressed higher levels of HPRT1 compared to $CD4^+CD25^{low/-}$ T cells in samples with the same number of cells, and therefore was not appropriate to use as house-keeping gene (paper IV). For this reason, the difference in gene expression between sorted $CD4^+CD25^{high}$ and $CD4^+CD25^{low/-}$ T cells was calculated from the difference in Ct values in equal numbers of cells without reference to the HPRT1 Ct value, according to a previously described method (Bustin 2002). Due to the low numbers of cells obtained by sorting from tumor tissue,

different cell numbers of sorted cells were acquired, and for this reason gene expression differences between various samples could not always be compared. Instead, comparisons between different genes within a sample were performed (paper IV). The results showed that $CD4^+CD25^{high}$ T cells had a higher ratio of IL-10 vs. IFN-γ expression compared to $CD4^+CD25^{low/-}$ T cells, which supports suppressive and effector functions of the $CD25^{high}$ and $CD25^{low/-}$ populations, respectively (fig. 7). Unexpectedly, TGF-β was expressed in higher levels in $CD4^+CD25^{low/-}$ cells in both tumor-free mucosa and PBMC compared to $CD4^+CD25^{high}$ cells. The relative increase in IFN-γ and TGF-β in the $CD4^+CD25^{low/-}$ cells was more pronounced in the tumor-free gastric mucosa compared to in PBMC, indicating a cell specific up-regulation of both the Th1-cytokine IFN-γ and the suppressive TGF-β in the mucosa (fig. 7).

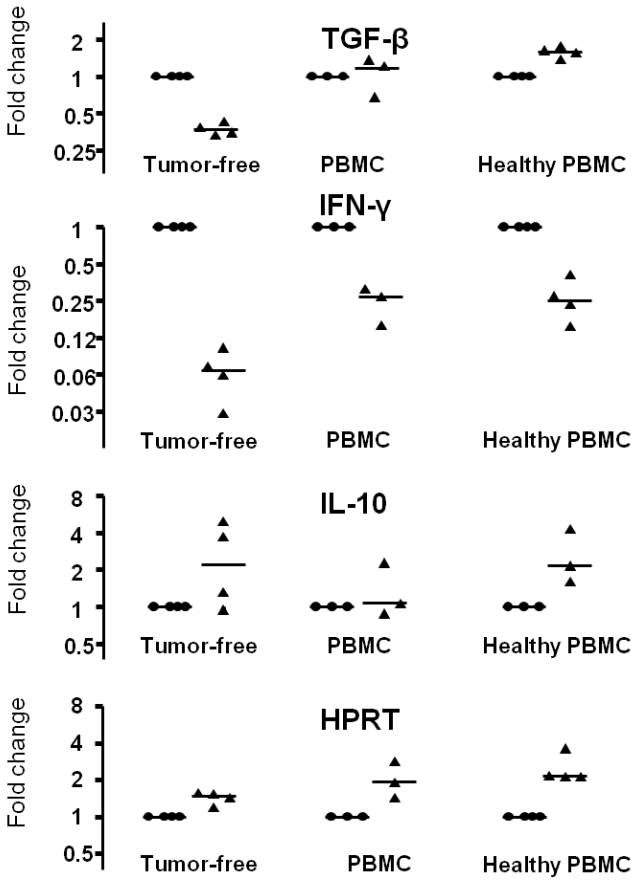

Figure 7: Fold-differences of expression levels in sorted $CD4^+CD25^{high}$ T cells in relation to $CD4^+CD25^{low/-}$ T cells. Gene expression levels of IFN-γ, IL-10, TGF-β and HPRT1 from isolated $CD4^+CD25^{high}$ and $CD4^+CD25^{low/-}$ cells of tumor free gastric mucosa and PBMC were analyzed with Real-time RT PCR. The graphs show the fold difference of expression in $CD4^+CD25^{high}$ (triangles) compared to the expression of $CD4^+CD25^{low/-}$ (circles) normalized to 1 for each paired sample, with equal numbers of counted cells from each individual.

The mRNA analysis of $CD4^+CD25^{high}$ T cells from gastric tumor-free mucosa show increased production of IL-10 but lower levels of IFN-γ compared to $CD4^+CD25^{low/-}$. This is opposite to the results of the in vitro stimulations, which failed to detect any cytokine production by the $CD4^+CD25^{high}$ T cells (paper II). However, the reason for the low or absent amounts of cytokines observed may be suboptimal *in vitro* culture conditions, and hence it is more relevant to study cells isolated directly from the inflamed tissue (paper IV). TGF-β is known to be an important cytokine for the regulatory T cells (Nakamura, et al. 2004; Zheng et al. 2004). However, in our analysis, $CD4^+CD25^{low/-}$ T cells from the mucosa expressed higher levels of TGF-β compared to $CD4^+CD25^{high}$ cells. This was surprising, but in line with this, it has been shown that the suppressive function of Treg cells from TGF-β-deficient mice can be abrogated by anti-TGF-β monoclonal antibodies, indicating that functional TGF-β can be provided by a non-Treg cell source (Fahlen, et al. 2005). Thus, our results show that $CD4^+CD25^{high}$ T cells are not the main producers of TGF-β in the gastric mucosa, and support the possibility that regulatory T cells may use TGF-β from other cell sources to induce suppression.

$CD4^+FOXP3^+$ cells expressing CTLA-4 and CD27 support a suppressive function

Since FOXP3 recently has been debated as a marker for cells with suppressive capacity (Allan et al. 2007; Tran et al. 2007), we were interested to study if the $CD4^+FOXP3^+$ T cells express other markers related to suppressive function. In paper IV we analyzed the expression of CTLA-4 and CD27 in $FOXP3^+$ T cells by flow cytometry, since these markers have been reported to be linked with suppressive activity (Duggleby et al. 2007; Levings et al. 2001b; Ruprecht et al. 2005; Wing et al. 2005; Zheng et al. 2008). We could confirm that 85 % of the analysed FOXP3 expressing cells in the tumor mucosa co-expressed CD27 and 100 % co-expressed CTLA-4. Taken together, this strengthens our hypothesis that the majority of the $CD4^+FOXP3^+$ T cells in the tumor and tumor-free gastric mucosa have a suppressive phenotype.

The unknown function of $CD25^-CD4^+FOXP3^+$

From the flow cytometry staining it was obvious that $CD4^+FOXP3^+$ T cells belong to a separate population of cells distinct from the $CD4^+FOXP3^-$ T cells. In contrast, the expression of CD25 in the $FOXP3^+$ population ranged from no expression at all to high expression of the receptor, without occurrence of any separate populations. We decided to group the $FOXP3^+$ cells into $CD25^-$, $CD25^{low}$ and $CD25^{high}$. It warrants further studies to investigate whether the

$CD25^-CD4^+FOXP3^+$ cells have a suppressive function or if they are $CD4^+$ $FOXP3^+$ T cells without a suppressive function. However, there are at the moment no surface markers to distinguish and analyze these cells and it is impossible to sort cells based on FOXP3 expression without killing them. Hence, today it is not possible to do a co-culture assay to measure the suppressive capacity of these $CD25^-CD4^+FOXP3^+$ cells. Another way to determine if these cells have a suppressive function might be to measure the level of demethylation in the FOXP3 promotor. This genomic region is highly demethylated in natural Treg, and only complete demethylation of the conserved FOXP3 promotor region support stable long term FOXP3 expression and a committed Treg phenotype in humans (Janson et al. 2008). Furthermore, it has been proposed that $CD4^+CD25^-$ T cells can be induced to demethylate the FOXP3 promotor region and/or other regulatory regions upstream of the FOXP3 gene (Lal, et al. 2009) and thereby up-regulate expression of FOXP3 and obtain a suppressive phenotype (Gavin et al. 2006; Long and Buckner 2008; Mahic et al. 2008b; Pillai et al. 2007; Walker et al. 2005; Walker et al. 2003). This is an important issue that may be investigated in future studies.

Immune-responses to *H. pylori*-stimulation in T cells from gastric cancer patients

To find evidence for immunosuppression in patients with gastric cancer, we studied the immune response to *H. pylori* in such patients. In PBMC, stimulation with *H. pylori* MP induced production of higher levels of IL-10 from both $CD4^+$ and $CD8^+$ T cells compared to cells from AS individuals and *H. pylori* negative patients with pancreatic cancer (Paper III). Production of IFN-γ from MP-stimulated $CD8^+$ T cells was equal between the different groups. In contrast, production of IFN-γ from $CD4^+$ T cells of AS individuals were 250-fold higher than cells of gastric cancer patients. Furthermore, the proliferation to MP was suppressed among $CD8^+$ T cells from gastric cancer patients. It was not possible to compare the responses of the gastric mucosa between AS individuals and gastric cancer patients, due to lack of sufficient cell numbers from the non-cancer patients. Nevertheless, the analysis of LPL from gastric cancer patients revealed a similarly high IL-10 production in the mucosal cells, among both $CD4^+$ and $CD8^+$ T cells. The production of IFN-γ was 10 fold higher among $CD4^+$ T cells in gastric tumor-free mucosa compared to blood, but equal amount of IFN-γ was produced in $CD8^+$ T cells in blood and mucosa.

To overcome the lack of isolated gastric T cells from AS individuals, gastric mucosal biopsies were taken from gastric cancer patients and AS for analysis of mRNA expression of IL-10 and IFN-γ. The results showed a 3.5 fold increase of IL-10 mRNA in biopsies from gastric cancer patients compared to AS individuals and these results confirm that gastric cancer is associated with an increased production of IL-10 in the stomach mucosa. There were no major differences in IFN-γ mRNA expression between gastric cancer patients and AS. Furthermore, when the IFN-γ mRNA expression was compared in samples from tumors and from the surrounding tumor-free area, an up-regluation of IFN-γ mRNA expression was found in the tumor (Paper IV).

A possible reason for higher levels of IL-10 in gastric mucosa and higher production of IL-10 in $CD4^+$ and $CD8^+$ T-cell cultures of *H. pylori*-infected gastric cancer patients is that these patients had a genetic predisposition for a high production of IL-10. However, this is unlikely, since people with a gene polymorphism leading to low IL-10 production runs a higher risk of developing gastric cancer (El-Omar, et al. 2003). A more likely explanation to the increased production of IL-10 is that the presence of the tumor induces an altered immune response that involves higher IL-10 production. In support of this, recent data from a mouse model of breast

cancer showed that as the tumor progressed there was induction of IL-10-producing cells both among IFN-γ secreting and non-secreting $CD4^+$ as well as $CD8^+$ T cells (Salazar LG et al, *in press*), In addition, there may also be induction of IL-10 producing $CD4^+$Tr1 cells (Groux et al. 1997) and $CD8^+IL10^+$ regulatory T cells (Wei, et al. 2005).

The IFN-γ production was higher in T cells from the gastric mucosa than in cells from peripheral blood of gastric cancer patients (paper III). These results are supported by mRNA analysis of sorted $CD4^+CD25^{high}$ and $CD4^+CD25^{low/-}$ cells, which showed a 5-fold increased expression of IFN-γ mRNA in $CD4^+CD25^{low/-}$ cells of gastric mucosa compared to cells of peripheral blood (paper IV). Further studies are needed to determine the identity of the IL-10 producing cell populations of GC patients, including cellular and epigenetic regulation of these cells.

$CD8^+$T cell activity and suppressive milieu

Since we showed that there are increased numbers of $CD4^+FOXP3^+$ T cells in gastric tumor mucosa compared to tumor-free mucosa (paper II), we were interested in the effect of the increased Treg-numbers on the total cytokine production and there by the anti-tumor immune response. Therefore, expression of Treg-associated TGF-β and IL-10 and the pro-inflammatory cytokine IL-8 and IFN-γ were measured. RNA was isolated from whole biopsies from gastric tumor- and tumor-free mucosa and quantified using real-time RT-PCR. We found that all analyzed genes had higher mRNA levels in the tumor compared to the corresponding tumor-free mucosa. Although FOXP3 gene transcripts were increased in the tumor compared to tumor-free mucosa in the same patient, supporting the results from FCM and immunofluorescence staining (paper II), there was also a significant increase in transcription of IFN-γ and IL-8 in the tumor (paper IV). This indicates an increased pro-inflammatory profile of the tumor compared to the tumor-free surrounding mucosa. The increase of a range of pro-inflammatory cytokines in gastric tumor tissue has also been detected in another recent study (Ellmark, et al. 2006). It is likely that an inflammatory response promote tumor development and progression (Karin 2006), while the observed increase of anti-inflammatory signals may be generated to counteract the tumor-specific response.

$CD4^+CD25^{low/-}$ and $CD8^+$ T cells express higher levels of activation marker CD69 in tumor mucosa compared to tumor-free mucosa

Effector $CD4^+CD25^{low/-}$ T cells and cytotoxic $CD8^+$ T cells are important cells for the anti-tumor immune response. We were therefore interested to determine if the activity of these cells was lower in Hp^+GC compared to Hp^+AS and in tumor mucosa in Hp^+GC compared to tumor-free mucosa, depending on increased numbers of Treg. In gastric tumor mucosa, $CD4^+CD25^{low/-}$ T cells expressed lower amounts of the activation marker CD69 compared to gastric tumor-free mucosa indicating a suppression of the effector CD4 T cells in the tumor (paper II), while gastric mucosal $CD8^+$ T cells expressed equal levels of perforin in both Hp^+ GC patients and Hp^+ AS controls, indicating that the cytotoxic capacity was equal in both groups (paper III). However, analysis of the CD25 expression on $CD8^+$T cells showed that Hp^+ AS have 4 times more activated $CD8^+$ cells compared to Hp^+ GC irrespective of whether the cells of the latter were isolated from gastric tumor or tumor-free mucosa. This demonstrates diminished levels of activated mucosal $CD8^+$ T cells both in the tumor and

outside the tumor of GC patients. Furthermore, there was a decrease in CD69 expression on the $CD8^+$ T cells from tumor mucosa, indicating a more marked reduction of CD8 activity in tumor compared to tumor-free mucosa.

The higher IL-10 mRNA expression and lower $CD8^+$ T cell activity in Hp^+GC compared to Hp^+AS indicate an inverse correlation between IL-10 expression and $CD8^+$ T-cell activity. In line with this, an IL-10 knockout mouse model revealed that the cytotoxic capacity is increased in the $CD8^+$ intraepithelial lymphocytes from IL-10-deficient compared to wild-type mice, supporting that IL-10 can reduce the cytotoxic function in $CD8^+$ T cells (Utermöhlen 2008). This matches well with the theory that the tumor cells up-regulate the expression of IL-10 to avoid clearance by an anti-tumor immune response.

The proportion of $CD4^+FOXP3^+$ T cells vs. $CD8^+$ T cells is increased in gastric tumors.

Furthermore, the proportion of $CD4^+FOXP3^+$ T cells was also analyzed in relation to $CD8^+$ cells in the tumor and tumor free mucosa. There was a three-fold increase of $CD4^+FOXP3^+$ T cells vs. $CD8^+$ T cells in the tumor mucosa compared to tumor-free mucosa, which had a similar ratio as in the peripheral blood (paper IV). This data again supports that the anti-tumor response is suppressed in the gastric tumor mucosa.

The clinical importance of the Treg/CD8 ratio is highlighted by the fact that ovarian cancer patients exhibiting a low such ratio had a better prognosis (Sato et al. 2005). In gastric cancer this connection has not been studied, and it could not be done in the present study due to insufficiently low patient numbers. However, it is possible that the *H. pylori* infection drives the Treg/CD8 ratio towards higher values since we have seen that $CD4^+$ T cells stimulated *in vitro* with heat killed *H. pylori* up-regulate expression of FOXP3, and that these cells have the capability to reduce $CD8^+$ T-cell proliferation that occur in response to heat killed *H. pylori* stimulation (unpublished data).

Our results suggest that the increased levels of IFN-γ is not due to an increase in $CD8^+$ T-cell numbers in the analyzed tumors, which also has been indicated by immunohistochemistry analysis by our group previously (Enarsson, et al. 2006). Instead, our present results demonstrate that the higher numbers of $FOXP3^+$ Treg and lower activity in the $CD8^+$T cells are concurrent with increased levels of pro-inflammatory cytokines in the tumor mucosa. This apparent contradiction may be explained by a contribution of pro-inflammatory cytokines to the enhancement of $CD4^+FOXP3^+$ T cells or other immunomodulating factors within the

tumor. IL-8 is known to favour angiogenesis (Li, et al. 2003) and it has been shown that IFN-γ can up-regulate the expression of FOXP3 in $CD4^{+}CD25^{-}$ T cells (Ouaked, et al. 2009; Wang, et al. 2006). This indicates that the role of IFN-γ in tumors may be more complex than previously anticipated. In addition, we found an indication of increase of TGF-β gene transcription when tumor tissue was compared with tumor-free tissue. Although the increase was not statistically significant, the results indicate that TGF-β may be increased in the tumors mucosa and play a role in tumor immunomodulation in gastric cancer, in line with previous studies (Hawinkels, et al. 2007)

Mechanisms responsible for increased levels of Treg in tumor mucosa

Recruitment of regulatory T cells

Although we had confirmed an increase of regulatory T cells at the site of *H. pylori*-infection and in the tumor mucosa, the underlying mechanisms of this increase were not determined. In theory, the increase of regulatory T cells within the tissue could be due to an increased recruitment of the cells (Curiel et al. 2004), an increased proliferation of the $FOXP3^+$ cells already present within the tissue (Cao, et al. 2007) or a induction of FOXP3 expression in $CD4^+CD25^-$ T cells present in the tissue (Mahic et al. 2008b). To study the mechanisms responsible we initially investigated the probability of active recruitment of circulating $CD25^{high}$ T cells by analyzing the homing receptors L-selectin and integrin α4β7.

We found increased frequencies of $CD4^+CD25^{high}$ Treg expressing L-selectin compared to $CD4^+CD25^{low}$ and $CD4^+CD25^-$ cells but decreased frequencies of $CD4^+CD25^{high}$ Treg expressing the integrin α4β7 compared to $CD4^+CD25^{low}$ and $CD4^+CD25^-$ cells in both tumor and tumor-free tissue as well as in blood (paper II). These results indicated that the secondary lymphoid tissue homing receptor L-selectin may contribute to recruitment of Treg to the gastric mucosa and that this receptor may be more important for mucosal recruitment than the homing receptor α4β7. In support of this, the L-selectin counter-receptor PNAd has been shown to be de novo expressed in *H. pylori*-associated gastritis (Kobayashi, et al. 2004) and data from our group has shown that PNAd expression is up-regulated in gastric tumor mucosa, compared to tumor-free mucosa of *H. pylori*-infected GC patients (Enarsson et al. 2006), indicating that both ligand and receptor are induced in the relevant cells types in *H. pylori* infected GC patients.

The chemokine (C-C motif) receptor 4 (CCR4) is expressed on leukocytes and is a specific receptor for chemokines CCL17 and CCL22. Tumor cells and macrophages produce the chemokine CCL22 (Curiel et al. 2004) and CCL17 can be produced by epithelial cells, fibroblasts and endothelial cells. Higher frequencies of $CCR4^+$ cells among the $CD4^+CD25^{high}$ population, compared to the $CD25^{low}$ and $CD25^-$ population was found in tumor, tumor-free mucosa and PBMC (paper II) and when the concentration of the CCR4 ligands CCL22 and CCL17 were analyzed in mucosal extracts, we found significantly higher concentrations of CCL22 than CCL17 in both tumor-free and tumor mucosa compared to PBMC, while there was no significant difference in CCL22 concentration in tumor compared to tumor-free mucosa. Recently, Mizukami et al. has shown that the frequency of $CCL17^+$ and $CCL22^+$

cells among $CD14^+$ cells within gastric tumors was significantly higher compared to normal gastric mucosa. Furthermore, there was a correlation between the frequency of $CCL17^+$ and $CCL22^+$ cells and $FOXP3^+$ Tregs in tumor infiltrating lymphocytes (Mizukami, et al. 2008). This study and our results support that CCR4 on Tregs increase the recruitment and that this could be a mechanism to increase the levels of $FOXP3^+$ Treg cells in the tumor mucosa.

Proliferation

To further investigate the mechanisms behind the increased levels of regulatory T cells we next investigated the level of proliferation of $FOXP3^+$ cells.

The rationale for looking at proliferation is that during tumor progression, a subset of dendritic cells (DC) as been proposed to be recruited to draining lymph nodes and selectively promote the proliferation of Treg cells in a TGF-β-dependent manner in mice and rats (Ghiringhelli, et al. 2005). In humans, $CD4^+CD25^{high}$ regulatory T cells increase in PBMC in hepatocellular carcinoma (HCC) patients and are positively correlated with tumor burden in this disease. When PBMC are co-cultured with human hepatocellular carcinoma cell lines, $CD4^+CD25^{high}$ T cell population increase in frequency because of natural Treg proliferation (Cao et al. 2007). Furthermore, Wolf et al claim that the increased frequencies of Treg in PBMC of cancer patient are due to active proliferation rather than due to redistribution from other compartments (Wolf, et al. 2006). They showed that Tregs had lower levels of T-cell receptor excision circles (TREC), which indicated that the cells had undergone many cell divisions, but the length of the telomere was preserved in Tregs from peripheral blood, probable due to induction of telomerase activity. It has also been shown that peripheral blood $CD4^+FOXP3^+$ T cells have a higher expression of the proliferation marker Ki67 (Tuovinen, et al. 2008). So, we were interested to study if $CD4^+FOXP3^+$ T cells in the gastric tumor mucosa have a higher proliferation compared to tumor-free mucosa and if this could be one explanation to higher number of $CD4^+FOXP3^+$ T cells in the tumor tissue.

Flow cytometric analysis of proliferation in different T-cell populations in PBMC by Ki-67 staining revealed that there was a high proportion of proliferating cells among $CD4^+FOXP3^+$ T cells in peripheral blood, and that 25-40% of all proliferating $CD4^+$ T cells were $FOXP3^+$; the levels were not different between cells from gastric cancer patients and age-matched healthy controls. Furthermore, the level of proliferation in $CD4^+FOXP3^+$ PBMC was 8-fold higher than the frequency of proliferating cells in the $CD4^+FOXP3^-$ population (paper IV).

In the mucosal compartment, there was around two-fold increase in proliferating $CD4^+FOXP3^+$ and $CD4^+$FOXP3- cells in the tumor compared to the tumor-free mucosa. Furthermore, the proliferation of the $CD4^+FOXP3^+$ cells in tumor mucosa was increased around 2-fold compared to the $CD4^+FOXP3^-$ cells in the same site. These results indicate that proliferation of $CD4^+FOXP3^+$ T cells in tumor mucosa may contribute to the increased frequency of this cell population.

Finally, we found by analyzing RNA extracted from equal numbers of sorted cells, from both gastric mucosa and PBMC, that the $CD4^+CD25^{high}$ cells had an increased expression of the house-keeping gene HPRT1 compared to $CD4^+CD25^{low/-}$ cells. We suggest that this may indicate a more active cell metabolism, which also support a higher proliferation of the $CD4^+CD25^{high}$ cells. Our study is, to our knowledge, the first to show that $CD4^+FOXP3^+$ T cells have a higher proliferation in the gastric tumor mucosa compared to the tumor-free mucosa and that proliferation can be one mechanism mediating the increased number of $CD4^+FOXP3^+$ T cells in the tumor.

CONCLUDING REMARKS

Based on the work presented in this thesis, we have been able to advance the understanding of Treg function in the human gastric mucosa in *H. pylori*-related duodenal ulcer disease and gastric cancer. We have demonstrated that *H. pylori* infection and infection-induced disease is associated with increased numbers of FOXP3-expressing T cells in the infected mucosa. The infection induces production of a range of cytokines and chemokines, such as IL-8, by the epithelial cells. The activation of innate immune activities will also lead to the recruitment of lymphocytes, including $CD4^+$ and $CD8^+$ T cells. From our results it is not possible to determine whether the increased FOXP3-numbers is caused directly by the presence of the bacteria, or indirectly, through the induction of inflammation. The increase of Treg is associated with increased numbers of bacteria both in the antral mucosa and in the duodenum in areas of DGM, and the expression of FOXP3 diminishes when the infection is eradicated. However, there is also a further increase of FOXP3-expressing T cells in the tumor mucosa of gastric cancer patients, and these patients either lack *H. pylori* entirely or have markedly diminished levels in their stomach. Since they still exhibit high and even increased levels of IL-8 and other pro-inflammatory cytokines, it is likely that the inflammation is the main factor that leads to increase of $FOXP3^+$ T cells due to *H. pylori* infection. Three possible explanations to the increased number of $CD4^+FOXP3^+$ T cells in the mucosa are recruitment, conversion of previously FOXP3-negative cells and proliferation of Treg. Our data indicates that both increased recruitment and increased mucosal proliferation of Treg are contributing, and based on other reports it is likely that conversion of FOXP3-negative to positive $CD4^+$ T cells is occurring as well. The recruitment of $CD4^+CD25^{high}FOXP3^+$ T cells into the mucosa is suggested to be mediated by L-selectin, that binds to PNAd, and CCR4 interacting with its ligand CCL22 (see "1" in fig. 8). CCL22 is expressed in the infected mucosa, and is increased further in tumor tissue, similar to PNAd on the stomach endothelium ("2" in fig. 8). Conversion of $CD4^+FOXP3^-$ into $CD4^+FOXP3^+$ may occur in association to inflammation. We hypothesize that this process may be mediated by PGE_2-release due to COX-2 expression ("3" in fig. 8) (Bartchewsky et al. 2009; Bryn, et al. 2008) or alternatively by induction of nitric oxide (Niedbala, et al. 2007). It is intriguing to note that physiological concentrations of ascorbate inhibit PGE_2 in response to *H. pylori* in gastric epithelial cells (Smith and Farthing 2005). It has also been reported that a low intake of vitamin C is associated to gastric cancer development (Riboli and Norat 2003). One mechanism accounting for this phenomenon may

IL-8
CCL22
PNAd
CCR4
L-selectin
FOXP3+ CD4+ T cell
FOXP3- CD4+ T cell
Endothelial cell (blood vessel)
Epithelial cell
Epithelial cell – intestinal metaplasia
Tumor cell
1
2
3
4
IL-8
COX-2
PGE2
IFN-γ
IL-10
IL-10

Figure 8

be a lower induction of FOXP3 through reduced PGE_2-levels, and thereby a more efficient anti-tumor immune responses.

Interestingly, the anti tumor cytokine IFN-γ has been proposed to amplify TGF-β-induced FOXP3-expression in $CD4^+$ T cells via STAT1 (Ouaked et al. 2009). The conversion into $FOXP3^+$ cells by IFN-γ is presumably a general way of inducing feed-back inhibition in areas of intense immune activation. In the context of gastric cancer this is detrimental: the high IL-8-production within the tumor is associated with angiogenesis and thereby acts as a tumor-promoting factor. Thus, certain aspects of inflammation are truly related to the "dark side" of the force of the immune system.

We also found that the proliferation of $CD4^+FOXP3^+$ cells is increased in stomach tumors ("4" in fig. 8). This proliferation might be induced directly by the tumor cells or the *H. pylori* infection, or indirectly by the inflammation induced by the tumor or the infection. The exact mechanisms for the observed induction of proliferation is not known, but it has been shown that tumor cells can have this effect *in vitro* (Cao et al. 2007), and we have observed similar results in peripheral blood $CD4^+CD25^{low/-}$ T cells stimulated with *H. pylori* antigens (data not shown).

It is still unknown whether the CD25-negative $CD4^+FOXP3^+$ T cells have a suppressive function or whether they are recently activated effector cells. Even if the FOXP3 expression is transiently upregulated, the cells may have a suppressive function and this has been suggested by other studies (Pillai et al. 2007). We intend to address the transient up-regulation by measuring the level of demethylation in the FOXP3 promotor which is a method that can be used to determine the origin of the different $FOXP3^+$ T-cell populations and if they have a suppressive function (Janson et al. 2008; Lal et al. 2009)

Many of the $CD4^+FOXP3^+$ T cells are located in aggregates/follicles of $CD4^+$ T cells, both in antral- and duodenal-infected mucosa and in tumors (illustrated in fig 8). The endothelial cells in lymphoid follicles that form in the stomach as a consequence of *H. pylori* colonization express PNAd (Kobayashi, et al. 2009). We suggest that $CD4^+CD25^{high}FOXP3^+$ expressing L-selectin can home to the PNAd expressing aggregates/follicles and that the *H. pylori* infection drives the formation of follicles and aggregates in both antral and duodenal mucosa.

At present we do not have any data that demonstrate whether or not the increased numbers of $CD4^+FOXP3^+$ in DGM in DU patients are involved in the development of duodenal ulcer.

Earlier findings in our group have shown that there is a markedly lower production of cytokines, specially IL-8, in the duodenal epithelial cells of DU patients, and it was suggested that this leads to accumulation of neutrophils in the mucosa since the lack of epithelial cytokines would lead to inhibition of transepithelial migration of neutrophils into the gut lumen (Stromberg et al. 2003a). Furthermore, there were indications that regulatory T cells can inhibit IL-8 production by epithelial cells and thereby contribute to ulcer development through this process (Stromberg, et al. 2005). However, our study is not supporting that the reduction in cytokine production by epithelial cells is caused by the infiltrating Treg (paper I). Thus, the increase of $CD4^+FOXP3^+$ is only 2-fold in DGM from DU patients compared to in DGM of AS individuals which seems too modest an increase to explain the total depletion of epithelial cytokines observed in DU patients. Furthermore, there are few $CD4^+FOXP3^+$ cells located in close contact to the duodenal epithelium, they are mainly located deeper within the lamina propria. In addition, the numbers of Treg are also increased markedly in the tumor tissue, and these Treg are not inducing a reduction of IL-8 secretion in the tumor, as IL-8 levels are very high in this location. We believe that the increase of regulatory T cells is an effect secondary to the local *H. pylori* colonization, and the Tregs act to reduce the degree of inflammation induced by the bacterium. Since the inflammation is maintained at a controlled level by the suppressive Treg, this effect may explain why *H. pylori* infections remain chronic.

It is evident from our results that $CD4^+CD25^{high}FOXP3^+$ T cells in the gastric mucosa suppress both proliferation and cytokine production of other T cells. However, the mechanism of suppression is less clear. Two likely candidate mediators are CTLA4 and IL-10, as both these were expressed by $CD25^{high}FOXP3^+$ T cells in the mucosa. Unexpectedly, TGF-β mRNA was expressed in lower levels in Treg than in the $CD4^+CD25^{low/-}$ population. This pattern was also seen in circulating cells from healthy controls, so it is not related to the presence of a tumor. However, since we could not sort cells from the stomach mucosa of individuals without cancer, we do not know if non-Treg-related TGF-β production in the stomach is altered in gastric cancer relative to non-cancer patients.

The observed increase in IL-10 production from *H. pylori*-stimulated $CD4^+$ and $CD8^+$ T cells from gastric cancer patients support that $CD4^+$ Tr1 and IL-10-producing $CD8^+$ regulatory T cells may be induced during tumor development, whereas in asymptomatic individuals we found high IFN-γ production from the $CD4^+$ T cells. This supports reduced anti-tumor immune responses in the gastric cancer, which may be associated to tumor survival and

progression. Similar findings were recently found in a mouse model of breast cancer, where IL-10 blockade led to clearance of the tumor. The mechanism for the induction of suppressive T-cell populations in gastric cancer could be further studied using a mouse model, such as the $gp130^{757F/F}$ model which spontaneously develops stomach cancer (Howlett, et al. 2005). However, it has not been described if the same phenomena of increased levels of IL-10 and increased infiltration and proliferation of $FOXP3^{+}$ T cells occur in this mouse model, so this issue first needs to be addressed.

In conclusion, the results reported in this thesis have added to the understanding of the process of immune regulation in relation to FOXP3-expressing regulatory T cells in *H. pylori*-associated diseases. Although a lot of work remains, some of the findings may be useful for development of vaccine against *H. pylori* or designing new therapies for gastric cancer. In particular, blocking of Treg infiltration by inhibition of L-selectin – PNAd binding, blockage of Treg proliferation in the mucosa, inhibiting the induction of FOXP3 expression by the use of COX-2 inhibitors, or finally inhibition of IL-10 secretion are all theoretically possible. Future studies may hopefully clarify the role of Tregs in the development of peptic ulcers and/or gastric cancer and we hope that increased knowledge of the mechanisms behind the immune response will contribute to reduce the cases of *H. pylori* induced diseases in the future.

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REFERENCES

Abnet CC, Freedman ND, Kamangar F, Leitzmann MF, Hollenbeck AR & Schatzkin A 2009 Non-steroidal anti-inflammatory drugs and risk of gastric and oesophageal adenocarcinomas: results from a cohort study and a meta-analysis. *Br J Cancer* **100** 551-557.

Alegre ML, Frauwirth KA & Thompson CB 2001 T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* **1** 220-228.

Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, Roncarolo MG & Levings MK 2007 Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* **19** 345-354.

Asseman C, Mauze S, Leach MW, Coffman RL & Powrie F 1999 An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* **190** 995-1004.

Bacchetta R, Passerini L, Gambineri E, Dai M, Allan SE, Perroni L, Dagna-Bricarelli F, Sartirana C, Matthes-Martin S, Lawitschka A, et al. 2006 Defective regulatory and effector T cell functions in patients with FOXP3 mutations. *J Clin Invest* **116** 1713-1722.

Baecher-Allan C, Brown JA, Freeman GJ & Hafler DA 2001 CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* **167** 1245-1253.

Bamford KB, Fan X, Crowe SE, Leary JF, Gourley WK, Luthra GK, Brooks EG, Graham DY, Reyes VE & Ernst PB 1998 Lymphocytes in the human gastric mucosa during Helicobacter pylori have a T helper cell 1 phenotype. *Gastroenterology* **114** 482-492.

Baratelli F, Lin Y, Zhu L, Yang SC, Heuze-Vourc'h N, Zeng G, Reckamp K, Dohadwala M, Sharma S & Dubinett SM 2005 Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. *J Immunol* **175** 1483-1490.

Bardel E, Larousserie F, Charlot-Rabiega P, Coulomb-L'Hermine A & Devergne O 2008 Human CD4+ CD25+ Foxp3+ regulatory T cells do not constitutively express IL-35. *J Immunol* **181** 6898-6905.

Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, de Waal-Malefyt R, Coffman RL, Hawrylowicz CM & O'Garra A 2002 In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* **195** 603-616.

Bartchewsky W, Jr., Martini MR, Masiero M, Squassoni AC, Alvarez MC, Ladeira MS, Salvatore D, Trevisan M, Pedrazzoli J, Jr. & Ribeiro ML 2009 Effect of Helicobacter pylori infection on IL-8, IL-1beta and COX-2 expression in patients with chronic gastritis and gastric cancer. *Scand J Gastroenterol* **44** 153-161.

Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF & Ochs HD 2001 The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* **27** 20-21.

Betts GJ, Clarke SL, Richards HE, Godkin AJ & Gallimore AM 2006 Regulating the immune response to tumours. *Adv Drug Deliv Rev* **58** 948-961.

Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, Heib V, Becker M, Kubach J, Schmitt S, et al. 2007 Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* **204** 1303-1310.

Braun D, Longman RS & Albert ML 2005 A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation. *Blood* **106** 2375-2381.

Brenner H, Rothenbacher D & Arndt V 2009 Epidemiology of stomach cancer. *Methods Mol Biol* **472** 467-477.

Bryn T, Yaqub S, Mahic M, Henjum K, Aandahl EM & Tasken K 2008 LPS-activated monocytes suppress T-cell immune responses and induce FOXP3+ T cells through a COX-2-PGE2-dependent mechanism. *Int Immunol* **20** 235-245.

Buckner JH & Ziegler SF 2008 Functional analysis of FOXP3. *Ann N Y Acad Sci* **1143** 151-169.

Bustin SA 2002 Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* **29** 23-39.

Calle EE, Rodriguez C, Walker-Thurmond K & Thun MJ 2003 Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* **348** 1625-1638.

Cao M, Cabrera R, Xu Y, Firpi R, Zhu H, Liu C & Nelson DR 2007 Hepatocellular carcinoma cell supernatants increase expansion and function of CD4(+)CD25(+) regulatory T cells. *Lab Invest* **87** 582-590.

Chandanos E & Lagergren J 2008 Oestrogen and the enigmatic male predominance of gastric cancer. *Eur J Cancer* **44** 2397-2403.

Chen JH, Liu TY, Wu CW & Chi CW 2001 Nonsteroidal anti-inflammatory drugs for treatment of advanced gastric cancer: cyclooxygenase-2 is involved in hepatocyte growth factor mediated tumor development and progression. *Med Hypotheses* **57** 503-505.

Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS & Vignali DA 2007 The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* **450** 566-569.

Crabtree JE, Peichl P, Wyatt JI, Stachl U & Lindley IJ 1993 Gastric interleukin-8 and IgA IL-8 autoantibodies in Helicobacter pylori infection. *Scand J Immunol* **37** 65-70.

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, et al. 2004 Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* **10** 942-949.

de la Rosa M, Rutz S, Dorninger H & Scheffold A 2004 Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur J Immunol* **34** 2480-2488.

Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka M, et al. 2007 Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* **204** 1257-1265.

Dickson AM, Schuss A, Goyal A & Katz DS 2004 Radiology-Pathology Conference: Calcified untreated gastric cancer. *Clin Imaging* **28** 418-421.

Duggleby RC, Shaw TN, Jarvis LB, Kaur G & Gaston JS 2007 CD27 expression discriminates between regulatory and non-regulatory cells after expansion of human peripheral blood CD4+ CD25+ cells. *Immunology* **121** 129-139.

El-Omar EM, Oien K, El-Nujumi A, Gillen D, Wirz A, Dahill S, Williams C, Ardill JE & McColl KE 1997 Helicobacter pylori infection and chronic gastric acid hyposecretion. *Gastroenterology* **113** 15-24.

El-Omar EM, Rabkin CS, Gammon MD, Vaughan TL, Risch HA, Schoenberg JB, Stanford JL, Mayne ST, Goedert J, Blot WJ, et al. 2003 Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. *Gastroenterology* **124** 1193-1201.

Ellmark P, Ingvarsson J, Carlsson A, Lundin BS, Wingren C & Borrebaeck CA 2006 Identification of protein expression signatures associated with Helicobacter pylori infection and gastric adenocarcinoma using recombinant antibody microarrays. *Mol Cell Proteomics* **5** 1638-1646.

Enarsson K 2005 T Lymphocytes Recruitment in *Helicobacter pylori*-Associated Diseases.

Enarsson K, Johnsson E, Lindholm C, Lundgren A, Pan-Hammarstrom Q, Stromberg E, Bergin P, Baunge EL, Svennerholm AM & Quiding-Jarbrink M 2006 Differential mechanisms for T

lymphocyte recruitment in normal and neoplastic human gastric mucosa. *Clin Immunol* **118** 24-34.

Enarsson K, Lundin BS, Johnsson E, Brezicka T & Quiding-Jarbrink M 2007 CD4+ CD25high regulatory T cells reduce T cell transendothelial migration in cancer patients. *Eur J Immunol* **37** 282-291.

Everhart JE 2000 Recent developments in the epidemiology of Helicobacter pylori. *Gastroenterol Clin North Am* **29** 559-578.

Fahlen L, Read S, Gorelik L, Hurst SD, Coffman RL, Flavell RA & Powrie F 2005 T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med* **201** 737-746.

Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, Belladonna ML, Fioretti MC, Alegre ML & Puccetti P 2003 Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* **4** 1206-1212.

Fontenot JD, Rasmussen JP, Gavin MA & Rudensky AY 2005 A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* **6** 1142-1151.

Fukaura H, Kent SC, Pietrusewicz MJ, Khoury SJ, Weiner HL & Hafler DA 1996 Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. *J Clin Invest* **98** 70-77.

Garin MI, Chu CC, Golshayan D, Cernuda-Morollon E, Wait R & Lechler RI 2007 Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* **109** 2058-2065.

Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A, Ocheltree EL, Greenberg PD, Ochs HD & Rudensky AY 2006 Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A* **103** 6659-6664.

Gershon RK & Kondo K 1970 Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology* **18** 723-737.

Ghiringhelli F, Puig PE, Roux S, Parcellier A, Schmitt E, Solary E, Kroemer G, Martin F, Chauffert B & Zitvogel L 2005 Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. *J Exp Med* **202** 919-929.

Goldstein JM, L. Marodon, G. 2009 Dynamic regulation of Foxp3 expression by STAT5-mediated signalling. In *WIRM 2009 Davos*.

Gonzalez CA, Pera G, Agudo A, Palli D, Krogh V, Vineis P, Tumino R, Panico S, Berglund G, Siman H, et al. 2003 Smoking and the risk of gastric cancer in the European Prospective Investigation Into Cancer and Nutrition (EPIC). *Int J Cancer* **107** 629-634.

Graham DY, Malaty HM & Go MF 1994 Are there susceptible hosts to Helicobacter pylori infection? *Scand J Gastroenterol Suppl* **205** 6-10.

Green DR, Flood PM & Gershon RK 1983 Immunoregulatory T-cell pathways. *Annu Rev Immunol* **1** 439-463.

Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP & Ley TJ 2004a Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* **21** 589-601.

Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP & Ley TJ 2004b Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* **104** 2840-2848.

Groux H, Bigler M, de Vries JE & Roncarolo MG 1996 Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* **184** 19-29.

Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE & Roncarolo MG 1997 A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389** 737-742.

Hamlet A, Thoreson AC, Nilsson O, Svennerholm AM & Olbe L 1999 Duodenal *Helicobacter pylori* infection differs in cagA genotype between asymptomatic subjects and patients with duodenal ulcers. *Gastroenterology* **116** 259-268.

Harris PR, Wright SW, Serrano C, Riera F, Duarte I, Torres J, Pena A, Rollan A, Viviani P, Guiraldes E, et al. 2008 Helicobacter pylori gastritis in children is associated with a regulatory T-cell response. *Gastroenterology* **134** 491-499.

Hawinkels LJ, Verspaget HW, van Duijn W, van der Zon JM, Zuidwijk K, Kubben FJ, Verheijen JH, Hommes DW, Lamers CB & Sier CF 2007 Tissue level, activation and cellular localisation of TGF-beta1 and association with survival in gastric cancer patients. *Br J Cancer* **97** 398-404.

HCC-Group 2001 Gastric cancer and Helicobacter pylori: a combined analysis of 12 case control studies nested within prospective cohorts. *Gut* **49** 347-353.

Holmen N, Lundgren A, Lundin S, Bergin AM, Rudin A, Sjovall H & Ohman L 2006 Functional $CD4^{+}CD25^{high}$ regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity. *Inflamm Bowel Dis* **12** 447-456.

Howlett M, Judd LM, Jenkins B, La Gruta NL, Grail D, Ernst M & Giraud AS 2005 Differential regulation of gastric tumor growth by cytokines that signal exclusively through the coreceptor gp130. *Gastroenterology* **129** 1005-1018.

Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H & Fujii H 2003 Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* **9** 4404-4408.

Jakszyn P & Gonzalez CA 2006 Nitrosamine and related food intake and gastric and oesophageal cancer risk: a systematic review of the epidemiological evidence. *World J Gastroenterol* **12** 4296-4303.

Janson PC, Winerdal ME, Marits P, Thorn M, Ohlsson R & Winqvist O 2008 FOXP3 promoter demethylation reveals the committed Treg population in humans. *PLoS ONE* **3** e1612.

Janzon A, Sjoling A, Lothigius A, Ahmed D, Qadri F & Svennerholm AM 2009 Failure to detect Helicobacter pylori DNA using highly sensitive real-time PCR assays in drinking and environmental water in Dhaka, Bangladesh. *Appl Environ Microbiol.*

Kamineni A, Williams MA, Schwartz SM, Cook LS & Weiss NS 1999 The incidence of gastric carcinoma in Asian migrants to the United States and their descendants. *Cancer Causes Control* **10** 77-83.

Kandulski A, Wex T, Kuester D, Peitz U, Gebert I, Roessner A & Malfertheiner P 2008 Naturally occurring regulatory T cells (CD4+, CD25high, FOXP3+) in the antrum and cardia are associated with higher H. pylori colonization and increased gene expression of TGF-beta1. *Helicobacter* **13** 295-303.

Karin M 2006 Nuclear factor-kappaB in cancer development and progression. *Nature* **441** 431-436.

Kido S, Kitadai Y, Hattori N, Haruma K, Kido T, Ohta M, Tanaka S, Yoshihara M, Sumii K, Ohmoto Y, et al. 2001 Interleukin 8 and vascular endothelial growth factor -- prognostic factors in human gastric carcinomas? *Eur J Cancer* **37** 1482-1487.

Kleinewietfeld M, Starke M, Di Mitri D, Borsellino G, Battistini L, Rotzschke O & Falk K 2009 CD49d provides access to "untouched" human Foxp3+ Treg free of contaminating effector cells. *Blood* **113** 827-836.

Knutson KL, Disis ML & Salazar LG 2007 CD4 regulatory T cells in human cancer pathogenesis. *Cancer Immunol Immunother* **56** 271-285.

Kobayashi M, Lee H, Nakayama J & Fukuda M 2009 Roles of gastric mucin-type O-glycans in the pathogenesis of Helicobacter pylori infection. *Glycobiology* **19** 453-461.

Kobayashi M, Mitoma J, Nakamura N, Katsuyama T, Nakayama J & Fukuda M 2004 Induction of peripheral lymph node addressin in human gastric mucosa infected by Helicobacter pylori. *Proc Natl Acad Sci U S A* **101** 17807-17812.

Kronenberg M, Steinmetz M, Kobori J, Kraig E, Kapp JA, Pierce CW, Sorensen CM, Suzuki G, Tada T & Hood L 1983 RNA transcripts for I-J polypeptides are apparently not encoded between the I-A and I-E subregions of the murine major histocompatibility complex. *Proc Natl Acad Sci U S A* **80** 5704-5708.

Kuipers EJ, Thijs JC & Festen HP 1995 The prevalence of *Helicobacter pylori* in peptic ulcer disease. *Aliment Pharmacol Ther* **9 Suppl 2** 59-69.

Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, Reid SP, Levy DE & Bromberg JS 2009 Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J Immunol* **182** 259-273.

Lauren P 1965 The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. An Attempt at a Histo-Clinical Classification. *Acta Pathol Microbiol Scand* **64** 31-49.

Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal Malefyt R & Roncarolo MG 2001a IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J Immunol* **166** 5530-5539.

Levings MK, Sangregorio R & Roncarolo MG 2001b Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* **193** 1295-1302.

Li A, Dubey S, Varney ML, Dave BJ & Singh RK 2003 IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol* **170** 3369-3376.

Lindholm C, Quiding-Jarbrink M, Lonroth H, Hamlet A & Svennerholm AM 1998 Local cytokine response in Helicobacter pylori-infected subjects. *Infect Immun* **66** 5964-5971.

Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, et al. 2006 CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* **203** 1701-1711.

Long SA & Buckner JH 2008 Combination of rapamycin and IL-2 increases de novo induction of human CD4(+)CD25(+)FOXP3(+) T cells. *J Autoimmun* **30** 293-302.

Lundgren A, Stromberg E, Sjoling A, Lindholm C, Enarsson K, Edebo A, Johnsson E, Suri-Payer E, Larsson P, Rudin A, et al. 2005a Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in Helicobacter pylori-infected patients. *Infect Immun* **73** 523-531.

Lundgren A, Suri-Payer E, Enarsson K, Svennerholm AM & Lundin BS 2003 Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to H. pylori in infected individuals. *Infect Immun* **71** 1755-1762.

Lundgren A, Trollmo C, Edebo A, Svennerholm AM & Lundin BS 2005b *Helicobacter pylori*-specific $CD4^+$ T cells home to and accumulate in the human *Helicobacter pylori*-infected gastric mucosa. *Infect Immun* **73** 5612-5619.

Lundin BS, Johansson C & Svennerholm AM 2002 Oral immunization with a Salmonella enterica serovar typhi vaccine induces specific circulating mucosa-homing CD4(+) and CD8(+) T cells in humans. *Infect Immun* **70** 5622-5627.

Mahic M, Henjum K, Yaqub S, Bjornbeth BA, Torgersen KM, Tasken K & Aandahl EM 2008a Generation of highly suppressive adaptive CD8(+)CD25(+)FOXP3(+) regulatory T cells by continuous antigen stimulation. *Eur J Immunol* **38** 640-646.

Mahic M, Yaqub S, Bryn T, Henjum K, Eide DM, Torgersen KM, Aandahl EM & Tasken K 2008b Differentiation of naive CD4+ T cells into CD4+CD25+FOXP3+ regulatory T cells by continuous antigen stimulation. *J Leukoc Biol* **83** 1111-1117.

Mahic M, Yaqub S, Johansson CC, Tasken K & Aandahl EM 2006 FOXP3+CD4+CD25+ adaptive regulatory T cells express cyclooxygenase-2 and suppress effector T cells by a prostaglandin E2-dependent mechanism. *J Immunol* **177** 246-254.

Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K & Schmidt-Weber CB 2006 Molecular mechanisms underlying FOXP3 induction in human T cells. *J Immunol* **176** 3593-3602.

Mattsson A, Tinnert A, Hamlet A, Lonroth H, Bolin I & Svennerholm AM 1998 Specific antibodies in sera and gastric aspirates of symptomatic and asymptomatic Helicobacter pylori-infected subjects. *Clin Diagn Lab Immunol* **5** 288-293.

Mbulaiteye SM, Hisada M & El-Omar EM 2009 Helicobacter Pylori associated global gastric cancer burden. *Front Biosci* **14** 1490-1504.

Miller A, Lider O, Roberts AB, Sporn MB & Weiner HL 1992 Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc Natl Acad Sci U S A* **89** 421-425.

Mizukami Y, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H & Fujii H 2008 CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3+ regulatory T cells in gastric cancer. *Int J Cancer* **122** 2286-2293.

Morgan ME, van Bilsen JH, Bakker AM, Heemskerk B, Schilham MW, Hartgers FC, Elferink BG, van der Zanden L, de Vries RR, Huizinga TW, et al. 2005 Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Hum Immunol* **66** 13-20.

Munn DH & Mellor AL 2007 Indoleamine 2,3-dioxygenase and tumor-induced tolerance. *J Clin Invest* **117** 1147-1154.

Nakamura K, Kitani A, Fuss I, Pedersen A, Harada N, Nawata H & Strober W 2004 TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. *J Immunol* **172** 834-842.

Nakamura K, Kitani A & Strober W 2001 Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* **194** 629-644.

Niedbala W, Cai B, Liu H, Pitman N, Chang L & Liew FY 2007 Nitric oxide induces CD4+CD25+ Foxp3 regulatory T cells from CD4+CD25 T cells via p53, IL-2, and OX40. *Proc Natl Acad Sci U S A* **104** 15478-15483.

Oderup C, Cederbom L, Makowska A, Cilio CM & Ivars F 2006 Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology* **118** 240-249.

Okwor I & Uzonna J 2008 Persistent parasites and immunologic memory in cutaneous leishmaniasis: implications for vaccine designs and vaccination strategies. *Immunol Res* **41** 123-136.

Ouaked N, Mantel PY, Bassin C, Burgler S, Siegmund K, Akdis CA & Schmidt-Weber CB 2009 Regulation of the foxp3 gene by the Th1 cytokines: the role of IL-27-induced STAT1. *J Immunol* **182** 1041-1049.

Parkin DM, Bray F, Ferlay J & Pisani P 2005 Global cancer statistics, 2002. *CA Cancer J Clin* **55** 74-108.

Peek RM, Jr. & Blaser MJ 2002 Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer* **2** 28-37.

Perrone G, Ruffini PA, Catalano V, Spino C, Santini D, Muretto P, Spoto C, Zingaretti C, Sisti V, Alessandroni P, et al. 2008 Intratumoural FOXP3-positive regulatory T cells are associated with adverse prognosis in radically resected gastric cancer. *Eur J Cancer* **44** 1875-1882.

Pillai V, Ortega SB, Wang CK & Karandikar NJ 2007 Transient regulatory T-cells: a state attained by all activated human T-cells. *Clin Immunol* **123** 18-29.

Rad R, Brenner L, Bauer S, Schwendy S, Layland L, da Costa CP, Reindl W, Dossumbekova A, Friedrich M, Saur D, et al. 2006 $CD25^+$/$Foxp3^+$ T cells regulate gastric inflammation and *Helicobacter pylori* colonization in vivo. *Gastroenterology* **131** 525-537.

Raghavan S, Fredriksson M, Svennerholm AM, Holmgren J & Suri-Payer E 2003 Absence of $CD4^+CD25^+$ regulatory T cells is associated with a loss of regulation leading to increased pathology in *Helicobacter pylori*-infected mice. *Clin Exp Immunol* **132** 393-400.

Read S, Malmstrom V & Powrie F 2000 Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* **192** 295-302.

Ren X, Ye F, Jiang Z, Chu Y, Xiong S & Wang Y 2007 Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. *Cell Death Differ* **14** 2076-2084.

Riboli E & Norat T 2003 Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *Am J Clin Nutr* **78** 559S-569S.

Roncador G, Brown PJ, Maestre L, Hue S, Martinez-Torrecuadrada JL, Ling KL, Pratap S, Toms C, Fox BC, Cerundolo V, et al. 2005 Analysis of FOXP3 protein expression in human $CD4^+CD25^+$ regulatory T cells at the single-cell level. *Eur J Immunol* **35** 1681-1691.

Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K & Levings MK 2006 Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* **212** 28-50.

Rothenbacher D & Brenner H 2003 Burden of Helicobacter pylori and H. pylori-related diseases in developed countries: recent developments and future implications. *Microbes Infect* **5** 693-703.

Ruprecht CR, Gattorno M, Ferlito F, Gregorio A, Martini A, Lanzavecchia A & Sallusto F 2005 Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. *J Exp Med* **201** 1793-1803.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M & Toda M 1995 Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* **155** 1151-1164.

Sarris M, Andersen KG, Randow F, Mayr L & Betz AG 2008 Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. *Immunity* **28** 402-413.

Sasada T, Kimura M, Yoshida Y, Kanai M & Takabayashi A 2003 CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. *Cancer* **98** 1089-1099.

Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C, et al. 2005 Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A* **102** 18538-18543.

Sawaoka H, Kawano S, Tsuji S, Tsuji M, Sun W, Gunawan ES & Hori M 1998 Helicobacter pylori infection induces cyclooxygenase-2 expression in human gastric mucosa. *Prostaglandins Leukot Essent Fatty Acids* **59** 313-316.

Seddiki N & Kelleher AD 2008 Regulatory T cells in HIV infection: who's suppressing what? *Curr HIV/AIDS Rep* **5** 20-26.

Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, et al. 2006 Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* **203** 1693-1700.

Sepulveda A, Peterson LE, Shelton J, Gutierrez O & Graham DY 2002 Histological patterns of gastritis in H. pylori-infected individuals with a family history of gastric cancer. *Am J Gastroenterol* **97** 1365-1370.

Shen LS, Wang J, Shen DF, Yuan XL, Dong P, Li MX, Xue J, Zhang FM, Ge HL & Xu D 2009 CD4(+)CD25(+)CD127(low/-) regulatory T cells express Foxp3 and suppress effector T cell proliferation and contribute to gastric cancers progression. *Clin Immunol*.

Shevach EM 2006 From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* **25** 195-201.

Smith GV & Farthing MJ 2005 The effect of ascorbic acid on Helicobacter pylori induced cyclooxygenase 2 expression and prostaglandin E2 production by gastric epithelial cells in vitro. *Helicobacter* **10** 83-89.

Steer HW 1984 Surface morphology of the gastroduodenal mucosa in duodenal ulceration. *Gut* **25** 1203-1210.

Strauss L, Bergmann C, Szczepanski M, Gooding W, Johnson JT & Whiteside TL 2007 A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. *Clin Cancer Res* **13** 4345-4354.

Stromberg E, Edebo A, Lundin BS, Bergin P, Brisslert M, Svennerholm AM & Lindholm C 2005 Down-regulation of epithelial IL-8 responses in *Helicobacter pylori*-infected duodenal ulcer patients depends on host factors, rather than bacterial factors. *Clin Exp Immunol* **140** 117-125.

Stromberg E, Edebo A, Svennerholm AM & Lindholm C 2003a Decreased epithelial cytokine responses in the duodenal mucosa of Helicobacter pylori-infected duodenal ulcer patients. *Clin Diagn Lab Immunol* **10** 116-124.

Stromberg E, Lundgren A, Edebo A, Lundin S, Svennerholm AM & Lindholm C 2003b Increased frequency of activated T-cells in the *Helicobacter pylori*-infected antrum and duodenum. *FEMS Immunol Med Microbiol* **36** 159-168.

Suerbaum S & Michetti P 2002 Helicobacter pylori infection. *N Engl J Med* **347** 1175-1186.

Takehara H, Iwamoto J, Mizokami Y, Takahashi K, Ootubo T, Miura S, Narasaka T, Takeyama H, Omata T, Shimokobe K, et al. 2006 Involvement of cyclooxygenase-2--prostaglandin E2 pathway in interleukin-8 production in gastric cancer cells. *Dig Dis Sci* **51** 2188-2197.

Thornton AM & Shevach EM 1998 CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* **188** 287-296.

Tran DQ, Ramsey H & Shevach EM 2007 Induction of FOXP3 expression in naive human $CD4^{+}$FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. *Blood* **110** 2983-2990.

Tuovinen H, Laurinolli TT, Rossi LH, Pekkarinen PT, Mattila I & Arstila TP 2008 Thymic production of human FOXP3(+) regulatory T cells is stable but does not correlate with peripheral FOXP3 expression. *Immunol Lett* **117** 146-153.

Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N & Schlemper RJ 2001 Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* **345** 784-789.

Utermöhlen O 2008. *Abstract Scandinavian Society of Immunology meeting 2008*.

Walker MR, Carson BD, Nepom GT, Ziegler SF & Buckner JH 2005 De novo generation of antigen-specific CD4+CD25+ regulatory T cells from human CD4+CD25- cells. *Proc Natl Acad Sci U S A* **102** 4103-4108.

Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH & Ziegler SF 2003 Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *J Clin Invest* **112** 1437-1443.

Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW & Toes RE 2007 Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* **37** 129-138.

Wang Z, Hong J, Sun W, Xu G, Li N, Chen X, Liu A, Xu L, Sun B & Zhang JZ 2006 Role of IFN-gamma in induction of Foxp3 and conversion of CD4+ CD25- T cells to CD4+ Tregs. *J Clin Invest* **116** 2434-2441.

Wei S, Kryczek I, Zou L, Daniel B, Cheng P, Mottram P, Curiel T, Lange A & Zou W 2005 Plasmacytoid dendritic cells induce CD8+ regulatory T cells in human ovarian carcinoma. *Cancer Res* **65** 5020-5026.

Vignali DA, Collison LW & Workman CJ 2008 How regulatory T cells work. *Nat Rev Immunol* **8** 523-532.

Wing K, Suri-Payer E & Rudin A 2005 CD4+CD25+-regulatory T cells from mouse to man. *Scand J Immunol* **62** 1-15.

Wolf D, Rumpold H, Koppelstatter C, Gastl GA, Steurer M, Mayer G, Gunsilius E, Tilg H & Wolf AM 2006 Telomere length of in vivo expanded CD4(+)CD25 (+) regulatory T-cells is preserved in cancer patients. *Cancer Immunol Immunother* **55** 1198-1208.

Wyatt JI, Rathbone BJ, Dixon MF & Heatley RV 1987 Campylobacter pyloridis and acid induced gastric metaplasia in the pathogenesis of duodenitis. *J Clin Pathol* **40** 841-848.

Yang ZZ, Novak AJ, Stenson MJ, Witzig TE & Ansell SM 2006 Intratumoral CD4+CD25+ regulatory T-cell-mediated suppression of infiltrating CD4+ T cells in B-cell non-Hodgkin lymphoma. *Blood* **107** 3639-3646.

Zaitoun AM 1995 The prevalence of lymphoid follicles in *Helicobacter pylori* associated gastritis in patients with ulcers and non-ulcer dyspepsia. *J Clin Pathol* **48** 325-329.

Zhang X, Izikson L, Liu L & Weiner HL 2001 Activation of CD25(+)CD4(+) regulatory T cells by oral antigen administration. *J Immunol* **167** 4245-4253.

Zheng SG, Wang JH, Gray JD, Soucier H & Horwitz DA 2004 Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol* **172** 5213-5221.

Zheng Y, Manzotti CN, Burke F, Dussably L, Qureshi O, Walker LS & Sansom DM 2008 Acquisition of suppressive function by activated human CD4+ CD25- T cells is associated with the expression of CTLA-4 not FoxP3. *J Immunol* **181** 1683-1691.

Zou W 2006 Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* **6** 295-307.