Approaches to Analyses of Cytotoxic Cells,

And studies of their role in *H. pylori* infection

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I **Natural Killer Cells and *Helicobacter pylori* Infection: Bacterial Antigens and Interleukin-12 Act Synergistically To Induce Gamma Interferon Production**

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In *manuscript*

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Helicobacter pylori infection causes chronic gastritis that may progress to peptic ulcers or gastric adenocarcinoma and thereby cause major world-wide health problems. Previous studies have shown that $CD4^+$ T cells and the production of the cytokine IFN-γ are important components of the immune response to *H. pylori* in humans. However, the roles of NK cells and $CD8^+$ T cells – which are major IFN-γ producers and have cytotoxic function - are less clear.

The aim of this thesis was to develop methods to study NK cells and $CD8^+$ T cells in the context of human *H. pylori* infection, and to study their role in the immune response to*H. pylori* in infected subjects.

To this end, we first evaluated ways to activate NK cells by *H. pylori* antigens. This was done by purifying NK cells from peripheral blood, and stimulating the cells by combinations of stimulatory cytokines such as IL-12 and lysate from *H. pylori*. Furthermore, parallel experiments were performed when the NK cells were separated from the bacterial antigens by an epithelial cell layer.

To develop methods to activate and study *H. pylori*-reactive $CD8^+$ T cells, we initially evaluated whether dendritic cells (DC), B cells or monocytes pulsed with *H. pylori* antigens could efficiently activate $CD8^+$ T cells from *H. pylori* infected or non-infected individuals. In order to study proliferation of $CD8^+$ T cells in cell cultures, the cells were stained using the fluorescent dye CFSE, which allows analysis of proliferation of single cells in a mixed cell population. Furthermore, the presence of cytotoxic activity among the *H. pylori*-reactive $CD8^+$ T cells was analysed mainly by the cytotoxicity-related molecules granzyme A and B, and IFN-γ. This was done using intracellular analysis of granzyme B and IFN-γ expression, and by analysing the secretion of granzyme A and B into the supernatants by *H. pylori*-activated $CD8^+$ T cells.

Our results show that highly purified NK cells can be activated by *H. pylori* antigens, and that there is a synergistic effect of *H. pylori* and IL-12 in the activation of NK cells. Furthermore, we show that in *H. pylori*-infected individuals, there are *H. pylori*-reactive memory $CD8^+$ T cells that proliferate, produce IFN-γ and secrete granzyme A after activation. We show that these cells can be activated both by B cells and DC pulsed with *H. pylori* antigens, but for practical purposes, B cells are preferable to use as APC.

In conclusion, in this thesis we show that cytotoxic cells may contribute to the immunity against *H. pylori* in infected individuals by production of IFN-γ; but there are also indications that cytotoxic activity is involved. These findings may be of importance for the further study of NK-cell and cytotoxic T lymphocytes (CTLs) activity in subgroups of *H. pylori*-infected individuals, especially in relation to protection against *H. pylori*-induced gastric cancer development.

Key words: *Helicobacter pylori*, mucosal immunology, stomach, duodenum, asymptomatic, duodenal ulcer, gastric adenocarcinoma, NK cells, T cells, B cells, dendritic cells, cytotoxic

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To my Love!

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Abstract

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ORIGINAL PAPERS

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

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Contents

Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
APC	antigen presenting cells
APC	allophycocyanin
AS	asymptomatic
Bab	blood antigen binding
BCR	B cell receptor
BrdU	bromodeoxyuridine
cag	cytotoxin associated gene
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CTL	cytotoxic T cell
CMV	cytomegalovirus
ELISA	enzyme-linked immunosorbent assay
DC	dendritic cell
DTT	dithiothreitol
DU	duodenal ulcer
EDTA	ethylenediamine tetraacetic acid
EIA	enzyme immunoassay
FITC	fluorescein isothiocyanate
FACS	fluorescence activated cell sorter
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HLA	human leucocyte antigen
Hp-	*H. pylori* non-infected individuals
Hp+	*H. pylori* infected individuals
Hpa	*Helicobacter pylori* adhesion molecule
HP-NAP	*H. pylori* neutrophil-activating protein
ICAM	intracellular cell-adhesion molecule
Ig	immunoglobulin
IL	interleukin
IFN	interferon
KIR	killer-cell immunoglobulin-like receptor
LFA	leucocyte function-associated antigen
LPS	lipopolysaccharide
MALT	mucosa associated lymphoid tissue
MHC	major histocompatibility complex
MP	membrane protein
NK	natural killer
NKT	natural killer T cell
PAI	pathogenecitiy island
PBMC	peripheral blood mononuclear cells
PBS	phosphate salin buffer

PE	phycoerytherin
PerCP	peridinin-chlorophil protein
PAMP	Pathogen Associated Molecular Pattern
PHA	phytohemagglutinin
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCR	T cell receptor
Th	T helper
TLR	tool-like receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	natural regulatory T cells
UBT	urease breath test
Vac A	vacuolating cytotoxin A

Helicobacter pylori

H. pylori are spiral-formed, gram-negative, microaerophilic and motile bacteria that colonize the gastric and duodenal mucosa of approximately 50% of the world's population, and causes a life-long infection. The infection is thought to be transmitted by oral-oral or fecal-oral routes.

Almost all individuals infected with *H. pylori* develop a variable degree of gastritis which in the majority of cases is asymptomatic with moderate inflammation detectable only in biopsies by histopathological analysis. However, an important minority of infected subjects (10–15%) develop severe gastroduodenal pathologies, including gastric and duodenal ulcers and gastric cancer during their life. The different outcomes of the infection are believed to be substantially influenced by an excessive or inappropriate reaction of the host, by bacterial and environmental factors.

Diagnosis of infection is preferably performed by three non-invasive tests, namely ELISA-based serology, ^{13}C- urea breath test (UBT) and stool antigen tests. The current eradication therapy consists of two antibiotics together with a proton-pump inhibitor, and is primarily given to infected individuals that develop symptoms, such as peptic ulcers. A problem that is occurring with greater frequency, however, is that *H. pylori* are developing resistance to some antibiotics, and therefore there is a need for development of other therapies, such as a vaccine that would induce clearance of the infection.

Virulence factors

H. pylori have developed several factors that permit colonization and survival in the hostile environment of the stomach. These include bacterial spiral shape, flagellae, urease and adherence factors. However, to date it is suggested that the most critical virulence factors of *H. pylori* are urease, the cytotoxin associated gene A (*cag*A), vacuolating cytotoxin A (VacA) and *bab*A.

Urease is one of the most abundant proteins produced by *H. pylori*, representing ~5-10% of the total bacterial cell protein *(Dunn 1990; Evans 1991)*. In mice studies, it has been shown

that urease is essential for colonization *(Eaton 1994)*. Furthermore, urease is chemotaxic for monocytes, neutrophils and T cells *(Nakamura 1998; Enarsson 2005)* and is capable to induce cytokine secretion by mucosal macrophages, monocytes and neutrophils *(Mai 1991; Mai 1992; Harris 1998)*.

The *cag*A gene is located in the *cag* pathogenicity island (*cag*PAI), which contain about 30 genes. The *cag*PAI encodes the components of a needle-like structure (type IV secretion system) that is involved in injection of CagA from bacteria into host cells *(Odenbreit 2000)*. The injection of CagA induces production of chemokines and cytokines by the epithelial cells. Also, it has recently been shown that CagA associates with epithelial tight-junction proteins, which lead to disruption of epithelial barrier function and dysplastic alterations in epithelial cells *(Amieva 2003)*.

The *vacA* gene encodes a vaculating toxin that induces large cytoplasmic vacuoles in epithelial cells. It has also been reported that VacA induces apoptosis in epithelial cells *in vitro (Kuck 2001; Cover 2003)* and has a role in inhibition of T-cell proliferation *(Kuck 2001; Gebert 2003)* and might thereby induce immune suppression *(Molinari 1998)*.

The *babA* (blood group antigen binding adhesin) gene encodes an adhesion protein, located in the outer membrane of *H. pylori* that mediate binding to Lewis b (Le^b) blood group antigen on the surface of epithelial cells (Green 1989).

H. pylori-associated diseases

The healthy, non-infected human stomach contains few inflammatory cells. Colonization by *H. pylori* induces an acute inflammatory response (acute gastritis) that is characterized by infiltration of neutrophils into the gastric mucosa. Infiltration of macrophages, T and B cells into the gastric mucosa constitutes the histological chronic gastritis that is asymptomatic in the majority of cases.

It is suggested that the chronic gastritis induced by *H. pylori* changes acid secretion according to the prevalent location of the gastritis in the gastric body or in the antrum. Gastritis in the stomach body causes hypochlorhydria that increases the risk of gastric ulceration and

development of gastric adenocarcinoma; whereas gastritis in the antrum causes hyperchlorhydria that increases the risk of duodenal ulcer *(Blaser 2004)*.

Duodenal ulcer

Hypersecretion of acid into the duodenum promotes development of duodenal gastric metaplasia, i.e. the presence of gastric-type mucus secreting cells in the surface epithelium of the duodenum *(Bode 1991)*. The appearance of gastric epithelial cells in the duodenum allows colonization by *H. pylori*, which subsequently induce a chronic inflammatory response (duodenitis) that may develop to duodenal ulcer (Dixon 2000).

Gastric adenocarcinoma

An adenocarcinoma is a type of cancer that involves the epithelial cells of glands. Gastric adenocarcinoma is one of the most common cancers in the world and it is the second leading cause of cancer-related death. A number of epidemiologic investigations and studies in animal models have reported an association between *H. pylori* infection and gastric adenocarcinoma *(Peek 2002)*. In 1994, the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) classified *H. pylori* as a carcinogen.

Gastric mucosa-associated lymphoid lymphoma

Another type of cancer that may be at least partially caused by *H. pylori* is gastric mucosa associated lymphoid tissue (MALT) lymphoma, a type of cancer of the lymphatic tissue in the stomach. This condition is rare, and eradication of *H. pylori* in MALT-lymphoma patients often cures the disease.

Immune responses in *H. pylori* infection

An Overview

Following the interaction of bacteria and epithelium, *H. pylori* inject CagA protein into epithelial cells through their type IV secretion system. CagA induces the epithelial cells to produce chemokines like IL-8 and pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α. These molecules cause leukocytes to be attracted to and activated at the site of infection. Initially neutrophils and macrophages accumulate in the gastric mucosa. The increased infiltration of neutrophils results in increased concentration of reactive oxygen species and, together with the epithelial damage caused by bacterial toxins (i.e. VacA), acute mucosal damage will arise.

Dendritic cells transport *H. pylori* antigens to the draining lymph nodes and present the processed antigen to T cells. Antigen-specific T cells ($CD4^+$ and $CD8^+$) are activated in the lymph nodes and infiltrate into the site of infection. Activated macrophages secrete IL-12 that drives the $CD4^+$ T-cell response towards a Th1 type that is characterized by production of IFN-γ. This cytokine activate many parts of the immune system, including phagocytosis and antigen presentation *(Boehm 1997; Xing 2001)*.

The role of $CD8^+$ T cells in *H. pylori* infections is not well known. So far, it has been shown that $CD8^+$ T cells infiltrate into the infected area *(Agnihotri 1998)* and produce high levels of IFN-γ in response to *H. pylori* stimulation *(Quiding-Jarbrink 2001)*. Furthermore, the role of NK cells has also been poorly studied.

H. pylori antigens and toxins also induce humoral immune responses. *H. pylori*-specific antibodies against urease, flaggellae and membrane preparations have been detected both locally and systemically in *H. pylori*-infected individuals *(Mattsson 1998)*. Despite the fact that the innate and adaptive immune responses in *H. pylori*-infected individuals are strong, the infection is usually not cleared but remains as a lifelong chronic infection in most individuals (Figure 1).

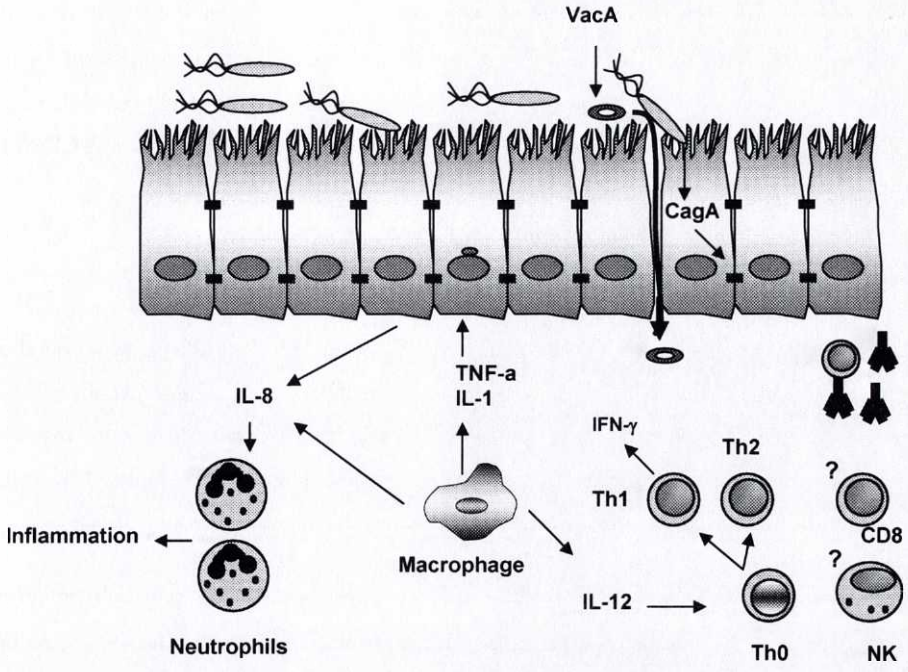

Figure 1. Bacterial spiral morphology, motility and urease enzyme, allow the bacteria to survive in the acidic stomach and swim along the mucus layer and reach the gastric epithelial cells, to which it binds to Lewis antigens, present on host gastric cells, through BabA adhesion molecules. *H. pylori* injects the CagA protein into the host cells by a type IV secretion system and releases other toxic factors such as *H. pylori* neutrophil-activating protein (HP-NAP) and vacuolating cytotoxin A (VacA). Injected CagA proteins signal the nucleus to release pro-inflammatory lymphokines; and induce alterations of tight junctions. VacA induces formation of large vacuoles. *H. pylori* components cross the epithelial lining and together with chemokines produced by epithelial cells recruit neutrophils and monocytes, and result in inflammation. The combined toxic activity of VacA and TNF-α & oxygen metabolites produced by phagocytes leads to tissue damage that is enhanced by loosening of the protective mucus layer and acid penetration. $CD4^+$ T cells are activated by IL-12 and produce IFN-γ. B cells produce *H. pylori*-specific IgA and IgG antibodies. The role of NK cells and $CD8^+$ T cells are not well known.

Cell-Mediated Effector Responses

Both antigen-specific and –non-specific cells contribute to the cell-mediated immune response. Non-specific cells include NK cells, macrophages, neutrophils and eosinophils, whereas specific cells include $CD8^+$ and $CD4^+$ T cells.

The role of NK cells and $CD8^+$ T cells in *H. pylori* infection are not well known; however, it has been documented that IL-12 and IFN-γ are key components of the immune response to *H. pylori*. IL-12 is a cytokine that activates NK cells and induces Th1 differentiation that has a critical role in $CD8^+$ T-cell activation. One of the main functions of NK cells and $CD8^+$ T cells is IFN-γ production.

NK Cells

The term "natural killer" (NK) was coined by Kiessling and co-workers, who found a population of lymphocytes in mouse with cytolytic specificity for Moloney leukemia cells *in vitro (Kiessling 1975)*. Like other hematopoietic cells, NK cells are derived from bone marrow precursors. They are large lymphocytes with numerous cytoplasmic granules. NK cells constitute 5-20% of the mononuclear cells in peripheral blood and spleen.

Natural killer (NK) cells are involved in early defenses against allogeneic cells, infected autologous cells and tumor cells through cytokine production, in particular IFN-γ, or by direct cytotoxic attack. NK cells discriminate target cells from normal cells by a combination of inhibitory and activating receptors.

In humans, inhibitory receptors belong either to "the killer cell immunoglobulin-like receptor (KIR) superfamily" or "the C-type lectin-like receptor superfamily". KIRs recognize self class I MHC molecules on target cells and lectin-like receptors (i.e. CD94 and NKG2) recognize non-classical or MHC-related molecules including human leucocyte antigen (HLA)-E (by CD94/NKG2) *(Moretta 1997; Lanier 1998)*. Activating receptors (also called "natural cytotoxicity receptors, NCR, i.e. NKp46, NKp30 and NKp44) recognize unknown ligands expressed by a variety of tumor cells or infected cells. When both activating and inhibitory receptors are engaged, the influence of the inhibitory receptor is dominant, and the NK cell is not activated. This mechanism prevents killing of normal host cells.

Infection often leads to an inhibition of class I MHC expression, and therefore the ligands for the inhibitory NK cell receptors are lost. As a result, the NK cells are released from their normal state of inhibition, and the infected cells are killed.

NK-cell-mediated cytolysis is mediated by directional delivery of cytotoxic proteins (perforin, granzymes and granulysin) or ligation of the membrane-bound Fas ligand (FasL) on CTLs with the Fas receptor on the surface of target cells.

Even without activation, NK cells express TNF-related apoptosis-inducing ligand (TRAIL), and can thereby activate apoptosis in cells that express TRAIL, including death receptor 4 and death receptor 5. Furthermore, NK cells induce Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) that can also be performed by neutrophils, eosinophils and macrophages. In this cytolytic system, Fc receptors (CD16) on the surfaces of these cells bind to the Fc portion of antibody molecules that have already bound to a target cell and that cell becomes lysed.

NK-cell characterization

In humans, NK cells are characterized by lack of the CD3 complex, and expression of CD16 and CD56 receptors. Subsets of NK cells can be distinguished by the density of the CD56 expression ($CD56^{bright}$ and $CD56^{dim}$). $CD56^{dim}$ cells (~90% of total peripheral NK cells) are the most cytotoxic subset *(Robertson 1990)*, while the $CD56^{bright}$ NK-cell subset (~10%) can produce a series of cytokines, including IFN-γ, TNF-α, TNF-β, GM-CSF. In addition, $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets show differences in their NK receptor repertoires. $CD56^{bright}$ cells express high levels of the C-type lectin-like CD94/NKG2 family with only small fractions expressing killer-cell immunoglobulin-like receptors (KIR) *(Farag 2002)*, while $CD56^{dim}$ NK cells express both KIR and C-type lectin-like receptors at high surface density.

T Cells

T cells are derived from bone marrow precursors and mature in the thymus. Thymic development involves random gene rearrangements within TCR germ-line DNA, expression of membrane markers CD3, CD4 or CD8, and selection of MHC-restricted T cells (positive selection) that are self-tolerant (negative selection). T cells that survive thymic maturation (~2%) bear a specific TCR for a particular antigen that are present in thousands of identical copies exposed at the cell surface. It is thought that the human body has the genetic ability to generate (10^{12}) diverse TCR and recognize 10^7 - 10^9 different epitopes (Berg 2002).

The matured naïve $CD4^+$ and $CD8^+$ T cells migrate to the circulatory system and circulate through the lymphoid system in order to be stimulated by DCs. Stimulation may lead to anergy or activation due to the absence or presence of co-stimulation, respectively. $CD4^+$ T cells recognize the antigen on class II MHC molecules, presented by antigen-presenting cells (APCs), while the $CD8^+$ T cells recognize the antigens on class I MHC molecules.

$CD8^+$ subsets

$CD8^+$ T cells compose 30% of peripheral lymphocytes and are divided into naive ($CD45RA^+CD27^+$), memory ($CD45RA^-CD27^{+/-}$) and effector subsets ($CD45RA^+CD27^-$) *(Hamann 1997).*

Naïve $CD8^+$ T cells have no ability to kill target cells and produce low level of cytokines. Memory $CD8^+$ T cells fail to kill target cells but can proliferate and produce cytokines such as IL-2 and IFN-γ in response to antigen stimulation. Effector cells have the capability to kill target cells through perforin/granzyme or Fas ligands; and produce cytokines.

CTL activation

Activation of naïve $CD8^+$ T cells and their subsequent proliferation and differentiation into effector CTL require at least three sequential signals: a primary signal, delivered when TCR complex and CD8 co-receptors interact with a foreign peptide-MHC molecule complex; a co-stimulatory signal, delivered by interaction between CD28 (on the T cells) and B7 molecules (on antigen-presenting cells), and a third signal which is induced by the interaction of IL-2 with the high-affinity IL-2 receptor (Goldsby). In contrast, antigen-experienced effector cells and memory cells (as apposed to naïve T cells) are able to respond to TCR-mediated signals with little, if any, co-stimulation.

Interaction of antigen-specific CTL with target cells follows conjugate formation through binding of the integrin receptor leucocyte function-associated antigen 1 (LFA-1) on the CTL with intracellular cell-adhesion molecules (ICAMs) on the target cell membrane. Formation of a CTL-target cell conjugate results in the appearance of granules that contain 65-kDA monomers of a pore-forming protein called perforin and several serine proteases called

granzymes (or fragmentins). These substances are released from the granules by exocytosis into the junctional space between the two cells. The effector CTLs can also kill infected cells via ligation of Fas ligand (FasL) with Fas receptors on target cells or via the perforin/granzyme pathway. Production of TNF-α by $CD8^+$ T cells affords an additional pathway for the induction of apoptosis.

Lysis of a target cell may occur over several minutes or hours, depending on the nature of the target cell and the activity of the CTL. Furthermore, activated $CD8^+$ T cells may produce cytokines such as IFN-γ and TNF-α that have immunomodulatory and anti-microbial effects.

Assays for evaluating CTL activity

Cytotoxic activity of CTLs may lead to target cell death. Cell death can occur by two pathways, necrosis and apoptosis. Necrosis is defined as a passive and non-specific process that is characterised by cell swelling and membrane loss, resulting in the death of cells due to hypoxia or ischemia, hyperthermia, or acute exposure to toxic chemicals, and exogenous insults or poisons. In contrast, apoptosis is an active programmed process of self-destruction of the cell and is associated with characteristic morphological and biochemical changes. Cell shrinkage, nuclear and cytoplasmic condensation, fragmentation of the dying cell into membrane-bound apoptotic bodies, and chromosomal DNA degradation into oligonucleosomal fragments upon the activation of specific nucleases are typical characteristics of apoptosis.

Apoptosis can be extrinsically imposed on target cells by CTLs through the interaction of the so-called death receptors with their corresponding ligands, such as Fas (also termed CD95 or Apo-1) with Fas-Ligand (FasL) *(Brunner 1995)*, TRAIL receptor (TRAIL-R) with TRAIL (Nagata 1997), tumor necrosis factor receptors TNF-R1 or 2 with TNF (Beutler 1990), or intrinsically, by the activation of some members of the BCL-2 family *(Adams 1998)*. Other inducers of apoptosis include the perforin/granzyme system, cytokine deprivation *(Matsui 2005)*, and irradiation *(Martin 1991)*.

Many different assay systems have been described for measuring and analysing apoptosis *in vitro*. In principle, killing of target cells is followed and quantified by changes occurring in cells during apoptosis. Apoptosis is a rapid process and at any given time the percentage of apoptotic cells within the population may be small. Therefore, in most assays, it is necessary to score large numbers of cells to accurately estimate the fraction that is undergoing apoptosis. Flow cytometric techniques are very useful in this regard in that large numbers of individual cells can be assayed rapidly.

Flow cytometric assays detect apoptotic cells on the basis of various properties, including DNA fragmentation, altered membrane permeability, decreased intracellular pH, decreased cell size, and altered phospholipid composition of the extracellular membrane. Several cytofluorometric dyes are available for detecting different aspects of cellular changes occurring during apoptotic processes.

The optimal method for identification of apoptotic events will depend on the cell assay system, tissue type, as well as the mode of induction of apoptosis. In one of our previous studies, we used two-color flow cytometry (usig Annexin V-FITC and propidium iodide-PI) for evaluation apoptosis in human leukocytes and cell lines after stimulation with *Haemophilus ducreyi* cytolethal distending toxin *(Wising 2005)*. The test principle was to label target cells with Annexin V and to follow the membrane disrupted targets cells by a DNA-labeling fluorochome, (i.e PI). Annexin V has high affinity for phosphatidylserine (PS), a phospholipid normally found in the inner leaflet of the plasma membrane. Upon induction of apoptosis, PS is externalized enabling phagocytic cells to clear apoptotic cells before they rupture. This externalization process also results in accessibility of PS to exogenous annexin V and therefore provides a convenient *in vitro* tool to measure apoptotic cell death (Vermes et al., 1995). PI is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Cells that stain positive for Annexin V-FITC and negative for PI are undergoing apoptosis. Cells that stain positive for both Annexin V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both Annexin V-FITC and PI are alive and not undergoing measurable apoptosis.

In another study, we used ^{51}Cr release assay to measure cytotoxic activity of CTL induced in mice after vaccination with DC pulsed with OVA conjugated CT *(Sun 2004)*. ^{51}Cr release assay has been the most popular method to study CTL activity *in vitro* (Brunner et al., 1968). Target cells are loaded with $Na_2{}^{51}CrO4$ which passively enters cells and binds to intracellular proteins. Upon target cell lysis, ^{51}Cr is released into the supernatant and the amount of release is quantified using a gamma counter. Although this method has the benefits of being reproducible and relatively easy to perform, it has several drawbacks including: (1) difficulty to achieve a low cytoplasm/nucleus ratio when labeling cells, (2) high spontaneous release of ^{51}Cr from target cells over time, (3) a delay between actual cell damage and release of ^{51}Cr-bound intracellular proteins into the supernatant, (4) measurement of lysis at the population (vs. single cell) level and (5) the care and handling associated with radioactive isotope usage.

Aims of the study

The overall aim of this thesis was to develop methods to activate cytotoxic lymphocytes by *H. pylori* antigens *in vitro*, and to investigate the presence and the role of such cells in human *H. pylori* infection. The specific aims were:

- To study the interaction between *H. pylori* antigens and NK cells and the possible influence of stimulating cytokines.
- To compare the efficiency of different antigen-presenting cells to activate *H. pylori*-reactive $CD8^+$ T cells *in vitro*.
- To characterize the peripheral $CD8^+$ T-cell response to *H. pylori* antigens in *H. pylori* infected and uninfected individuals.
- To investigate whether *H. pylori*-reactive $CD8^+$ T cells are cytotoxic.

Material and methods

Volunteers and diagnosis of H. pylori infection

In total, 61 volunteers were included in the studies. Among these 26 were infected with *H. pylori* (but asymptomatic), and the remaining subjects were non-infected and healthy. The infected asymptomatic carriers were recruited either among patients were subjected to gastroscopy or among blood donors that had been registered as *H. pylori*-infected at the Blood Bank list (Department of Gastroentrology and Blood Bank, Sahlgrenska University Hopspital, Göteborg, Sweden). They had not taken any antibiotics or any other medication for at least three weeks before sampling. The non-infected individuals were recruited among blood donors, who were screened for *H. pylori*-specific antibodies in serum, and they had no history of gastroduodenal disease.

The studies were approved by the Human Research Ethical Committee of the Medical Faculty, Göteborg University, Göteborg, Sweden.

H. pylori-infected and non-infected volunteers were identified by at least two independent tests. Tests that have been used in the studies are: Bacterial culture, Serum ELISA, stool ELISA and UBT.

Culturing: Biopsies from the gastric antrum were homogenized in 1 ml physiological saline and cultured on Columbia Iso Agar plates at 37 °C in microaerobic conditions for three days, and subsequently screened for *Helicobacter*-like colonies.

Serum ELISA: *H. pylori* elicit a specific serological response in the infected person. IgM antibody levels may be detectable early in the course of an active infection. Levels of IgG and IgA rise with infection and remain high or drop gradually over time. We have used an in house ELISA based on coating with a membrane preparation (MP) of *H. pylori* as solid phase *(Mattsson 1998)* or the Pyloriset EIA-G III ELISA (Orion Diagnostica, Espoo, Finland).

Urea Breath Test (UBT): In the UBT test, the patient drinks a urea suspension containing radioactive carbon (C-13). If *H. pylori* are present in the stomach, they will break down the

urea and form ammonia and carbon dioxide gas. The carbon dioxide gas is quickly absorbed through the lining of the stomach and brought into the blood, eventually being expelled into the breath. Samples of exhaled breath are collected at various points and the level of radioactive carbon dioxide is measured. If this level raises above a set amount, *H. pylori* is determined to be present. The urea breath test is about 96 to 98% accurate.

Stool antigen test: We have used a commercial *H. pylori* stool antigen test (Amplified IDEIATM Hp StARTM, DAKO, Denmark) that is an accurate method for diagnosis of infected individuals. The European "Maastricht 2-2000 Consensus Report" suggested that the stool antigen test may be an alternative to the urea breath test after treatment *(Malfertheiner 2002)*. This test is a sandwich-type enzyme immunoassay (EIA) in a microplate format for the direct, non-invasive detection of *H. pylori* antigens in human stool specimens. It is based on monoclonal antibodies (specific for *H. pylori* antigens) that are bound to the wells of the microplate.

Bacterial preparations

Live *Helicobacter pylori* bacteria: Strain Hel305, isolated from a duodenal ulcer patient and being *cag*A$^+$ and *vac*A$^+$, was grown from -70 °C stock cultures on Columbia Iso Agar plates, under microaerobic conditions for 3 days, followed by culture in liquid Brucella broth liquid culture. Bacteria were diluted in the appropriate cell culture medium to OD_{600} = 1 (equals $5x10^9$ bacteria/ml) and used for further experiments.

The expression of *cag*A and *vac*A genes were detected by PCR, as previously described *(Thoreson 2000)*.

Inactivated *H. pylori*: strain Hel 305 bacteria were prepared as above. Then, formaldehyde was added to a final concentration of 0.01 M. After incubation at 37 °C on a slow shaker for 2 h, followed by overnight shaking at room temperature, the bacteria were washed three times in PBS and then resuspended in PBS to an OD_{600} of 1.5 (corresponding to 7.5×10^9 cells/ml) and stored at 4 °C until used. Complete inactivation of the culture was confirmed by the lack of growth on horse blood agar plates *(Raghavan 2002)*.

Lysates: Lysates of *H. pylori* strain Hel 305 and *E. coli* strain E11881/99, respectively, were prepared as previously described *(Raghavan 2002)*. The protein contents were determined using a spectrophotometer. Each lysate was snap frozen in liquid nitrogen and was stored in aliquots at -70 °C until used.

Membrane preparation (MP): strain Hel 305 was grown as described above. Membrane preparation was prepared by sonication followed by differential centrifugation as previously described *(Bolin 1995)*. Gel electrophoresis of *H. pylori* MP showed that it contained more than 20 different proteins; among these, urease, the neutrophil activating protein (NAP), *H. pylori* adhesin A (HpaA), and flagellin were identified by Western blotting using monoclonal antibodies (mAbs) specific for the different antigens *(Lindholm 1997; Thoreson 2000)*. The MP contained <50% (wt/wt) lipopolysaccharide (LPS), as determined by the Limulus test.

Urease: This antigen was purified from *H. pylori* strain E32, which is a good producer of urease. A combination of the methods described by Dunn et al. and Evans et al. *(Dunn 1990; Evans 1992)* was used for purification of urease. The purity of the preparation was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with polyclonal anti-sera and urease-specific antibody, and urease activity was confirmed by using a commercial urease test.

rHpaA: The antigen kindly provided by Astra Zeneca was recombinantly produced and purified as previously described *(Lundstrom 2003)*. Briefly, the *HpaA* gene was cloned from *H. pylori*, transferred to two different expression vectors, and transformed into *E. coli* and purified by affinity chromatography.

rCag antigen: *H. pylori* rCag antigen (Austral Biologicals, California, USA), was produced in genetically engineered *E. coli* and covers Glu 748 to Glu 1015 of the *H. pylori* CagA antigen.

Sampling of mucosal biopsies and collecting cells:

Gastroduodenal endoscopies were performed under local anaesthesia after intake of antifoaming agent (Minifoam^{R}). Biopsy samples were obtained from the antrum and duodenum. The epithelium and the intraepithelial lymphocytes were removed by stirring the biopsies for 4x15 min (duodenal biopsies) or 6x15 min (antral biopsies) in Hank's balanced

salt solution (HBSS) without calcium or magnesium containing 1 mM EDTA and 1 mM DTT, followed by two incubations in HBSS without EDTA, at room temperature. To isolate lymphoid cells the remaining tissue was thereafter subjected to enzymatic digestion – the biopsies were stirred for 2.5 h at 37 °C in 5 ml collagenase/DNAse solution (100 U/ml collagenase, Sigma C-0255, and 0.1 mg/ml DNAse, Sigma D-5025). The resulting suspension was filtered through a nylon mesh and the cells were counted under the microscope. This cell isolation regimen gave maximal yield of cells, with very little of the epithelium remaining in the lamina propria fraction.

Isolation and fractionation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were collected by density gradient centrifugation on Ficoll-Hypaque (Amersham Bioscience, Sweden). $CD14^+$ cells were purified by positive selection using anti-CD14 conjugated magnetic microbeads (MACS, Miltenyi Biotec, Bergish Gladbach, Germany), according to the instructions of the manufacturer. The isolated cells were 96-98% $CD14^+$, as determined by flow cytometry, and were either used as monocytes or cultured to generate dentriric cells (DCs). To prepare DCs, the isolated $CD14^+$ cells were cultured at 10^6 cells/ml in Iscove's complete medium (3 µg/ml L-glutamin, 50 µg/ml gentamicin, and 5% human AB^+ serum) supplemented with 800 U/ml GM-CSF (Leucomax, Molgramostim, Shering-Plough) and 500 U/ml IL-4 (R&D Systems Europe Ltd, Oxon, UK), for 7 days. Every second day half the volume of medium was replaced by fresh medium containing GM-CSF and IL-4. Determination of the purity of the DCs using anti-CD11c revealed that 96-98% was $CD11c^+$.

NK cells ($CD56^+CD3$-) were isolated from PBMCs by negative selection using a magnetic bead isolation kit (Human NK isolation kit, Miltenyi Biotech, Germany) according to the recommendations of the manufacturer; the cells had a purity of more than 90 %.

$CD4^+$ and $CD8^+$ T cells were further purified from PBMCs by incubation together with magnetic beads coated with antibodies to CD4 or CD8 receptors, respectively (Dynal AS, Oslo, Norway). The beads were subsequently released from the cells by incubation with a tailor-made F(ab) preparation (Detachabead; Dynal). The mean purity of $CD4^+$ T cells and $CD8^+$ T cells were 95% and 96%, respectively.

B cells were purified by positive selection, using anti-CD19 conjugated magnetic microbeads (Dynal AS), according to the instructions of the manufacturer. The mean purity of $CD19^+$ cells were 90%.

Identification and sorting of CD8 T-cell subsets

PBMCs were stained with anti-CD3 conjugated to Peridinin-chlorophyll-protein-PerCP, anti-CD8 conjugated to allophycocyanin-APC, anti-CD45RA conjugated to fluorescein isothiocyanate-FITC and anti-CD27 conjugated to Phycoerythrin-PE. $CD8^+$ T-cell subpopulations were identified in gated lymphocytes, following $CD8^+$ gated cells. The $CD45RA^+CD27^-$ cells were recognized as effector cells; $CD45RA^-$ as memory cells and $CD45RA^+CD27^+$ as naïve T cells *(Hamann 1997; Sallusto 1999)* . For isolation of enriched $CD8^+$ sub-populations of interest (memory, effector or naïve), the other two populations were depleted from PBMCs suspensions using a FACSVantage SE (BD, San Jose, CA, USA) operating at a sheath pressure of 22 psi. The purity of the different sub-populations varied between 80-99 % in the $CD8^+$ T-cell analysis gate.

Stimulation of T cells and analysis of proliferation

DCs, B cells and monocytes were cultured at a concentration of $5x10^4$ cells/ well in round-bottomed 96 well plates and pulsed with *H. pylori* antigens overnight. After washing, CFSE-labelled responder cells ($2x10^5$ / well) were added to the pulsed APCs. After 6 days of cultivation, the supernatants were collected and the cell proliferation was measured by flow cytometry.

As positive controls, phytohemagglutinin (PHA; 1 μg/ml; Murex Diagnostics Ltd., Temple Hill, United Kingdom) and anti-CD3 mAb (1 μg/ml; OKT-3, Ortho-McNeil Pharmaceutical, Raritan, NJ, USA) were used to stimulate CFSE-labelled responder cells. As negative controls, responder cells were stimulated with antigen non-pulsed APC.

For CFSE-labeling of PBMCs, purified $CD8^+$ and $CD8^+$ subset-depleted cells (memory, effector and naïve), 10^7 cells/ml were incubated with 2 μM CFSE (Vybrant CFDA SE cell

tracer kit; Molecular Probes, Leiden, The Netherlands) according to the instructions of the manufacturer.

5-(6)-Carboxy-fluorescein succinimidyl ester (CFSE) is a non-fluorescent, non-polar fatty acid ester that diffuses passively into cells. Within the viable cells CFSE is hydrolyzed by estrases to produce free polar carboxy-fluorescein, a fluorescent compound which binds covalently to proteins and is well retained within the cell. During cell division, CFSE is shared equally between daughter cells. The fluorescent intensity of the viable CFSE-labeled cell population is linearly proportional to the number of viable cells present. CFSE is non-toxic in low concentration.

Determination of IFN-γ and granzymes

The supernatants from stimulated cells were frozen at -80 °C until assayed for IFN-γ content by an enzyme-linked immunosorbent assay (ELISA), as previously described *(Lundin 2002)*.

The amount of Granzyme A and B were measured in supernatant of *H. pylori*-stimulated PBMCs by the same ELISA assay principle, according to the instructions of the manufacturer (PeliKine Granzyme Kit, Sanquin Reagents, Netherlands). Briefly, anti-human Granzyme A or B antibodies were bound onto the wells of a polystyrene 96-well plate to capture any Granzyme A or B present in the sample. Thereafter incubation of biotinylated anti-Granzyme A or B antibodies was followed by horseradish peroxidase-conjugate streptavidine and substrate. A standard curve was generated using recombinant Granzyme A or B.

Intracellular staining for IFN-γ and Granzyme B, using flow cytometry

Intracellular staining was performed as previously described *(Jung 1993; Prussin 1997)*. Briefly, stimulated PBMCs were resuspended in 200 µl medium containing GolgiStop (BD Biosciences) (4 µl in every 6 ml). Six hours later, cells were harvested and resuspended in 200 µl/well PBS supplemented with 10% AB^+ serum and incubated for 15 min at 4° C, to avoid non-specific binding. After washing, the cells were stained with anti-CD8 (PerCP), for 20 min at 4°C. After washing, the cells were resuspended in 200 µl of 4% paraformaldehyde

and incubated at 4° C for 15 min. After washing, the cells were resuspended in FACS-buffer and stored in 4° C overnight. After centrifugation and removal of the supernatant, the cells were resuspended in 200 µl Perm/wash buffer (BD Biosciences) and incubated at 4 °C for 15 min. The cells were then stained with anti-IFN-γ-PE and/or anti-Granzyme B-Alexa Fluor 647. Mouse IgG2b and IgG1 were used as isotype controls, respectively.

All antibodies were obtained from BD Biosciences

Generation of CTL-line and ^{51}Cr-Release Cytotoxicity Assay

CTL cell lines were prepared from PBMCs isolated from heparinized blood and resuspended in complete medium at a concentration of $2x10^5$ /ml in the presence of *H. pylori* MP (5 µg/ml). The cells were then distributed in 24-well plate (2 ml/well) and incubated at 37 °C, 5% CO_2 for 6 days. At day three, 30 IU/ml of recombinant human interleukin-2 (rhIL-2) (Proleukin; Chiron, Emeryville, CA) was added. At day 7, cells were washed twice with medium and used as CTL cell line.

Both fresh BPMCs and CTL-lines were used as effector cells. Overnight MP-pulsed B cells and non-pulsed B cells were labelled with 100 µCi ^{51}Cr per 10^6 cells and used as target cells. Labeled B cells were co-cultured at 5×10^4 per well with serially diluted effector cells (from 75:1 effector:target ratio to 2.7:1) in 200-µl volumes in round-bottomed, 96-well plates. After 4 hours incubation at 37 °C in 5% CO_2, 100 µl of supernatant was analyzed using a γ-counter. Percent specific lysis was calculated as: 100 × [(mean sample release − mean spontaneous release) / (mean maximal release B − mean spontaneous release)].

Flow cytometric analysis

Cells were microscopically analyzed for viability, using Trypan blue (>98% viable cells) and then stained with anti-CD3, anti-CD8 and anti-CD4 antibodies and analyzed in FACS in presence of TruCount beads (BD Biosiences). A Becton Dickinson FACSCalibur, dual-laser, equipped with computer software CellQuest (BD, Biosciences) was used to assess the percentage or number of proliferated T cells *(Hasbold 1999)*. Briefly, the flow cytometer settings were established for linear amplification of light scatter and logarithmic

amplification of fluorescence channels. The threshold was defined in side scatter to include cells and TruCount beads. For all samples, at least 100,000 events were collected. Proliferation was determined by the loss of CFSE staining in the cell populations of interest.

RT-PCR

Reverse transcriptase-polymerase chain reaction was used to evaluate mRNA coding for cytokines and cytotoxic mediators. Total RNA was isolated from the cells of interest, using Total RNA Extraction Kit (Sigma Aldrich, St.Louis, MO) and cDNA was synthezised by using an oligo-dT primer and the Omniscript RT-PCR Kit (Qiagen, Hilden, Germany) as described by the manufacturer. A specific primer set was used for each cytokine. Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was used as housekeeping gene. The PCR products were run on 3% agarose gels, stained with ethidium bromide and visualized under UV-light.

Statistical analysis

The Mann-Whitney test was used to evaluate differences in proliferation and cytokine secretion by cells from Hp^+ and Hp^- individuals, respectively. A paired *t* test was used to analyze differences between the $CD8^+$ T-cell proliferation induced by B cells, DCs and monocytes, and for comparisons of proliferation and IFN-γ secretion in the presence and absence of $CD4^+$ cells or exogenous IL-2. A P value < 0.05 was considered statistically significant.

Results and Comments

NK cells become activated by a combination of H. pylori lysate and IL-12 in vitro (paper I)

Classically, NK cells have been regarded to be activated either by virus infected/cancer transformed cells or by activating cytokines like IL-12 or type I interferons produced during acute infections *(Cerwenka 2001; Smyth 2001; Moser 2002)*. However, there is evidence that NK cells also can be activated by direct action of bacterial products *(Kirby 2002)*. Apart from their cytotoxic capacity, NK cells can also secrete different cytokines. A key cytokine produced by NK cells is IFN-γ, and since IFN-γ dominates both local and systemic immune responses to *H. pylori*, we have investigated whether NK cells may play a role in the immune response to *H. pylori* infection.

We began to investigate whether NK cells are present in the mucosa and if their proportion differs between *H. pylori* infected and non-infected subjects. Biopsies from antrum (of stomach) and duodenum from *H. pylori*-infected and non-infected individuals were obtained and lamina propria cells were analyzed for the presence and proportion of NK cells. The results showed that NK cells are present in both antrum and duodenum of *H. pylori*-infected and non-infected subjects; however in the antrum of *H. pylori*-infected individuals the frequency of NK cells was lower, compared to non-infected subjects. Identification of other immune cell populations, such as $CD4^+$, $CD8^+$, B cells and NKT cells, present in the antrum of *H. pylori*-infected individuals, indicates that the decrease in NK cells frequency was due to a large infiltration of $CD4^+$ T cells and B cells into the infected mucosa. Although the frequency of NK cells was lower, counting of the total number of isolated cells from each biopsy revealed that there was no difference in NK-cell numbers between *H. pylori*-infected and non-infected mucosa (data not shown).

The presence of NK cells in mucosa of non-infected individuals is not surprising. It is known that NK cells normally mainly reside in the blood, spleen and liver, but also in the stomach and intestinal mucosa. However, the presence of NK cells in *H. pylori*-infected mucosa has not been previously studied. From studies of viral infections, it could be

expected that the number of NK cells would increase after *H. pylori* infection. Recent *in vivo* studies using intracellular bromodeoxyuridine (BrdU) staining as a direct measure of proliferation have demonstrated NK-cell proliferation, early during the course of murine cytomegalovirus infection *(Dokun 2001)*. In *H. pylori* infection, the initial non-specific proliferative response of NK cells is induced by release of pro-inflammatory cytokines like IL-12 and IL-15 from epithelial cells. Although we found no evidence of increased NK-cell numbers in the chronically infected mucosa, it is still possible that the numbers increase during the early course of infection, in a similar way as in cytomegalovirus (CMV) infection. However, this could not be studied since all our volunteers were adults and probably infected since many years.

After showing that NK cells are present in the stomach mucosa, we decided to study the interaction between NK cells and *H. pylori* antigens. Since we did not have access to enough mucosal NK cells, we purified NK cells from peripheral blood and stimulated them with *H. pylori* preparations (lysate, inactivated bacteria and live bacteria). After 48 hours, the supernatants were collected and the concentration of IFN-γ was measured using ELISA. The results showed that highly purified NK cells produced IFN-γ in response to *H. pylori* stimulation. In further experiments we compared the responses of NK cells obtained from *H. pylori*-infected and non-infected individuals. These responses were comparable which was not unexpected since NK cells are part of the innate immune system and have no antigen-specific receptor or memory for *H. pylori* products.

Next, we analysed whether the response to *H. pylori* by NK cells could be influenced by IL-12, a cytokine which is produced by innate cells in the *H. pylori*-infected mucosa. When NK cells were stimulated with low levels of *H. pylori* lysate or IL-12 the production of IFN-γ was low. However, by adding IL-12 together with lysate for stimulation of NK cells, a strong synergistic effect on the IFN-γ production was induced (Figure 2). A similar synergistic effect was seen in NK-cell cultures stimulated with live or inactivated *H. pylori* bacteria given together with IL-12. This is of importance, since both IL-12 and components of *H. pylori* bacteria are present in the gastric lamina propria, where the NK cells are residing. Stimulation of NK cells with *E. coli* lysate showed a similar pattern, indicating that the IFN-γ

production by NK cells is not specific for *H. pylori* but can also be induced by other bacterial species if present in the gastric mucosa.

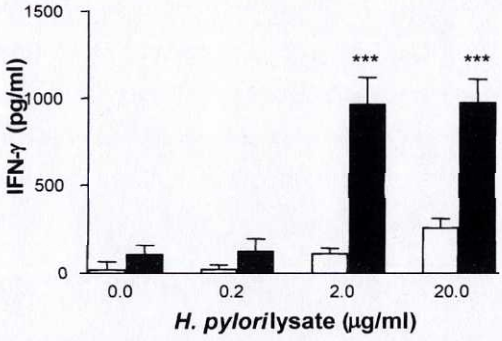

Figure 2. NK cells were stimulated with *H. pylori* lysate and/or IL-12 and the production of IFN-γ was measured. Adding IL-12 together with lysate induced a synergistic effect on the IFN-γ production by NK cells.

The synergistic effect of *H. pylori* antigens and IL-12 was confirmed by IFN-γ intracellular staining and analysis of IFN-γ mRNA. Thus, flow cytometry data revealed that 12-25% of NK cells produced IFN-γ in response to different *H. pylori* antigens together with IL-12. Furthermore, RT-PCR data at 15 hours showed that stimulation of NK cells with only lysate induced expression of mRNA for IFN-γ, but mRNA expression was higher when NK cells were stimulated with IL-12 and lysate.

The activation of NK cells after stimulation with *H. pylori* preparations was confirmed by analysis of expression of the activation markers CD25 and CD69 by FACS.

It would be interesting to complement these results using NK cells obtained from the gastric mucosa. It has previously been shown that NK cells isolated from human intestinal mucosa exhibit functions similar to those of peripheral blood NK cells *(Tarkkanen 1993)*. Preliminary data from our laboratory indicate that mucosal NK cells do indeed produce IFN-γ after stimulation with *H. pylori* antigens (data not shown).

We then performed experiments in a more *in vivo*-like situation, using a trans-well system, to investigate whether NK cells can be activated even when an epithelial cell line separate them from *H. pylori* bacteria. The results showed that even under these circumstances, NK cells produced IFN-γ in response to bacteria. In this setup, it is possible that the activation was mediated by the engagement of bacterial pathogen-associated molecular patterns (PAMPs) with toll-like receptors (TLRs) on the surface of the epithelial cell line, resulting in secretion of pro-inflammatory cytokines that lead to NK-cell activation. In addition, components of *H. pylori* may have diffused across the epithelial cell layer, allowing a direct activation of the NK cells.

Next, we wanted to rule out that the NK-cell activation seen by *H. pylori*-stimulated NK cells was not due to high IL-12 production by contaminating monocytes in the culture. Therefore, we analyzed the supernatants of wells containing NK cells stimulated with *H. pylori* preparations alone for content of IL-12, using ELISA. Also, we analyzed the supernatant from the trans-well system and in both cases there was no detectable level of IL-12 (data not shown). We could then conclude that the NK-cell response to *H. pylori* products was not induced by endogenous IL-12 production *in vitro*.

To investigate whether recognition of *H. pylori* lysate by NK cells was due to binding of *H. pylori* LPS to toll-like receptors we stimulated NK cells with purified LPS from both *H. pylori* and *E. coli*. The results showed that in contrast to lysate, neither *H. pylori* LPS nor *E. coli* LPS could activate NK cells in the absence of IL-12. Furthermore, when the effects of LPS were blocked, only partial (30%) inhibition of lysate-induced IFN-γ could be seen. Taken together, these results indicate that the activation of NK cells by *H. pylori* is largely independent of LPS.

We continued the study to investigate whether the NK-cell activation after stimulation with *H. pylori* antigens was limited to IFN-γ production or enhanced the cytotoxic capacity of the NK cells. RT-PCR data at 15 hours after stimulation showed an increased level of mRNA for granzyme B and perforin in NK cells stimulated with bacterial lysate, IL-12, and lysate plus IL-12. However, no synergistic effect could be seen. These results indicate that NK cells that have been activated by *H. pylori* lysate show an increased potential for cytotoxic activity.

In an attempt to understand mechanism behind NK cells activation and the synergistic effect of *H. pylori* antigens and IL-12, the expression of interlukine-12 receptors (IL-12Rβ1 and IL-12Rβ2) were analyzed after stimulation. The results showed upregulation of IL-12Rβ2 but not of IL-12Rβ1 in $CD56^{bright}$ NK cell population after stimulation with lysate and/or IL-12 (Figure 3). We suggest that the upregulation of IL12Rβ2 after stimulation with lysate alone may explain the synergistic effect of lysate and IL-12. This possibility was further supported when NK cells were first stimulated with *H. pylori* antigens and IL-12 was added later, and compared to the results with alternative stimulation (first IL-12 and addition of *H. pylori* antigens later).

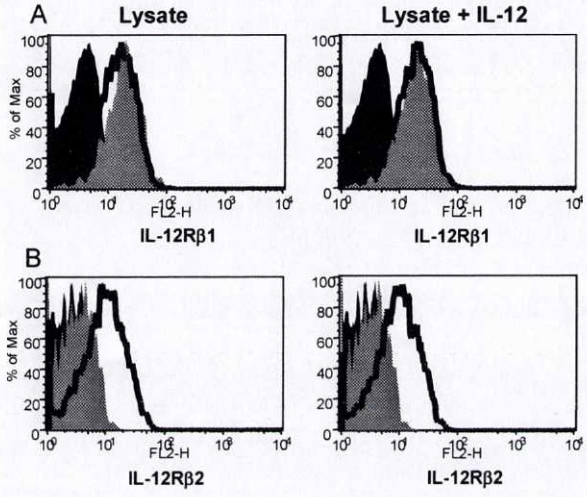

Figure 3. Flow cytometric analysis of IL-12 receptor expression in $CD56^{bright}$ NK cells after stimulation with lysate or lysate+IL-12 for 15 h. The solid black shaded area shows isotype control, the gray area shows unstimulated cells, and the bold line shows the indicated stimulation.

An optimal/practical in vitro system for stimulation of H. pylori-reactive CD8⁺ T cells (paper II)

H. pylori infection induce both mucosal and circulating specific $CD4^+$ and $CD8^+$ T-cell responses *(Bamford 1998; Lindholm 1998; Quiding-Jarbrink 2001)*. Previously, it has been shown that *H. pylori* colonization is associated with a significant increase of $CD8^+$ lymphocytes in the epithelium of the crypts *(Hood 1993)* and intraepithelial area *(Bedoya 2003)*. Also, it has been documented that $CD8^+$ T cells isolated from the gastric mucosa of *H. pylori* infected subjects, show a significant increase in expression of the activation marker HLA-DR *(Ihan 1995)*. A study in knockout mice has shown that mice lacking functional $CD8^+$ T cells had increased levels of *H. pylori* in the stomach mucosa compared to normal animals *(Pappo 1999)*. Furthermore, a previous study from our laboratory showed that both local and circulating purified $CD8^+$ T cells produce a large amounts of IFN-γ after stimulation with *H. pylori* antigens, both in patients suffering from duodenal ulcer and asymptomatic carriers *(Quiding-Jarbrink 2001)*. The presence of high numbers of activated cytokine producing $CD8^+$ T cells in the infected mucosa *(Hood and Læsna 1993; Bedoya 2003)*, suggests that $CD8^+$ T cells may also contribute to the T-cell response against *H. pylori*.

$CD8^+$ T cells require antigen-presenting cells for activation; therefore, studies aiming to define a way to induce an optimal/practical $CD8^+$ T cell response need to find a suitable antigen-presenting cell. It has been generally accepted that DCs are the most potent of the different professional APC types. However, for human studies of T-cell responses *in vitro*, usage of DCs is not practical, due to the laboursome and time-consuming procedure for generation of blood-derived DCs. For practical purposes, researchers usually prefer to use monocytes that are more easily obtained.

B cells – especially memory B cells - are known to be very efficient APCs *(Liu 1995)*, but the capacity of B cells for stimulation of $CD8^+$ T cells has not been well defined. Furthermore, to our knowledge, no one has previously compared the capacity of different APCs to activate *H. pylori*-specific $CD8^+$ T cells.

In an attempt to determine the optimal culture and stimulating conditions necessary to activate $CD8^+$ T cells in response to *H. pylori* antigens *in vitro*, we pulsed DCs, monocytes and

B cells from both *H. pylori*-infected and non-infected individuals with different concentration of *H. pylori* preparations overnight. After washing, the APCs were co-cultured with CFSE-labeled PBMCs or purified CFSE-labeled $CD8^+$ T cells. This setup was used in different conditions in order to find an optimal way of analyzing $CD8^+$ T-cell proliferation. Thus, different ratios of APCs and responder cells were used, analysis was performed at different time-points after stimulation, and varying concentrations of *H. pylori* antigens were used. Furthermore, CFSE-labeled PBMCs without any added APCs were stimulated with *H. pylori* antigens without the washing procedure. The use of CFSE for tracing the cellular division pattern of *H. pylori*-reactive $CD8^+$ or $CD4^+$ T cells, made it possible to follow the T-cell proliferative response to *H. pylori* antigens on a single-cell basis (Figure 4).

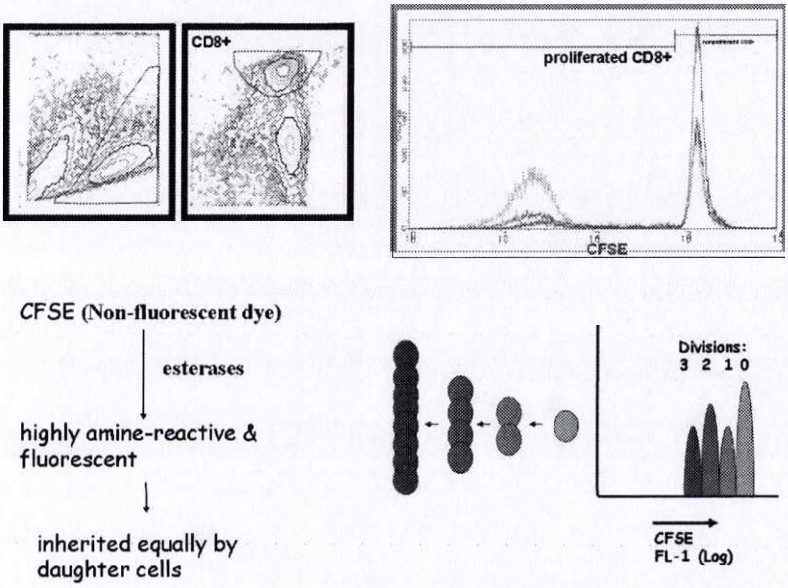

Figure 4. During cell division, CFSE is equally shared between the daughter cells by which the intensity of CFSE will be halved compared to the mother cells. By gating lymphocytes in forward scatter vs. side scatter plot following by $CD8^+$ T cells gating respectively (upper left), the frequency of proliferated cells (upper right) and number of cell divisions can be calculated (lower right).

Analysis of the proliferation pattern in $CD8^+$ or $CD4^+$ T cell populations and secretion of IFN-γ revealed that both *H. pylori*-reactive $CD8^+$ and $CD4^+$ T cells are present in peripheral blood of both *H. pylori*-infected and non-infected individuals. Stimulation of purified $CD8^+$

T cells with APCs pulsed with *H. pylori* antigens neither induced proliferative response nor IFN-γ production, whereas adding purified $CD4^+$ T cells or IL-2 resulted in proliferation and IFN-γ production, demonstrating that $CD8^+$ T cells require help from $CD4^+$ T cells or cytokines in order to become activated. These data demonstrate that in order to analyze the proliferation of $CD8^+$ T cells in a relatively non-artificial way – without addition of exogenous cytokines – there is a need of a method that detects cell division of $CD8^+$ T cells in a mixed cell population. Therefore, the use of CFSE-stained PBMC for analysis of $CD8^+$ T-cell proliferation, as outlined in this study, is very well suited for this purpose.

Consistent $CD8^+$ as well as $CD4^+$ T-cell responses was induced by pulsing any of the three APC types with 5 μg/ml MP. A ratio between pulsed APCs and responder cells of 1:5 gave strong proliferative responses without consuming excessive amounts of APCs. Furthermore, kinetic experiments showed that 6 days co-culturing of MP-pulsed APCs with CFSE-labeled PBMCs gave the highest proliferation of $CD8^+$ or $CD4^+$ T cells. Moreover, DCs and B cells were most efficient in inducing both $CD8^+$ and $CD4^+$ T-cell responses to *H. pylori* MP in both *H. pylori*-infected and non-infected individuals. The responses were similar between *H. pylori* infected and non-infected. In contrast, MP-pulsed monocytes induced low $CD8^+$ T-cell responses in both *H. pylori*-infected and non-infected individuals, whereas MP-pulsed monocytes induced five-fold higher proliferative response in $CD4^+$ T cells from non-infected subjects compared to infected individuals.

The high efficiency of DCs in activation of *H. pylori*-reactive $CD8^+$ cells observed is not surprising. The DCs' size and structure supply a large surface area allowing an efficient interaction between the APC and T cells. Furthermore DCs have ability to engulf antigens through both phagocytosis and macropinocytosis and process high amounts of antigens. Also, DCs can activate T cells (especially naïve T cells) very efficiently due to high expression of MHC molecules, costimulatory receptors and adhesion molecules *(Banchereau 2000; Guermonprez 2002)*. Moreover, DCs' ability to route internalized antigens into the MHC class I pathway (a process termed cross-presentation), leading to cross-priming of $CD8^+$ T cells is well known *(Heath 2001; Lizee 2003)*.

B cells can also work as efficient antigen-presenting cells. The B-cell receptors (BCRs) enable antigen-specific B cells to bind soluble target antigens with high affinity and in

concentrations thousands of times smaller than a macrophage would need. B cells internalize the antigen by receptor-mediated endocytosis and process it into small peptide fragments and present these peptides on the context of MHC molecules *(Rock 1984; Lanzavecchia 1985)*. Furthermore, engagement of Ig receptors by specific Ag has been found to activate B cells and thus trigger up-regulation of costimulatory molecules on B cells both *in vitro (Lenschow 1994)* and *in vivo (Constant 1995)*. Thus, at least two criteria required for the activation of naive $CD4^+$ T cells, e.g., TCR ligation and co-stimulation, appear to be met by Ag-specific B cells. Moreover, it has also been shown that some microbial constituents induce co-stimulatory responses, resulting to expression of B7.1 and especially B7.2 receptors that enhance B cells ability to work as APCs *(Lenschow 1994)*. This has been recognized as B cells adjuvant activity (microbial components enhance their response). However, little is known as to whether B cells can cross-present Ag via MHC class I to naive $CD8^+$ T cells, and whether cross-presentation leads to cross-priming. Our results indicate that B cells are able to cross-present *H. pylori* antigens on MHC I, since $CD8^+$ T-cell proliferation was induced to similar levels by antigen-pulsed B cells and DCs. Furthermore, this is also supported by data from our group, which shows that B cells can be used for stimulation of *H. pylori*-reactive $CD4^+$ and $CD8^+$ T cells from *H. pylori*-vaccinated mice (Nyström et.al., unpublished).

To explain why monocytes are not as efficient APCs as DCs or B cells for activating $CD8^+$ T cells, it may be suggested that the strength of the signals given by monocytes were not as strong as signals provided by DCs or B cells to $CD8^+$ T cells. It has been reported that the ratio of antigen-presenting cells and stimulator cells can influence monocytes' ability to supply required signals for activating T cells *(Roth 1996)*. For practical purposes, we chose to use 1:5 ratio in the present study. The results may have varied if we had used another ratio.

One may ask why *H. pylori*-reactive $CD8^+$ T cells are present in the peripheral blood of *H. pylori* non-infected individuals. However, it is not the first time that the presence of *H. pylori*-reactive T cells is demonstrated in peripheral blood of non-infected individuals *(Fan 1994; Di Tommaso 1995)*. It has previously also been shown that *H pylori* uninfected subjects have circulating IgG antibodies to several *H pylori* antigens *(Karttunen 1991; Fan 1994; Duchmann 1997)*. One possible explanation is non-specific response, meaning that there is one or more clones of $CD8^+$ T cells in the peripheral blood of both *H. pylori*-infected and

non-infected individuals that have specific receptors for those *H. pylori* antigenic peptide(s) that are not just specific for *H. pylori*, due to cross-reacting epitopes common to *H. pylori* and other microorganisms. It could also be argued that the comparable proliferative response of T cells to *H. pylori* antigens in *H. pylori*-infected and uninfected subjects may be due to the presence of some mitogenic components in *H. pylori* preparations, which act by unspecific activation of T cells regardless of TCR specificity. Such effects have not been reported previously. In contrast, there are reports indicating that *H. pylori* may inhibit mitogen-induced proliferation of human peripheral blood mononuclear cells *(Knipp 1993; Knipp 1994)*. Furthermore, in a mouse-model it has been demonstrated that *H. pylori* cell-free extract has similar effects on murine lymphoid cells *(Chen 2000)*. Our results do likewise not support mitogenic effects of *H. pylori* components on $CD8^+$ T cells. Analysis of precursor frequency of the responding $CD8^+$ T-cell population showed that only a small minority (1-5 %) of the $CD8^+$ T cells were proliferating in response to antigen-pulsed APC. In a polyclonal response there is a much higher precursor frequency of responding cells (Figure 5).

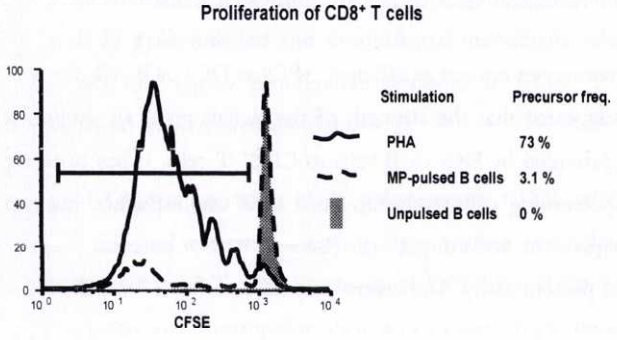

Figure 5. CFSE-stained PBMC were stimulated with PHA, MP-pulsed B cells or un-pulsed B cells, and analysed by flow cytometry. The precursor frequency was calculated by considering the frequency of dividing cells and the number of divisions each cell had gone through, using the computer software FlowJo. The figure shows results of gated $CD8^+$ T cells.

In order to examine the proliferative response of $CD8^+$ T cells in *H. pylori*-infected individuals in greater detail, and to possibly detect a $CD8^+$ T-cell response limited to only *H. pylori*-infected subjects, we examined the distribution and activation of memory $CD8^+$ T

cells. To examine whether the proliferative response of $CD8^+$ T cells in *H. pylori*-non-infected individuals was due to a *H. pylori*-specific response, we stimulated $CD8^+$ subsets (memory / effector / naïve) with B cells pulsed with more specific *H. pylori* antigens (i.e urease, rHpaA and rCag) and evaluated the response of memory $CD8^+$ T cells.

First, we characterized the distribution of naïve, memory and effector cells among $CD8^+$ T cells in peripheral blood and infected gastric mucosa. To this end, we stained the cells with anti-CD3-PerCP, anti-CD8-APC, anti-CD45RA-FITC and anti-CD27-PE. The results showed that both the concentration of $CD8^+$ T-cells and the frequencies of the subpopulations were similar in the peripheral blood of *H. pylori*-infected and non-infected individuals. In contrast, the frequencies of naïve and effector cells were low in the gastric mucosa, while the large majority of cells were memory cells.

After showing that in infected tissue, high frequency of $CD8^+$ T cells were memory cells, we sorted enriched memory $CD8^+$ T cells from PBMCs by depletion of effector and naïve $CD8^+$ T cells and stimulated these cells with B cells pulsed with different *H. pylori* antigens.

The results showed that urease-pulsed B cells induced 15-fold higher proliferative response in memory $CD8^+$ T cells from *H. pylori*-infected compared to non-infected individuals (Figure 6). Furthermore, 3 out of 5 *H. pylori*-infected individuals showed a strong proliferative response to rHpaA, whereas none of the non-infected subjects responded to this antigen. These data demonstrate that there are $CD8^+$ memory cells specific for *H. pylori* antigens in infected subjects. These are novel findings, and they indicate that $CD8^+$ T cells do take part in the immune response to *H. pylori* infection. Previously published studies have shown either comparable or lower proliferative response of $CD8^+$ T cells to *H. pylori* antigens in *H. pylori*-infected compared to non-infected individuals (Karttunen 1991). We believe that this discrepancy is due to that in previous studies, the use of bulk assays for proliferation assessment (thymidin incorporation assay) hampered the ability to analyse the $CD8^+$ T-cell responses. Furthermore, using our approach we were also able to specifically study memory $CD8^+$ T cells, allowing a detailed evaluation of the proliferative response of $CD8^+$ T cells.

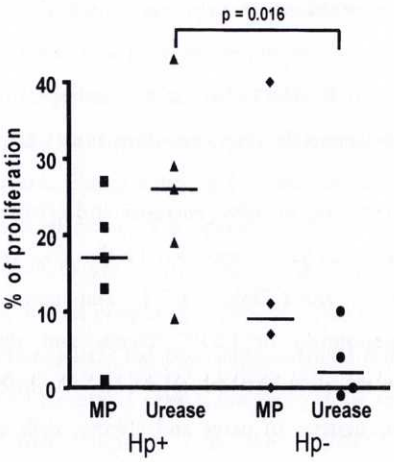

Figure 6. Enriched memory CD8+ T cells from *H. pylori* infected and non-infected were stimulated with B cells pulsed with *H. pylori* MP or urease. The frequency of proliferated CD8+ T cells were evaluated using flow cytometry.

Characterization of cytotoxic activity of H. pylori-reactive CD8⁺ T cells (paper III)

As we had shown that *H. pylori*-reactive $CD8^+$ T cells are present in the peripheral blood of *H. pylori*-infected individuals, we aimed to investigate whether or not these $CD8^+$ T cells possess cytotoxic activity. To this end, we tested different approaches to cytotoxic analysis. CFSE-stained PBMC were cultured in the presence of different *H. pylori* antigens, and analyzed using flow cytometry. This allowed simultaneous detection of IFN-γ and granzyme B production in individual cells proliferating in response to *H. pylori* antigens. Furthermore, the secretion of granzyme A and B by *H. pylori*-stimulated cells was studied using ELISA. Moreover, granzyme A and B secretion was also measured in cultures with enriched memory or effector $CD8^+$ T cells co-cultured with autologous *H. pylori*-pulsed B cells.

The method for cytotoxicity analysis outlined in this thesis is based on the fact that the most dominant mechanism of CD8-mediated cytotoxicity is the perforin /granzyme mediated killing. Cytoplasmic granules from NK cells and CTLs contain a pore forming protein, perforin and several homologous serine proteases called granzymes. Granzyme A and B are known to be present in the cytotoxic granules of NK cells and activated CTLs with cytotoxic potential. Thus a methodology, based on measurement of cell mediated cytotoxicity as a function of granzyme A or B release by effector cells, and simultaneous analysis of effector cell phenotype as well as viability in the same sample by flow cytometry under conditions that ensure reliable discrimination of target and effector cells would be ideal in monitoring immune response. Since production of IFN-γ is recognized as a hallmark function of cytotoxic cells, we also measured this simultaneously.

The method consists of two parts, flow cytometric (FACS) and ELISA assays. In the FACS-based part, the proliferation pattern of $CD8^+$ T cells was evaluated using CFSE; and the production of intracellular IFN-γ and/or expression of granzyme B measured using monoclonal antibodies. In the ELISA assays, the level of secreted IFN-γ, granzyme A and/or granzyme B were determined in supernatants.

The results showed that neither the intracellular levels nor the secretion of granzyme B by $CD8^+$ T cells was affected by *H. pylori* stimulation. In contrast, both granzyme A (Figure 7) and IFN-γ was secreted by *H. pylori*-stimulated cells.

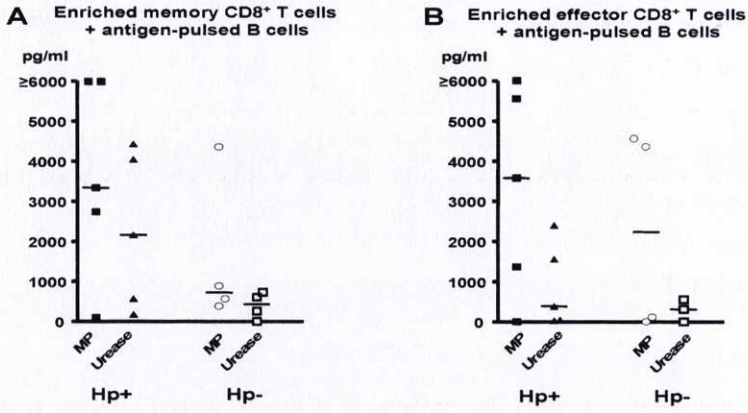

Figure 7. Granzyme A secretion after stimulation with *H. pylori*. Enriched memory or effector $CD8^+$ T cells *H. pylori*-infected and non-infected individuals were co-cultured with B cells pulsed with *H. pylori* MP or urease.

Analysis of supernatants for secreted granzyme A by memory $CD8^+$ cells stimulated with antigen-pulsed B cells showed that memory cells from *H. pylori*-infected individuals produce granzyme A, a marked indication for presence of *H. pylori*-specific CTLs

There are some hypotheses that may explain this. One possibility is that, for some unknown reason, granzyme B was not secreted at all, or it was secreted but its level was not detectable, despite the secretion of high levels of granzyme A. Another possibility rely on the fact that granzyme B induces rapid apoptosis. It is suggested that granzyme B may be produced early during incubation period and disappear during the 5 days incubation time. Rapid DNA fragmentation induced via granzyme B is supposed to be dependent on the activation of caspase(s) *(Darmon 1996; Anel 1997)*. Furthermore, it has been shown that the contribution of granzyme B to induce apoptosis is dictated by the quality of the target cell *(Pardo 2002)*. Our result may indicate that target cells in our system, for some unknown reasons, could not activate expression or secretion of granzyme B.

We have currently no definite proof that the granzyme A present in the supernatants was produced only by $CD8^+$ T cells, since both NK cells and $CD4^+$ T cells may also secrete granzyme A. However, $CD4^+$ T cells express lower levels of granzyme A than $CD8^+$ T cells (Figure 8). Furthermore, NK cells are more likely to be activated in the cultures without antigen-pulsed APC, and in these cultures lower levels of granzyme A were present (paper III).

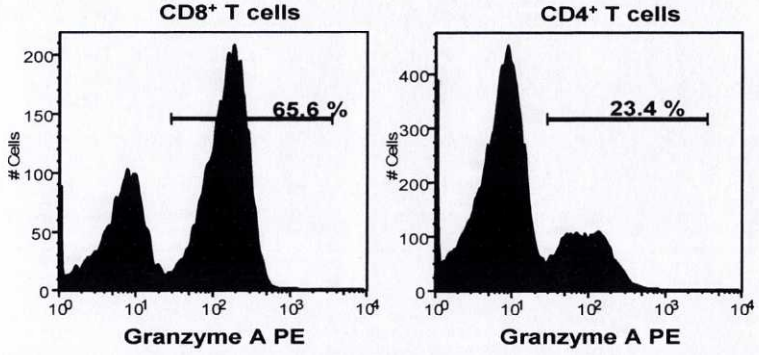

Figure 8. Granzyme A expression by peripheral blood T cells. PBMCs were stained for analysis of intracellular expression of Granzyme A, and analysed by flow cytometry. The T cells were identified by expression of CD3 and CD4 or CD8. The figure shows representative results from 6 experiments.

Moreover, we demonstrated that $CD8^+$ T cells can be activated by *H. pylori*-pulsed B cells (paper II), and on the single-cell level we show that *H. pylori*-activated $CD8^+$ T cells do produce IFN-γ (Figure 9). Since IFN-γ production is a marker for cytotoxic activity by $CD8^+$ T cells, it is therefore likely that at a major part of the granzyme A emanates from $CD8^+$ T cells.

Analysis of the frequency of intracellular IFN-γ producing $CD8^+$ T cells in a mixed lymphocyte population stimulated with MP or urease showed an increased production of IFN-γ among proliferating $CD8^+$ T cells of *H. pylori*-infected individuals in response to *H. pylori* antigens; this increase was absent in resting $CD8^+$ T cells in the same cultures, and also in the cultures of non-infected individuals. Interestingly, measurement of secreted IFN-γ in the supernatants by ELISA revealed no differences between infected and non-infected individuals. It is unclear to what extent total IFN-γ secretion correlates with IFN-γ

secreting by $CD8^+$ T cells; however, staining for intracellular IFN-γ and using FACS-based assay gives a more correct image of IFN-γ secreting $CD8^+$ T cells than ELISA-analysis in which the total amount of IFN-γ secreted by different cell populations is measured. Secretion of IFN-γ by $CD8^+$ T cells has been recognized as a typical function of CTLs as a result of their interaction with target cells *(Morris 1982)*. Therefore, enhanced secretion of granzyme A and increased expression of IFN-γ by proliferating memory $CD8^+$ T cells of *H. pylori*-infected subjects, after stimulation with B cells pulsed with *H. pylori* antigens, is a strong indication of potential cytotoxic activity of *H. pylori*-reactive $CD8^+$ T cells.

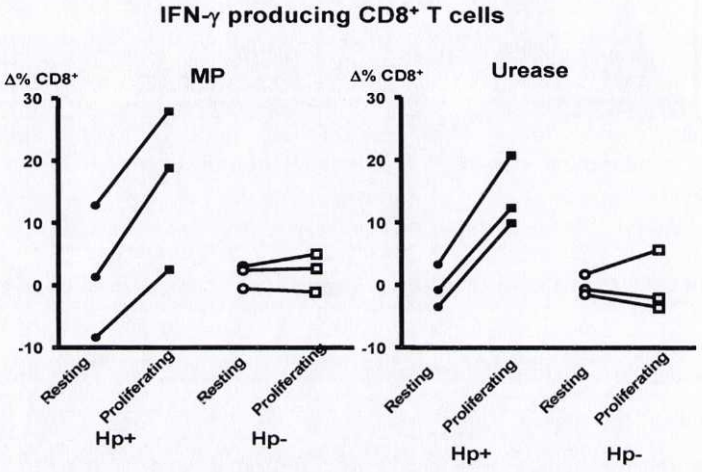

Figure 9. The frequency of IFN-γ producing $CD8^+$ T cells in the resting and proliferating populations from Hp^+ and Hp^- individuals are shown. CFSE-stained PBMCs from Hp^+ or Hp^- individuals were stimulated by MP or urease for 5 days and analysed by flow cytometry. The frequency of IFN-γ producing $CD8^+$ T cells without antigen stimulation has been subtracted.

To confirm direct cytotoxic function of *H. pylori*-reactive $CD8^+$ cells, we used both fresh PBMCs and CTL-lines as effector cells, co-incubated them with chromium-treated B cells pulsed with *H. pylori* MP and evaluated the cytotoxicity using chromium release assay. In both *H. pylori*-infected and non-infected individuals, cytotoxicity by CTL-lines was demonstrated. Fresh PBMCs also induced cytolysis in target cells, and this activity could be seen by cells from both the *H. pylori*-infected and the non-infected individuals (figure 10).

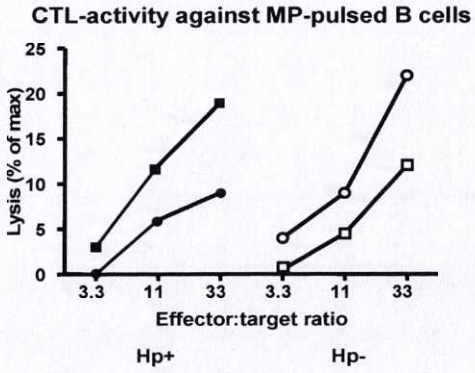

Figure 10. Direct cytotoxic activity *of H. pylori*-reactive cells were tested by co-incubation of chromium-treated MP-pulsed B cells with fresh PBMCs or CTL-lines, in different ratios, for 4 hours. The results show one Hp^+ and one Hp^- subject. No lysis of un-pulsed B cells was observed.

Killing of B cells pulsed with *H. pylori* MP by *H. pylori*-reactive $CD8^+$ T cells again confirm our hypothesis concerning the presence of effector *H. pylori*-reactive CTLs. However, to investigate which antigen specificity the *H. pylori*-reactive CTLs have and to what extent their responses differ in infected and non-infected individuals, larger numbers of subjects need to be analyzed.

In conclusion our data support a scenario in which at least some *H. pylori*-reactive $CD8^+$ T cells in Hp^+ subjects possess a cytotoxic function. We believe that these findings may be of importance for the further studies of CTL-activity in different categories of *H. pylori*-infected patients, with special emphasis on CTL-activity in relation to protection against *H. pylori*-induced gastric cancer development.

Concluding remarks

Natural *H. pylori*-infection fails to yield protective immunity, the infection is often acquired during early childhood, re-infection is very common, and current treatment strategies are associated with significant side effects, high cost and emergence of resistance. Therefore, it is necessary to find effective alternative ways to eliminate or eradicate an established *H. pylori*-infection. Evidently, immune-based strategies require a broad knowledge about the immune response to *H. pylori*. So far, studies have demonstrated that T cells and IFN-γ are key components of the immune responses to *H. pylori (Eaton 2001; Akhiani 2002)*; however, the role of NK cells and $CD8^+$ T cells have previously been poorly understood.

Presence of activated IFN-γ producing *H. pylori*-reactive $CD8^+$ in mucosa and blood of *H. pylori*-infected individuals *(Hood and Lesna 1993; Ihan 1995; Agnihotri 1998; Quiding-Jarbrink 2001; Bedoya 2003)* suggested that $CD8^+$ T cells also contribute to the immune response to *H. pylori*. The demonstration that *H. pylori* insert the virulence factor cagA into epithelial cells through a type IV secretion system *(Odenbreit 2000; Higashi 2002)* and that the bacteria can reside both inside gastric epithelial cells *(Kwok 2002)* and in the gastric lamina propria *(Jhala 2003)* focused our attention to cytotoxic-cell responses to *H. pylori*, However, previous studies have reported difficulties in detecting and evaluating *H. pylori*-specific T-cell responses in peripheral blood of *H. pylori*-infected individuals. It has been shown that peripheral T cells from non-infected individual also respond equally or even better than *H. pylori*-infected individuals to *H. pylori* antigens *in vitro (Karttunen 1990; Birkholz 1993); (Fan 1994; Sharma 1994; Ren 2000; Quiding-Jarbrink 2001; Lundgren 2003)*.

Our experiments revealed that the optimal activation of *H. pylori*-reactive $CD8^+$ T cells responses are achieved in the presence of APCs and $CD4^+$ T cells. The use of thymidin incorporation assay and ELISA when characterizing the profiles of *H. pylori*-reactive $CD8^+$ T cells in a mixed cell population was not a viable option; these assays only provide information on the overall proliferative response and cytokines production and gives no insight into specific cell subsets involved. Therefore, we evaluated the $CD8^+$ T-cell responses on an individual cell basis. Simultaneous detection of $CD8^+$ T-cell proliferation, IFN-γ production, and granzyme expression by single cells, and evaluation of the secreted IFN-γ

and granzymes in supernatants, gave a more accurate insight about T-cell responses to *H. pylori* antigens. Furthermore, the ability to study the cytokine production of resting or proliferating cell subsets, on an individual cell basis, finally made it possible to reveal the differences in the adaptive immune response to *H. pylori* antigens in *H. pylori*-infected and non-infected individuals.

Furthermore, we could show that B cells can be used as efficient antigen-presenting cells for activation of *H. pylori* antigen-reactive $CD8^+$ T cells, *in vitro*. This finding together with the fact that *H. pylori*-infected individuals have an increased number of B cells in gastric mucosa *(Mattsson 1998; Mattsson 1998)* suggest that B cells may also act as APCs for $CD8^+$ T cells *in vivo*.

Our results show that some *H. pylori*-reactive $CD8^+$ T cells in *H. pylori*-infected subjects possess a cytotoxic function. Furthermore, our data show that NK cells are present in the gastrointestinal mucosa and can be activated to produce IFN-γ by *H. pylori* components in synergy with IL-12. These are important findings, and improve our understanding for immune response to *H. pylori* infection.

Based on our results as well as previously published data, we speculate that components of bacteria that reside in the gastric lumen of the host are recognized by NK cells early in *H. pylori* infection. This may leads to IFN-γ production by NK cells. Epithelial derived chemokines and cytokines activate macrophages and induce IL-12 production. IL-12 and components from *H. pylori* lead to a synergistic activation of NK cells, enhancing the IFN-γ production. Furthermore, IL-12 promotes Th1 polarization of infiltrated $CD4^+$ T cells; resulting to Th1 differentiation; leading to more IFN-γ production by $CD4^+$ T cells.

Bacterial antigens in the presence of Th1 cytokines activate $CD8^+$ T cells that may act as cytotoxic cells. This cytotoxic activity may be directed against epithelial cells into which *H. pylori* antigens have been injected through the type IV secretion system, and possibly against B cells or other APCs in the gastric lamina propria, which have been ingesting *H. pylori* antigens. The killing of "*H. pylori*-modified" epithelial cells may either have an enhancing effect on the development of gastric cancer, due to increased apoptosis and cell

turnover of the epithelial cells, or alternatively the killing may have a protective effect, through increased killing of potentially abnormal cells.

In conclusion, this thesis reveal the early influence of NK cells and IFN-γ in the immune response to *H. pylori* infection and support a scenario in which both antigen-specific and non-specific cytotoxic effector cells are involved. We believe that these findings may be of importance for the further study of NK- and CTL-activity in different categories of *H. pylori*-infected patients, with special emphasis on the relation to protection against *H. pylori*-induced gastric cancer development.

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