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THE MUCOSAL IMMUNE RESPONSE AGAINST *HELICOBACTER PYLORI* INFECTION

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To Jing and Haoran

To my parents

ABSTRACT

H. pylori infection affects more than half of the world's population. The clinical consequences range from asymptomatic gastritis to peptic ulceration and gastric malignancy. *H. pylori* infection triggers strong humoral and cellular immune responses, both systemically and locally in the gastric mucosa. These responses, however, do not result in eradication of the bacteria.

The aims of our studies were to identify genes associated with *H. pylori*-induced inflammation in the human and mouse gastric mucosa and to investigate the mechanism underlying vaccination-induced protection.

We have studied the gene expression profiles in gastric tissue in 12 *H. pylori*-infected patients and 9 *H. pylori*-negative controls using cDNA arrays. We found a remarkably consistent pattern of genes differentially expressed in normal and *H. pylori*-infected gastric mucosa and identified a "Helicobacter-infection signature", which includes a set of genes encoding Toll-like receptors, adhesion molecules, metalloproteinases, chemokines and interleukins.

We subsequently studied the role of one of the genes identified in the first study, matrix metalloproteinase-9 (MMP-9), in *H. pylori*-induced gastritis. We demonstrated a large, on average 19-fold, increase of MMP-9 expression in the antrum of *H. pylori* infected patients, as compared to controls. In contrast, there was no difference in the levels of tissue inhibitors of metalloproteinases (TIMP)-1 or -2 between the groups. The increase in MMP-9 activity in the gastric mucosa, was probably caused by tissue-resident macrophages. The net increase in gastric MMP activity is likely to contribute to tissue damage in *H. pylori*-associated gastritis.

The inflammatory gene expression profiles in three groups of patients infected with triple-negative strains (lacking *CagA*, *BabA2* and *VacAs1*, but expressing *VacAs2*), double-positive strains (type I strains, expressing *CagA* and *VacAs1*) and triple-positive strains (expressing *CagA*, *VacAs1* and *BabA2*) were subsequently investigated. The gene expression pattern in the antrum mucosa from patients infected with different *H. pylori* strains was similar and no differentially expressed genes could be identified by pair-wise comparisons, suggesting a lack of correlation between the host inflammatory responses in the gastric mucosa and expression of the *BabA2, CagA and VacAs1* genes.

We have also explored the potential mechanism underlying the BabA-induced protection by gene expression profiling in antrum gastric mucosa in a set of naïve, unimmunized/challenged and immunized/challenged mice. The protection was neither associated with an altered Th1/Th2 cytokine expression pattern, nor with an up-regulation of expression of adipocyte-specific cytokines. Severe gastric inflammation was not associated with protection. Furthermore, we could not identify any gene that was specifically induced by the immunization procedure, as the putative "immunization signature" identified was part of the "*H. pylori*-infection signature". Thus, the specific immune response elicited is most likely underlying the protective mechanism.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals I-IV:

I. **Wen S**, Felley CP, Bouzourene H, Reimers M, Michetti P and Pan-Hammarström Q. Inflammatory gene profiles in gastric mucosa during *Helicobacter pylori* infection in humans. J Immunol. 2004.172 (4): 2595-606.

II. Bergin PJ, Edebo A, **Wen S**, Johnsson E, Andersson J, Lönroth H, Michetti P, Pan-Hammarström Q and Quiding-Järbrink M. Increased production of matrix metalloproteinases in *Helicobacter pylori*-associated human gastritis. Helicobacter. 2004. 9 (3): 201-10.

III. **Wen S**, Hultberg A, Borén T, Hammarström L and Pan-Hammarström Q. Gene expression profiles in gastric mucosa in mice immunized with BabA and challenged with *Helicobacter pylori.* 2007. Submitted.

IV. **Wen S**, Michetti P, and Pan-Hammarström Q. Correlation of *Helicobacter pylori* genotypes with inflammatory gene expression profiles in human gastric mucosa. 2007. Manuscript.

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LIST OF ABBREVIATIONS

BabA	Blood group antigen binding adhesin
cag PAI	cag pathogenicity island
CagA	Cytotoxin-associated gene A
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FDR	False discovery rate
Grb-2	Growth factor receptor bound 2
H. pylori	*Helicobacter pylori*
LCM	Laser capture microdissection
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MM	Mismatch
MMPs	Matrix metalloproteinases
NF-κB	Nuclear factor-κB
PM	Perfect match
SabA	Sialic acid binding adhesin
SAM	Significance analysis of microarrays
TIMPs	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
VacA	Vacuolating cytotoxin A

1 GENERAL INTRODUCTION

1.1 History

Spiral-shaped bacteria were observed both in human and animal stomachs as early as the end of the 19th century. However, these bacteria were thought to be a result of contamination rather than being gastric colonizers or pathogens involved in gastric diseases. It was not until April 1982, that this spiral-shaped bacterium was successfully isolated and cultured from human gastric biopsies by Barry Marshall and Robin Warren. After 34 failures, these spiral-shaped bacteria were finally cultured on plates that had been left by accident in the incubator over the Easter weekend (extending the incubation period from two to six days). They later demonstrated a relation between this spiral bacterium and inflammation of the gastric mucosa and peptic ulcer (1-3). The bacterium was initially called *Campylobacter pyloridis*, then renamed as *Campylobacter pylori* (4) and later as *Helicobacter pylori* (*H. pylori*) (5). The findings of Marshall and Warren had an enormous impact. Peptic ulceration, a disease of worldwide occurrence whose definitive treatment was surgical, became a disease that could be treated and cured with antibiotics. Moreover, the discovery led to recognition of *H. pylori* as a major cause of gastric cancer and the bacterium was subsequently designated as a Class I carcinogen by the World Health Organization (6). In 2005, Marshall and Warren were awarded the Nobel Prize in Physiology or Medicine for their "discovery of the bacterium *H. pylori* and its role in gastritis and peptic ulcer disease". The milestones in research on *H. pylori* and related diseases are summarized in Table 1 (7-10).

1.2 *H. pylori* associated diseases

1.2.1 Gastritis

The normal human stomach is a relatively sterile organ and devoid of inflammatory cells. *H. pylori* colonization results in inflammation in virtually all infected individuals. The epithelial response to infection includes mucin depletion, cellular exfoliation, regenerative changes and an accompanying hypochlorhydria (11). Following *H. pylori* infection, an acute inflammatory response develops which is characterized by infiltration of polymorphonuclear leukocytes into the gastric mucosa. If the initial host

Table 1. Milestones in research on *H. pylori* and related diseases

Years	Events
1586	Pnatus described gastric ulcer.
1688	Muraults observed duodenal ulcers.
1875	Bottcher and Letulle found spiral bacteria in animal stomachs.
1889	Jaworski described spiral organisms in human gastric washing.
1905	Reigel suggested that ulcers are caused by excess acid.
1906	Krienitz found spiral bacteria in human cancerous stomachs.
1910	Schwartz published the excess acid theory of the ulcer, "no acid, no ulcer."
1915	Antacids were first recommended for the treatment of peptic ulcer disease.
1924	Luck and Seth discovered urease in the human stomach.
1951	Allende first described the treatment of gastric ulcers with penicillin.
1982	*April 8* - first successful culturing of *H. pylori*.
1983	Marshall and Warren identified Campylobacter like bacteria associated with gastritis - beginning of the modern era.
1990	Rauws and Tytgat described cure of duodenal ulcer by eradication of *H. pylori* using a triple-therapy regimen.
1990	World Congress of Gastroenterology recommended eradicating *H. pylori* in order to cure duodenal ulcers.
1992	Covacci *et al*. sequenced the *CagA* gene. This was the first description of a virulence factor of *H. pylori* determined by molecular techniques.
1993	Bazzoli *et al*. started the proton-pump inhibitor-based triple therapy.
1994	Michetti *et al*. tested a urease-based vaccine in a mouse model.
1994	The World Health Organization's International Agency for Research on Cancer declared *H. pylori* as a class 1 carcinogen.
1997	Tomb *et al*. sequenced the entire *H. pylori* genome.
1998	Ilver *et al*. identified BabA.
1999	Michetti *et al*. tested urease vaccine in humans (although it failed).
2005	Marshall and Warren were awarded the Nobel Prize in Physiology or Medicine for their work on *H. pylori* and peptic ulcer disease.

response fails to clear the infection, lymphocytes, plasma cells and macrophages gradually accumulate in the gastric mucosa and secrete a wide range of cytokines, including IL-1, IL-6, IL-8 and TNF-α (11, 12). Among those, IL-8 is one of the important chemokines for recruitment of inflammatory cells (12). Continuous inflammatory reaction in the gastric muocsa eventually leads to a histological picture of active chronic gastritis. The persistent colonization by *H. pylori* and the associated chronic active gastritis can lead to development of gastric ulceration and gastric cancer. An antrum-predominat gastritis and a high acid secretion predispose for duodenal ulcer, whereas pangastritis or corpus- predominant gastritis and a lower acid secretion rate are considered risk factors for gastric ulcer and gastric cancer (11, 13).

1.2.2 Peptic ulcer

Peptic ulcers are defects in the integrity of the mucosa with a diameter of at least 0.6 cm, penetrating through the muscularis layer (14). Gastric ulcers mostly occur along the lesser curvature of the stomach and duodenal ulcers usually occur in the duodenal bulb. *H. pylori* has been found to be present in 90-100% of patients with duodenal ulcers and in 60-100% of patients with gastric ulcers (14). Infected individuals have 3 to 10 times higher risk for the development of duodenal or gastric ulcers than non-infected subjects and 15-20% of *H. pylori*-positive subjects developed ulcer disease during long-term follow-up (14, 15). Moreover, after eradication of the infection, the risk of recurrence of ulcer disease is dramatically reduced (16, 17), clearly suggesting that the development of duodenal and gastric ulcer disease is strongly associated with *H. pylori* infection. Duodenal ulcers are often associated with excess gastric acid secretion. The increased amount of acid induces gastric metaplasia in the duodenum, leading to inflammation and ulceration (11, 13, 18). In contrast, the pangastritis or corpus-predominant gastritis leads to an ongoing proliferation of gastric epithelial cells and progressive loss of gastric glands. These atrophic changes are associated with gastric ulcers and non-cardia gastric adenocarcinomas (11, 13, 18).

1.2.3 Gastric cancer

Gastric cancer is the second most frequent cause of cancer-related death after smoking-related lung cancer and the third most common cancer after lung and breast cancer. Since the discovery of *H. pylori* in 1982, there have been many studies suggesting that *H. pylori* increases the risk of gastric cancer (19-21). In 1994, the International Agency for Research on Cancer (IARC) identified *H. pylori* as a first class carcinogen with an

etiological role in the development of gastric cancer (6). Later, much more evidence has accumulated, including results from animal models (22). *H. pylori* can be detected in 70-90% of patients with gastric adenocarcinoma (23, 24). About 1-2% of *H. pylori*-infected individuals develop adenocarcinoma (24, 25) and *H. pylori* infection is estimated to increase the cancer risk 8 to 9 fold (24). Eradication of *H. pylori* decreases the incidence of gastric cancer in infected subjects (26). However, the effects of *H. pylori* on tumor development vary by anatomical site. A very recent, large sample sized, long-term prospective case-control study, has shown that *H. pylori* is a strong risk factor for noncardia gastric cancer but is inversely associated with the risk of gastric cardia adenocarcinoma (27), thus confirming previous reports (28-30). A number of studies have also shown that infection with *H. pylori* may reduce the risk of esophageal adenocarcinoma (29, 31). However, it has been reported that infection with CagA-positive strains of *H. pylori* may increase the risk for esophageal squamous-cell carcinoma (32). Thus, the benefits of eradication of *H. pylori* still need to be investigated further.

Gastric mucosa-associated lymphoid tissue (MALT) lymphoma (non-Hodgkin's gastric lymphoma) represents a small percentage (3%) of gastric malignant diseases (33). This type of B cell lymphoma is thought to arise from lymphoid follicles that develop due to *H. pylori* infection in the lamina propria of the stomach. Patients with gastric lymphoma were also shown to have been infected with *H. pylori* more often than matched controls (33, 34). Furthermore, eradication of the *H. pylori* resulted in a complete regression of low-grade gastric lymphomas in a large proportion of the patients (35, 36). These data suggest a strong association between *H. pylori* and development of MALT lymphoma.

1.3 *H. pylori* virulence factors

1.3.1 Vacuolating cytotoxin (VacA)

One of the major virulence factors of *H. pylori* is the vacuolating cytotoxin (VacA), which was initially found to induce formation of large cytoplasmic vacuoles in cultured eukaryotic cells (37). VacA is secreted as a soluble protein into the extracellular space or remains localized on the surface of *H. pylori* (38, 39).

The *VacA* gene is present in virtually all *H. pylori* strains, however, genetic variability in the *VacA* gene results in a different cytotoxic properties. Two diversified regions in the gene have been identified (40): one in the signal peptide coding region (allelic types s1a, s1b, s1c and s2) and the other in the mid-region which encodes part of the 58-kDa domain of VacA (allelic types m1, m2a and m2b). *H. pylori* strains that possess a type s1/m1 *VacA* allele enhance gastric epithelial cell injury (41) and are associated with an increased risk of peptic ulcer disease and gastric cancer (42), when compared to strains expressing *VacA* s2/m2. However, there are some reports suggesting that neither the presence of *VacA*, nor its subtypes shows any disease specificity (43, 44).

In addition to vacuolation, it was previously found that VacA induced apoptosis in gastric epithelial cells, which may play an important role in the development of ulcer disease and gastric cancer (45). Furthermore, a high concentration of VacA has also been shown to suppress T-cell proliferation and activation *in vitro*, and thus results in immunosuppression, although the mechanism is still not clear (46-48). One recent study has shown that the direct inhibition of T-cells by *H. pylori* is not mediated by VacA (49).

1.3.2 Cag pathogenicity island and *CagA*

The cag pathogenicity island (cag PAI) is a 40 kilobase segment of DNA, which contains 31 genes (50-52) and carries a set of genes with homology to type IV secretion systems (53). Approximately 60% of *H. pylori* strains isolated in Western countries carry the cag PAI, and almost all of the East Asian isolates are cag PAI-positive. Cytotoxin associated antigen (CagA), is an immuno-dominant protein, encoded by one of the genes (*CagA*) within the cag PAI. It is commonly used as a marker for the entire cag locus. Strains expressing *CagA* and *VacAs1* are present at a higher frequency in patients with duodenal ulcer, atrophic gastritis and gastric carcinoma (41, 54, 55), and are referred to as type I strains. In contrast, type II strains, which lack the *CagA* gene, present a nontoxic form of VacA and are considered less virulent (56, 57).

The molecular basis for the pathogenic activity of CagA on gastric epithelial cells has been, in part, elucidated. CagA has been shown to be translocated via a type IV secretion system into the membrane fraction of gastric epithelial cells (58). Once inside the gastric epithelial cell, CagA is phosphorylated on tyrosine residues and binds to SHP-2 tyrosine phosphatase, resulting in morphological changes with cytoskeletal

rearrangements and elongation (59). However, it has also been shown that the unphosphorylated CagA could interact with growth factor receptor bound 2 (Grb-2), leading to induction of a similar response in host cells (60).

In vitro studies have shown that several genes within the cag PAI, including *CagE*, C*agG*, *CagH*, *CagI*, *CagL* and *CagM*, are required for induction of production of proinflammatory cytokines such as IL-8 from gastric epithelial cells, whereas the *CagA* gene itself is not required (52, 61-63). Inactivation of these genes not only attenuates IL-8 expression but also results in decreased expression of NF-κB and mitogen-activated protein kinase (MAPK) which regulate proinflammatory cytokine production (62, 64, 65). These *in vitro* observations mirror *in vivo* events, as strains carrying only parts of the cag PIA, whether CagA positive or negative, are less likely to be associated with severe gastroduodenal pathology (66). The classification of strains into type I and type II should thus probably be based on presence of the entire cag PAI rather than the *CagA* gene alone (66).

1.3.3 Adhesins

Adherence is an important contributor to bacterial virulence. The intimate attachment may facilitate *H. pylori* colonization and efficient delivery of virulence factors such as VacA or CagA to the host cells, resulting in a severe gastric inflammation. However, tight adherence may also be harmful for the bacteria, when strong host responses are induced.

1.3.3.1 Blood group antigen binding adhesin (BabA)

The blood group antigen binding adhesin (BabA), encoded by the *BabA2* gene, has been shown to mediate adherence of *H. pylori* to the Lewis b blood group antigen on human gastric epithelial cells (67-69). Analysis of the *BabA* gene has shown that there are two *BabA* alleles (*BabA1* and *BabA2*) and one highly homologous gene, *BabB* (69, 70). However, only the *BabA2* gene is functionally active. The only difference between *BabA1* and *BabA2* is that the former lacks a 10-bp insertion, encoding a signal peptide, which results in the *BabA1* gene being silent (69). However, *H. pylori* was found to be capable of altering expression of its adhesins during infection. The expression of the *BabA* gene can be modulated (switch from “on to off ”or from “off to on”) through a recombination event between *BabA* and *BabB* or by a change in the number of CT

dinucleotide repeats in the 5' region during infection (71-74). Therefore, the ability to modulate adhesion may contribute to the adaptation to the gastric environment and persistent colonization in the stomach.

It has previously been shown that *BabA2* positive strains, particularly when present together with *CagA* and *VacAs1* (referred to as triple-positive strains) are more often associated with duodenal ulcer and adenocarcinoma than double-positive strains (75). Moreover, the triple positive strains are detected more frequently in patients with severe histological alterations (76-78). These results suggest that BabA may play an important role in the pathophysiology of *H. pylori* infection.

1.3.3.2 Sialic acid binding adhesin (SabA)

The other well-characterized *H. pylori* adhesin-receptor interaction is between the sialic acid binding adhesin (SabA) and the sialyl-Lewis x or a antigens (79). The levels of sialyl-Lewis x or a are low in the healthy gastric mucosa. It has been shown that persistent colonization of the human stomach with *H. pylori* could induce chronic inflammation and expression of sialyl-Lewis x or a antigens in the gastric mucosa (79, 80). The intimate adherence mediated by SabA binding to sialyl-Lewis x or a, may lead to an enhanced inflammatory response. Once the host inflammatory responses become too strong, the expression of SabA may be switched off, allowing the bacteria to escape from the intimate contact with the inflamed epithelium, assuring long-term persistence of the infection (79).

In addition, SabA was found to mediate binding of *H. pylori* to sialylated structures on neutrophils, which is a prerequisite for the nonopsonic activation of human neutrophils (81). A very recent report shows that *H. pylori* can be found on erythrocytes in capillaries and post-capillary venules in the gastric mucosa of infected humans and Rhesus monkeys. The binding of *H. pylori* to red blood cell is mediated by SabA (82). These results indicate that the bacteria may disseminate into the circulation by way of gastric mucosal capillaries.

As the adhesin-receptor recognition interaction between *H. pylori* and gastric mucosal tissue plays a critical role in bacterial colonization and persistence of infection, a vaccine strategy based on adhesins such as BabA or SabA may be used to target the infection.

1.4 Host responses to *H. pylori*

Mechanisms of host defense against invading microbial pathogens consist of innate immunity, which provides the initial non-specific defense against infection, and adaptive immunity, which develops the specific, more effective defense against infection (83). *H. pylori* infection induces both strong innate and adaptive immune responses (84, 85). These responses, however, do not result in eradication of the bacteria in a majority of cases and under certain circumstances, may actually contribute to the pathogenesis of the diseases (86-88).

1.4.1 Innate immunity

The principal components of innate immunity in stomach include an acid environment, mucus overlaying the gastric epithelium, an intact gastric epithelium, a variety of soluble factors such as complement and lysozyme, phagocytes and natural killer cells (83, 89). Many studies have shown that the initial colonization of *H. pylori* induces production of different proinflammatory mediators by the gastric epithelial cells, leading to subsequent recruitment of monocytes/macrophages (11, 12, 90, 91).

Toll-like receptors (TLRs) have recently been found to play an essential role in the innate immune system by recognition of microbial components (92). Both gastric epithelial cells and phagocytes express TLRs. Individual TLRs recognize distinct structural components of pathogens, thus, by this way TLRs mediate production of proinflammatory cytokines and chemokines, resulting in inflammation (93, 94). TLR2 is essential in the recognition of microbial lipopeptides and peptidoglycans from gram-positive bacteria. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides respectively, whereas TLR4 recognizes lipopolysaccharide (LPS), the major outer membrane component of gram-negative bacteria. TLR3 is implicated in the recognition of viral dsRNA, whereas TLR7 and TLR8 are implicated in the recognition of viral-derived ssRNA. TLR5 recognizes flagellin. TLR9 is essential in the recognition of CpG DNA (93, 94).

Several studies have shown that TLR4 is involved in the response to *H. pylori* LPS or infection in the human gastric mucosa, gastric epithelial cell lines (AGS and MKN45) or guinea pig gastric pit cells (95-97). However, recent evidence has suggested that *H.*

pylori infection activates innate immunity via TLR2 or TLR5 rather than TLR4 (98-101). As *H. pylori* is a gram-negative bacterium, recognition of bacteria should theoretically be mediated by TLR4. Studies have shown that *H. pylori* contains two types of lipid A, a major tetraacyl and a minor hexaacyl variety with exceptional fatty acid chains. The tetraacyl lipid A structure of *H. pylori* is very similar to that of *Porphyromonas gingivalis* which is recognized by TLR2 (102, 103). Thus, induction of an immune response by *H. pylori* LPS might be mediated by TLR2. Regarding the role of TLR5 signalling by *H. pylori* flagellins, studies have shown that the latter have a very low capacity to stimulate immune response mediated by TLR5 as compared to other bacteria (104-106). In summary, TLRs seem to play only a minor role in the induction of innate immune responses against *H. pylori* (101, 104, 107).

1.4.2 Adaptive immunity

1.4.2.1 Antibody responses

Earlier studies have shown that not only increased specific IgG in serum, but also high levels of *H. pylori* specific IgM and IgA could be detected in the gastric mucosa of *H. pylori* infected patients (108-111). Specific IgA antibodies against VacA and urease in maternal milk delay *H. pylori* colonization in infants (112, 113). *In vitro* studies have shown that binding of IgG to *H. pylori* promotes phagocytosis and killing by polymorphonuclear leukocytes (114). Thus, specific antibodies might play a role in limiting *H. pylori* infection. However, the systemic and local mucosal humoral responses do not result in eradication of *H. pylori* and may even contribute to the pathogenesis of *H. pylori* infection by induction of anti-Lewis$^{x/y}$ antibodies which recognize parietal cells, resulting in gastritis (87, 88, 115).

1.4.2.2 T cell responses

There are very few T cells in healthy stomach tissue. *H. pylori* infection induces both $CD4^+$ and $CD8^+$ T cell responses in gastric mucosa (116). T cells isolated from infected gastric tissue produce Th1 cytokines such as IL-12, INF-γ and TNF-α but rarely Th2 cytokines (IL-4 and IL-5), suggesting a Th1 predominance in the infected gastric mucosa (116-118). The severity of *H. pylori* induced gastritis correlated with mucosal expression of INF-γ and TNF-α (119). Although the Th1 response was inadequate to clear *H. pylori* infection, it is important for development of the inflammatory response,

as mice deficient in T cells or IFN-γ develop little or no gastritis upon *H. pylori* infection. For instance, the number of Helicobacter colonizing the stomachs of IFN-γ knockout C57BL/6 mice was higher than that of wild-type mice, and without an accompanying inflammatory reaction in the gastric mucosa (120, 121). In contrast, a more severe gastric inflammation and a lower level of colonization of bacteria was observed in IL-4 knockout C57BL/6 mice, as compared to wild type mice (121, 122). These results demonstrate that Th1 type responses (e.g. IFN-γ) may play an important role in protection against Helicobacter infection. Th2 type responses (e.g. IL-4) on the other hand, could limit the pathological consequences of infection. In addition, recent findings suggest that regulatory T cells ($CD4^+$ $CD25^+$) may suppress T cell immune responses to *H. pylori* and thereby contribute to the persistence of the infection (123-125).

The sustained Th1 response cannot eradicate the infection and may lead to pathogenic effects. The increased production of IL-12, IFN-γ and other proinflammatory cytokines leads to stimulation of epithelial cells, macrophages and polymorphonuclear cells to release inflammatory mediators which may in turn lead to tissue lesions (126, 127). Furthermore, TNF-α, IFN-γ and IL-1β up-regulate the expression of gastric mucosal Fas antigen, resulting in apoptosis of epithelial cells (128, 129).

1.4.3 Matrix metalloproteinases in *H. pylori* infection

The extracellular matrix (ECM) plays an important role in tissue architecture and homeostasis. ECM of the gastric mucosa is composed of a number of macromolecules such as collagen, laminin, proteoglycan, elastin, fibronectin and hyaluronic acid. The ECM of the gastric wall forms a physical barrier against cell migration and the movement of large macromolecules. Matrix metalloproteinases (MMPs) are a family of zinc-dependent metalloendopeptidases that are able to degrade ECM components. MMPs consist of more than 24 members in humans. Based on substrate specificity, MMPs can be broadly classified into collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -7, -10 and -11), elastases (MMP-12) and membrane-type MMPs (MT-MMPs, MMP-14, -15, -16, -17, -24 and -25) (130). MMPs are mostly secreted as inactive precursors, or zymogens. Activation of MMPs requires proteolytic removal of the propeptide domain (131). Most of the MMPs are activated outside the cell by other activated MMPs and serine proteases (132).

MMP activity is tightly regulated at different levels (131): at the level of transcription, at the point of activation from the precursor zymogens, by interaction with specific ECM components and by tissue inhibitors of metalloproteinases (TIMPs). Four kinds of TIMPs (TIMP-1, -2, -3 and -4) are known to bind to MMPs in a 1: 1 stoichiometric ratio and reversibly block MMP activity (133, 134). If the production of MMPs exceeds what can be regulated by TIMPs, there will be an imbalance in the ECM breakdown and repair system.

One of the main functions of MMPs is cleavage of structural ECM constituents. Many studies have shown that MMP substrates also include non-ECM molecules such as cytokines, growth factor binding proteins or receptors and cell surface adhesion molecules (135-138). For instance, cleavage of IL-8 by MMP-9 results in an increase in chemotactic activity (139). Conversely, monocyte chemoattractant protein-3 (MCP-3), becomes inactive when cleaved by MMP-2 (140). Thus, MMPs are not only involved in tissue remodelling, wound healing and malignant cell migration (141-143), but also in modulation of inflammation and immunity. Studies have shown that migration of T cells and dendritic cells is associated with gelatinases (144-146). The transendothelial migration of lymphocytes across high endothelial venules into lymph nodes is also affected by MMPs (147). Therefore, by degrading components of the ECM and by modulating cytokine and chemokine activity, MMPs play an important role in the successful execution of inflammatory reactions. However, excess MMPs activity following infection may lead to tissue damage and favours pathogen dissemination or persistence.

It has previously been shown that MMP-2 and MMP-9 activtiy may be important in gastric ulcer formation (148). An increasing amount of evidence suggests that *H. pylori* infection can influence the level of MMPs (MMP-1, -2, -3, -7 and -9), but the mechanisms involved are largely unknown (149-154). Some studies have shown that induction of MMP-7 and MMP-9 by *H. pylori* infection is regulated by activation of the NF-κB and extracellular signal-regulated kinase (ERK) pathways respectively and this induction is associated with cag PAI positive *H. pylori* strains (150, 154). A very recent study has however shown that up-regulation of MMP-2 and MMP-9 secretion due to *H. pylori* infection is independent of the cag PAI (152). The role of MMPs in *H. pylori* induced gastritis thus needs to be further investigated.

1.5 Immune response to *H. pylori* immunization

The immune response induced by natural infection does not confer protection. However, since the first successful Helicobacter vaccine based on urease was described (155), a number of studies have shown that immunization with whole-bacteria lysates or purified antigens (VacA or CagA), in the presence of an appropriate mucosal adjuvant, provides protection against Helicobacter infection in mouse models (155-161).

1.5.1 The role of antibodies in *H. pylori* immunization

In several animal models, protective effects have been associated with elevation of specific IgA and IgG antibody levels (162-164). Protection of germ-free mice from Helicobacter infection by passive IgA immunization has also been described (165). However, IgA deficient patients do not have an increased susceptibility to infection as compared to controls (166). More importantly, protection against Helicobacter infection could also be achieved by prophylactic or therapeutic vaccinations in B cell deficient mice (μMT mice), suggesting that antibodies may not be required (167-169).

1.5.2 The role of T cell responses in *H. pylori* immunization

It has previously been suggested that protection was associated with recruitment of $CD4^+$ and $CD8^+$ T cells in the gastric mucosa (160, 170). Moreover, studies using MHC class I and II knockout mice that are deficient in $CD8^+$ or $CD4^+$ T cells, have shown that protection against *H. pylori* infection required $CD4^+$ T cells rather than $CD8^+$ cells (168, 171). A Th2 response induced by immunization has been proposed to be important for achieving protection against *H. pylori* infection. In a mouse model of *H. felis* infection, passive transfer specific Th2 cells from donor mice immunized against *H. felis* into infected recipient mice resulted in reduction of the bacterial load, whereas transfer of Th1 cells had no effect on bacterial colonization after challenge (172). In addition, successful therapeutic immunization has also shown to be associated with a switch from a Th1 to a Th2 response after immunization (173). However, other studies have shown that a specific Th1 response was important for protection (174-177). These observations indicate that both Th1 and Th2 responses might mediate protection. However, studies based on cytokine knockout mice have shown that IL-4, IL-5, IL-13, IL-12, IL-18, TNFα and IFN-γ, all appear to be dispensable for the

protective mechanism (122, 178-180). Thus, the role of Th1 and Th2 immune responses in protective immunity against Helicobacter infection remains elusive.

As protection against *H. pylori* infection was observed in immunized IL-12 deficient BALB/c mice, but not in immunized IL-12 deficient C57BL/6 mice (122, 180), the role of cytokines may vary in mice with different genetic backgrounds.

1.6 Microarray

Microarray technology was first introduced by Schena and colleagues in 1995 (181). As this technology is a tremendously powerful method for simultaneously monitoring the expression of thousands of genes in a single experiment, it has become widely applied in biological and medical research, including the discovery of drug-sensitive genes (182), development of biomarkers for classification of disease subgroups and monitoring of disease progression (183-185). For example, using micrarray analysis, Weigelt et al found a set of gene markers that can be used as a more accurate predictor for breast cancer mortality than a set of National Institutes of Health-defined clinical guidelines (186).

There are two major types of microarray platforms that are commonly used, cDNA arrays and oligonucleotide arrays. cDNA (200~5,000 bases long) is immobilized onto a solid surface such as a glass slide or nylon membrane. Probes for cDNA arrays are usually products of polymerase chain reactions (PCR) generated from cDNA libraries or clone collections. Using this technique, arrays consisting of more than 30,000 cDNAs can be fitted onto the surface of a conventional microscope slide. The use of cDNA probes increases the sensitive of the assay because they are typically much longer than oligonucleotide probes. However, sensitivity comes with a loss of specificity due to cross-hybridization. For oligonucleotide arrays, a typical array is Affymetrix's GeneChip, where the short oligoucleotide (20-25 mers) probes are synthesized *in situ* using a photolithographic method. A probe pair consists of a perfect match (PM) probe and a mismatch (MM) probe. The PM probe has a sequence exactly complimentary to the particular gene and thus measures the expression of the gene. The MM probe differs from the PM probe by a single base substitution at the center base position, disturbing the binding of the target gene transcript. MM is used as a control to detect the background and nonspecific hybridization from unrelated sequences. A

major advantage of *in situ* synthesized probes, is that it eliminates the possibility of misidentified tubes, clones, cDNAs or spots, thereby avoiding the chance of mistakes during production of the chips. Affymetrix's short oligonuclelotides should in theory provide a better discrimination between related sequences. However, with complex eukaryotic genomes, they often have poor hybridization properties and might create problems with cross-hybridization because of the short length of the probe. The use of several oligonucleotide probes per gene may help to eliminate this problem. In addition, the Affymetrix approach has been commercially available for several years and the company provides strong support for data analysis. Arrays with 40-80 mer oligonuecleotides probes provide an enhanced sensitivity over 25 mers in part due to the larger area available for hybridization (187). However, longer probes provide greater sensitivity at the expense of a reduced specificity.

The process of expression analysis can be divided into several stages (Fig. 1): (1) experimental design; (2) sample collection and RNA extraction; (3) sample labeling; (4) hybridization, detection and scanning; (5) identification of differentially expressed genes; (6) cluster analysis, pathway identification and functional classification; (7) validation of the data.

Apart from the advantage of array-based methods in analyzing gene expression in clinical samples, a number of pitfalls may also be encountered. First, there are numerous regulatory events at the translational level that are not reflected by analysis of gene expression as measured by array hybridization. The protein array may thus be a suitable complementary method for large-scale analysis at the translational level. Second, the cellular sources of the gene expression changes are not identified, for instance, separate contributions by epithelial cells and leukocytes cannot readily be distinguished (in paper I). However, by using the laser capture microdissection (LCM) technique, samples from single cells can be collected. By using RNA amplification, as little as 100 ng of total RNA could generate enough RNA for microarray experiments. Third, there may be false-positive or negative results due to the sensitivity of the array assay and intrinsic problems related to the design of the array. Different methods, such as quantitative real-time PCR, are therefore needed to verify the array experiments. Fourth, a huge amount of data is generated and cooperation between bioinformatists and biologists is required to complete the data analysis. Fifth, high cost of array experiments. Sixth, the reproducibility between platforms and across laboratories, or

even within the same laboratory may be poor (188). The use of standardized protocols would thus help to increase reproducibility.

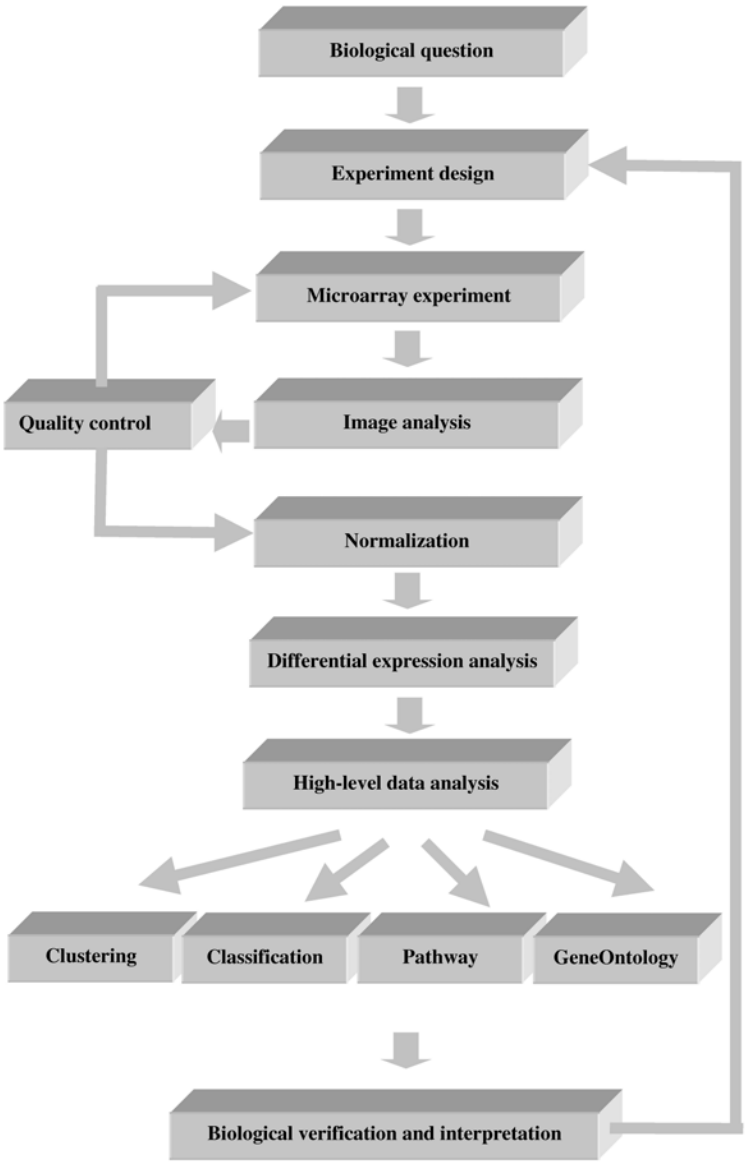

Fig. 1 The workflow of the microarray study

2 AIMS OF THE STUDY

General aim

The overall aim of my thesis work is to study the host immune responses in the gastric mucosa during *H. pylori* infection.

Specific aims

1. To identify genes associated with *H. pylori*-induced inflammation by microarray analysis.
2. To evaluate the role of a selected gene identified in the microarray study, MMP-9, in *H. pylori*-induced gastritis.
3. To understand the mechanism underlying protective immunity against *H. pylori* by studying the gene profile in the mouse gastric mucosa after vaccination.
4. To explore the correlation of *H. pylori* genotypes with the inflammatory gene expression profiles in human gastric mucosa.

3 MATERIALS AND METHODS

Detailed methods and materials are described in each paper. Selected methods /models are discussed here.

3.1 Transgenic mouse model (paper III)

Lewis b transgenic FVB/N mice, expressing the human α-1,3/4-fucosyltransferase gene in their gastric epithelial cells (189), were used in paper III. The transgenic mice express the histo-blood group Lewis b (Fucα1,2Galβ1,3(Fuc α1,4) GlcNAcβ) antigen, which serves as a receptor for the *H. pylori* adhesin BabA and mediates its attachment to gastric pit and surface mucous cells. Thus, these transgenic mice provide a useful tool for *H. pylori* research as it may constitute a "physiological" model for vaccine studies. The mice were bred and kept at the animal facility of the Swedish Institute for Infectious Disease Control in Stockholm, Sweden. As we found that *H. pylori* colonized the stomachs of female mice very poorly (data not shown), only male mice, aged 6-8 weeks, were included in the study.

3.2 *H. pylori* strain used for challenge in mice (paper III)

Low passage (maximum passages=5) *H. pylori* J99^{StrR} (streptomycin-resistant J99) were cultured on C-plates containing 500 mg/l of streptomycin and used for challenging the mice in this study. The *H. pylori* J99 strain was originally isolated in USA in 1994 from a patient suffering from duodenal ulcer. The strain has been completely sequenced and expresses *CagA, BabA2* and *VacAs1* genes (51). To get a clear background on the culture plates when performing quantitative culturing of *H. pylori* from stomach tissue, a streptomycin resistance gene was transfected into J99. The colonization of the gastric mucosa by J99 and J99^{StrR} was similar in our preliminary experiments (data not shown). For the challenge experiments, the bacteria were harvested from plates, washed once in Brucella broth (Acumedia Manufacturers Inc., Baltimore, MD) and the concentration of bacteria was adjusted to 10^9 CFU/ml. Before use, the *H. pylori* J99^{StrR} was analyzed in wet mounts to assess motility and gram stained to observe the morphology. Using low passage *H. pylori* is important, as colonization in the stomach is decreased if the mice are challenged with a high passage strain of *H. pylori*.

3.3 Quantitative culturing of *H. pylori* from stomach tissue (paper III)

Culturing of *H. pylori* is a specific and reliable golden standard for analyzing colonization as compared to other methods such as histopathology or urease testing. Histopathological examination is sensitive, but is influenced by the size of the tissue and the location from which they are taken (190, 191). Urease testing requires a high density of bacteria and anything that reduces the bacterial load may produce false-negative tests (190, 191). Moreover, in rodent models, presence of a contaminating microflora that may contain urease producers can lead to false-positive results (192). Quantitative culture of *H. pylori* was used in our study to evaluate the level of protection afforded by vaccines. Half of the stomach from each animal in our study was homogenized and cultured on C-plates containing 500 mg/l streptomycin. The bacteria were cultured under microaerophilic conditions at 37°C for 5-7 days until colonies were visible and the CFU could be quantified. Three colonies from each animal were selected for PCR amplification of *BabA* and *16S rRNA* to establish that they were of $J99^{StrR}$ origin.

3.4 *H. pylori* genotyping (papers I and IV)

The genotypes of *H. pylori* (presence of *16S rRNA*, *CagA*, *VacA1*, *VacA2*, and *BabA2* genes) were detected directly in the gastric biopsies using RT-PCR. The primers for *16S rRNA*, *CagA*, and *BabA2* were designed based on published sequences for the respective genes (U00679, AF033654 and AF247651). The primers for the *VacA* gene were described previously (40). Studies have shown that RT-PCR is a sensitive and specific method for detection of *H. pylori* genotypes in gastric tissue (193, 194). The results obtained from DNA and RNA amplification of *16S rRNA*, *CagA*, *VacA* and *BabA2* genes are highly consistent (75, 193, 194). Moreover, detection at the transcriptional level could reflect the actual expression of these genes in the bacteria. In our study, *H. pylori 16S rRNA* was detected in all the samples belonging to the *H. pylori* positive group as determined by histology and/or a positive rapid urease test in the biopsy samples.

3.5 RNA extraction from gastric biopsies (papers I-IV)

Total RNA was extracted from antrum biopsies using the RNeasy mini kit (Qiagen, Hilden, Germany). To remove trace amounts of genomic DNA, it was treated with Rnase-free Dnase I (Qiagen). The RNA samples were quantified by measuring the absorbance at 260 nm and 280 nm and the integrity was assessed by agarose gel

electrophoresis. Any trace amount of genomic DNA will result in a heavy background during the microarray experiment and real-time PCR analysis, therefore, removing genomic DNA completely from the RNA sample is very important.

3.6 Microarray (papers I, III and IV)

3.6.1 Human cytokine expression array

Human cytokine expression arrays (R&D Systems Europe Ltd, UK) were used in papers I and IV. PCR products derived from sequence-verified cDNA clones were printed onto charged nylon membranes. The array consists of 847 different genes, representing a comprehensive collection of cytokines, chemokines and other immunomodulatory factors, and their receptors. Nine positive control "housekeeping" genes and six negative controls are included in the arrays.

3.6.2 Mouse Genome 430A 2.0 GeneChip (Affymetrix)

In paper III, Mouse Genome 430A 2.0 arrays (Affymetrix, Santa Clara, CA) were used to study the potential mechanism underlying the BabA-induced protection by gene expression profiling in the antrum gastric mucosa. This array contains over 22,600 probe sets, representing transcripts and variants from over 14,000 well-characterized mouse genes.

3.6.3 Identification of differentially expressed genes

To identify genes that are differentially expressed is a primary goal for most microarray studies. The common methods for assessing differential expression include fold change, *t-test*, nonparametric test and ANOVA. To obtain a reliable judgment, a sufficient number of replicates is one of the basic requirements. Due to the cost of microarray experiments, the number of replicates is usually limited. Moreover, a microarray experiment is a multi-step process that may yield variable raw data in each experiment. Therefore, unlike traditional experiment designs with many replicates and few variables, expression arrays have few replicates and thousands of variables (genes). This creates problems for accurate estimates of variance. Furthermore, the power of statistical tests to differentiate between "regulated" and "non-regulated" genes might be very low (195, 196). Thus, a high false positive error rate often occurs during processing of the data. In our studies, we performed a pair-wise analysis using stringent criteria (requiring consistency in the expression values across a majority of the

samples) to identify differentially expressed genes between samples. For instance, in paper I, the normalized signal of each individual gene in a given sample in the *H. pylori* positive group (n=12) was compared to the data of the corresponding gene in each of the control samples in the *H. pylori* negative group (n=9). If the signal intensity was higher than in the control sample, i.e. showing at least a twofold increase or a change from being undetectable to detectable in more than 80% of these comparisons (n=108), the gene was considered being up-regulated. We noticed that all the "regulated" genes identified by this method have the lowest statistical *p* value when the Mann-Whitney test was applied. Moreover, there was a good correlation between the expression data from arrays and real-time PCR. Therefore, pair-wise analysis with stringent criteria is a reliable method that could decrease false-positive errors during microarray data analysis. Similar methods for identification of differentially expressed genes were used in papers III and IV.

3.6.4 SAM

The Significance Analysis of Microarray (SAM) (197) was used to identify those genes that showed statistically significant differences in expression. According to the SAM algorithm, the method identifies genes with statistically significant changes in expression by assimilating a set of gene-specific *t* tests. Each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene. Genes with scores greater than a threshold are deemed potentially significant. In addition, SAM calculates an estimated false discovery rate (FDR), which is the percentage of genes expected to be falsely identified as differentially expressed. The threshold can be adjusted to identify different sets of significantly differentially expressed genes. In our study, a FDR of <5% in each analysis and a minimum of twofold difference in expression level were chosen to identify genes with statistically significant changes in expression.

3.6.5 Cluster analysis

The basic concept in clustering is to try to identify and group together similarly expressed genes and then try to correlate the observations to biology (198). Clustering methods can be classified as supervised and unsupervised. Supervised methods assign some predefined classes to a data set, whereas in unsupervised methods, no prior assumptions are applied. In our studies, hierarchical clustering was performed by using

the CLUSTER program (198) and the results were displayed using TREEVIEW (http://genome-www4.stanford.edu/MicroArray/SMD/restech.html).

3.7 Real-time RT PCR (papers I-IV)

Real-time PCR is one of the most widely used methods for quantitative detection of gene expression (199). The real-time PCR system is based on detection and quantitation of a fluorescent reporter (200, 201). The fluorescent signal increases in proportion to the amount of PCR products in a reaction. In comparison to conventional RT-PCR, real-time PCR offers a much wider dynamic range of up to 10^{7-8}-fold (compared to 1000-fold in conventional RT-PCR) (202), and it is a 1000-fold more sensitive than dot blot hybridization (203). There is no post-amplification processing in real-time PCR. This helps to increase the throughput and reduces the chance of carryover contamination.

Taqman and SYBR Green are the most common systems for performing real-time PCR. In the Taqman system, a target gene specific probe is designed. This probe contains a reporter dye at the 5'- end and a quencher dye at the 3'- end of the probe, respectively. During the elongation step of the PCR reaction, the probe is cleaved by the Taq polymerase, which results in separation of the reporter dye and the quencher dye. This leads to an increased fluorescence of the reporter. SYBR Green is a non-sequence specific fluorescent intercalating dye. It emits a strong fluorescent signal when bound to double-stranded DNA (202). Thus, as PCR products accumulate, fluorescence increases.

As SYBR Green provides an inexpensive, easy to use, and sensitive format for detecting and quantitating PCR products in real-time reactions, it was used to detect the gene expression in all the studies included in this thesis. However, the disadvantage of this system is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which may result in an over-estimation of the target concentration. Therefore, well-designed primers and extensive optimization of PCR running conditions are very important. Furthermore, to identify specific amplification, follow-up assays (melting point or dissociation curve analysis) are needed (204). In our studies, we found that even a single nucleotide change in the PCR product could result in alteration of the dissociation curve. Thus, the

dissociation curve analysis is a sensitive method for detecting the formation of non-specific products or primer dimers.

Recently, new techniques such as locked nucleic acid (LNA) mediated real-time PCR (205, 206) and real-time PCR array (Superarray, Maryland USA) that combines the profiling capabilities of microarrays with the performance of real-time PCR have been developed. They hold promise for more sensitive, specific, accurate, rapid, and convenient methods for quantitative detection of gene expression levels.

4 RESULTS AND DISCUSSION

4.1 Paper I

To investigate the host immune responses in gastric mucosa during *H. pylori* infection, we analyzed the gene expression profiles in 12 *H. pylori* infected patients and 9 *H. pylori* negative controls using an inflammatory cDNA array.

Gene expression profile in *H. pylori* infected antrum gastric mucosa

Two main gene expression profiles were identified in normal and *H. pylori*-infected gastric mucosa based on a hierarchical cluster analysis (Fig. 2 in paper I). It was clearly shown in the cluster diagram that all the *H. pylori* positive samples were grouped into one branch whereas all the *H. pylori* negative samples were grouped in another branch, suggesting that the gene expression profiles were indeed different between *H. pylori*-positive and *H. pylori*-negative samples. The data obtained from the cluster analysis suggested a strong involvement of selected TLRs, adhesion molecules, MMPs, chemokines and interleukins in mucosal responses, which were identified as a set of "*H. pylori*-infection signatures".

Activation of TLRs in *H. pylori* infection

One notable finding of our study is that *H. pylori* infection induces expression of several genes that are associated with the innate immune system. The expression of several TLRs, including TLR1, 4, 5 and 6, were up-regulated in *H. pylori* infected gastric mucosa. Moreover, other factors related to the LPS signalling pathway such as CD14, LPS-binding protein (LBP) and RP105 (related to proliferation response of B cells to LPS) were also strongly induced by *H. pylori* infection. Thus, LPS, through interaction with TLR4, may still be a potent stimulator of innate immune responses although LPS from *H. pylori* has been considered to be less toxic than LPS from other gram-negative bacteria. Our data is consistent with previous reports showing that TLR4 is involved in the response to *H. pylori* infection in the stomach (95-97). However, it has been reported that *H. pylori* LPS or infection itself activates innate immunity via TLR2 rather than TLR4 (98-101). In our study, TLR2 was expressed at a very low level (below the threshold) in a majority of the samples and was not analyzable by microarray analysis. A more sensitive method such as real-time PCR may be able to address this question.

T cell responses in *H. pylori* infection

We found that a set of genes encoding receptors for IFN-γ, IL-2, IL-3, IL-12, and IL-18 were up-regulated in the *H. pylori*-infected gastric mucosa. Moreover, several genes such as IL-2, IL-12 p35 and IFN-γ were undetectable in our microarray experiments but were found to be up-regulated in *H. pylori*-infected samples by real-time PCR assay. Thus, our data support the previous notion of a predominant Th1 response.

Two members of the B7 family of costimulatory factors, B7-H1 and GL50/B7-H2, were found to be strongly up-regulated in *H. pylori*-infected gastric mucosa. In contrast to the classical B7.1/B7.2-CD28 interactions, which are important for priming naïve T cells, these novel costimulatory interactions appear to be crucial in regulating effector lymphocytes in the periphery (207, 208). The inducible costimulator on activated T cells provides a positive signal by interaction with its ligand B7-H2, whereas programmed cell death-1 and its ligand, B7-H1, provide negative signals to the activated T cells and are crucial for the regulation of peripheral tolerance and autoimmunity. Our findings suggest an important role for effector T cells at the sites of infection/inflammation.

In addition, recent findings suggest that regulatory T cells ($CD4^+CD25^+$) may suppress T cell responses to *H. pylori* and thereby contribute to the persistence of the infection (123-125). However, as the markers of regulatory T cells, Foxp3 and CD25, were not included in our array, we are not able to evaluate the role of regulatory T cells on *H. pylori* infection.

Expression of chemokines and chemokine receptors in *H. pylori* infection

We also found that expression of a set of selected chemokines and chemokine receptors were up-regulated in *H. pylori* infected gastric mucosa. These include several CXC chemokines, ENA78, GROγ, GROβ, GROα, and BLC/BCA-1 which have previously been implicated in *H. pylori* induced inflammation (209-211). These chemokines all bind to neutrophils, except BLC/BCA-1, which binds to naïve B cells and activated T cells. In addition, two CC chemokines, pulmonary and activation-regulated chemokine (PARC) and MIP-3α, three chemokine receptors, CCR6, CCR7 and CXCR4, which

have not been studied previously in this regard, were also up-regulated in the *H. pylori* infected samples. PARC and MIP-3α have shown to be involved in recruitment of naïve T cells, immature dendritic cells and memory $CD4^+$ T lymphocytes (212-214). Our data thus suggest that several CXC chemokines are involved in neutrophil trafficking that in turn contribute to the activity of *H. pylori*-induced gastritis, whereas PARC, MIP-3α and BCA-1 may contribute to the recruitment of other inflammatory cells, including lymphocytes and dendritic cells.

Correlation of chronic inflammation with the gene expression profile

We observed that the number of up-regulated genes seem to correlate with the degree of chronic inflammation (Table V in paper I). Moreover, we also identified 21 genes that were associated with a more severe chronic inflammation induced by *H. pylori* infection. Some of these genes have already been shown to be related to ulcerogenesis and carcinogenesis. For instance, cyclin B1, a factor that determines cellular apoptosis, has been associated with the progression of gastric MALT lymphoma (215). Maspin expression is related to metaplasia and gastric carcinogenesis (216). MMP-1 has been shown to be related to gastric uclerogenesis (217). Further analysis of these genes might enable us to develop a set of makers that can identify infected patients with a risk for developing a more severe disease.

Correlation of the *H. pylori* genotype with the gene expression pattern

We also investigated the correlation of the genotype of *H. pylori* with the gene expression profiles. There was no significant correlation between the presence of *BabA2 or CagA* and the gene expression pattern in the gastric mucosa based on hierarchical clustering and multidimensional scaling analysis. However, due to the limited number of *H. pylori* infected samples, no detailed analysis of double- (expressing *CagA* and *VacAs1*) or triple- (expressing *CagA*, *VacAs1* and *BabA2*) positive samples could be made.

In summary, in this study, we present the first global assessment of expression of immunological/inflammatory genes in gastric biopsies in patients with *H. pylori* infection. We found a remarkably consistent pattern of genes differentially expressed in normal and *H. pylori*-infected gastric mucosa. Further analysis of a large number of samples with different clinical phenotypes and/or infected by bacteria with different

genotypes, might help to better understand the pathophysiological mechanisms involved.

4.2 Paper II

In our first study, we found that several genes related to the MMPs family may be involved in *H. pylori* induced gastritis. We subsequently studied the role of one of these genes, matrix metalloproteinase 9 (*MMP9*), in *H. pylori*-induced gastritis.

MMP activity in the gastric mucosa

MMP activity in tissue extracts from gastric biopsies of *H. pylori*-infected and uninfected volunteers was assayed using SDS-PAGE zymography. We found that there was a large increase in MMP-9 (19-fold, $p<0.001$), and a moderate increase in MMP-2 activity (3 fold, $p<0.05$) in the gastric mucosa of infected individuals as compared to uninfected individuals. The increased expression of MMP-9 in the *H. pylori*-infected gastric mucosa was confirmed by real-time RT-PCR and Western blot. Our data is in agreement with previous and later reports showing that expression of MMP-2 and MMP-9 in gastric mucosa is induced by *H. pylori* infection (151, 152, 154). However, no significant activity of other MMPs such as MMP-1, -3 or -7, was found in the tissue extracts using casein gel zymography. This is in contrast to other studies, where an increased expression of MMP-7 in the gastric muocsa of *H. pylori* infected individuals has been shown (149, 150, 153). However, in those reports only the expression level of MMP-7, but not its biological activity, was studied.

TIMPs in the gastric mucosa

MMP activity in the tissue is associated with the expression level of TIMPs that are specific inhibitors of MMPs (133, 134). We therefore analyzed the level of TIMP-1 and TIMP-2 in the gastric mucosa by using ELISA. Interestingly, the increase in MMP activity was not accompanied by changes in the levels of TIMP-1 or TIMP-2 in *H. pylori*-infected individuals. Therefore, the imbalance of MMPs and TIMPs results in a net increase in gastric MMP activity, which probably contributes to tissue damage in *H. pylori*-associated gastritis.

Cellular source of MMP-9 in the gastric mucosa

MMP-9 was shown to be present in the lamina propia of *H. pylori*-infected individuals using an immunohistochemical assay. However, MMP-9 staining could not be detected

in the epithelium. Furthermore, using a double immunofluorescence assay, it clearly localized the MMP-9 staining to macrophages within the lamina propria. Our data is partly contradictory to the results from Mori et al, who suggested that MMP-9 is produced from both macrophages and epithelium in *H. pylori*-infected gastric samples (154). In our study, the staining of MMP-9 in the epithelium was undetectable. These differences might result from the use of different anti-MMP-9 antibodies and different detection systems. Furthermore, the use of formaldehyde-fixed sections or frozen cryosections for the assays may also contribute to the differences. In addition, we also found that macrophages collected from gastric lamina propria and those generated from peripheral blood mononuclear cells spontaneously secrete MMP-9, and that the MMP-9 activity was further increased by stimulation with *H. pylori*. These data demonstrate that macrophages may spontaneously secrete MMP-9 by an autocrine loop; however, it can be induced by *H. pylori* stimulation.

In conclusion, *H. pylori* infection results in a large increase in MMP-9 activity in the gastric mucosa, while there is no increase in the levels of TIMPs. The substantial increase in MMP-9 activity was probably caused by tissue-resident macrophages. The net increase in gastric MMPs activity is likely to contribute to tissue damage during *H. pylori*-associated gastritis.

4.3 Paper III

Many studies have shown that both prophylactic and therapeutic vaccinations can successfully protect from Helicobacter infection in animal models. However, the mechanism is still not fully understood. In this study, using the Lewis^{b} transgenic FVB/N mouse model, intranasal immunization with BabA and CTA1-DD as an adjuvant reduced *H. pylori* colonization in the mouse stomach and was accompanied by an enhanced specific antibody response both systemically and locally in the gastric mucosa. The gene expression profiles in the antrum gastric mucosa in untreated, unimmunized/challenged and immunized/challenged mice were subsequently studied in order to explore the mechanism underlying protective immunity against *H. pylori* by BabA-immunization.

Differentially expressed genes in unimmunized/*H. pylori*-infected and BabA immunized/*H. pylori*-infected antrum gastric mucosa

Using pair-wise analysis, we identified 193 genes that were differentially expressed between the untreated and unimmunized/challenged groups that might serve as a "signature for *H. pylori* infection" in the mouse gastric mucosa. Using the same method, we identified 79 genes that were up-regulated in the immunized/challenged samples as compared to those from untreated mice. Notably, almost all these genes are also up-regulated in the unimmunized/challenged mice. Thus, the "immunization signature" is likely to be a part of the "signature for *H. pylori* infection" and is not specific for the vaccine response. We also found that 10 genes were up-regulated in unimmunized/challenged samples as compared to the immunized/challenged samples (Table 2 in paper III). Moreover, 9 of the 10 genes were directly or indirectly related to immunity, in particular to innate immunity.

The role of Th cell response on *H. pylori* immunization

Until now, studies have shown that both Th1 and Th2 responses are required for vaccine-induced protection against *H. pylori* infection (172-177). In our study, the expression levels of the "classical" Th1 or Th2 cytokines such as IL-4, IL-5, IL-12, IL-13, IFN-γ and TNF-α, were too low to be analyzed by array or real-time PCR. However, 30 other detectable genes that are related to Th1 and Th2 responses were not differentially expressed in the three groups analyzed (Table S3 in paper III). Thus, our findings suggest that Th1 or Th2 type immune responses could neither be shown to be associated with the infection status nor with BabA-induced protection. Our results partly agree with previous studies, based on vaccination experiments in cytokine knockout mice, showing that IL-4, IL-5, IL-13, IFN-γ and TNF-α, are all dispensable for the protective mechanism (122, 178-180).

The role of adipocytokines in *H. pylori* immunization

A recent study using *H. felis* sonicate as a vaccine has shown that a set of adipose tissue-specific genes are up-regulated in the gastric mucosa in protected mice, but not in unprotected mice (218). These genes encode adipocyte-specific cytokines (adipocytokines), such as adipsin, adiponectin and resistin. However, in our study, the expression of *adipsin* and *adiponectin*, together with other adipocyte-related factors such as *stearoyl-coenzyme A deasaturase 1* and *carbonic anhydrase 3*, were up-regulated in the gastric mucosa from unimmunized/challenged mice, but not in those from immunized/challenged or untreated mice. Depending on the experimental conditions (BALB/c vs. FVB/N mice, *H. felis* vs. *H. pylori* infection, whole-cell vs.

BabA vaccine, 22 months vs. 1 months after challenge, whole stomach vs. antrum part of stomach analyzed), up-regulation of adipocyte-specific cytokines may thus either be associated with vaccine-induced protection (218) or persistence of the infection (our study).

Effect of vaccination on post-immunization gastritis

Previous reports have shown that vaccine-induced protection is associated with post-immunization gastritis (162, 219). However, in our study, post-immunization gastritis could not be observed although the number of bacteria was decreased more than 100 fold in the immunized/challenged mice. These findings suggest that a strong gastric inflammation is not associated with the BabA-vaccine-induced protection in our transgenic mice.

Effect of different mouse and Helicobacter strains on immune responses to *H. pylori* infection

It should be mentioned that the degree of gastric inflammation induced by Helicobacter infection and the post-immunization inflammation in mice is associated both with the bacterial strain and the mouse strain (122, 220-222). C57BL/6 mice exhibit a severe gastric inflammation and a strong post-immunization inflammation after *H. felis* infection. However, they only show a mild response if infected by *H. pylori SS* (222). *H. felis* infection also induces a severe gastritis in C3H/He mice, but not in other mouse strains such as BALB/c and CBA (223). In our Lewis^{b} transgenic mouse model, the *H. pylori* J99^{StrR} strain has been shown to colonize the stomach at a high density, while it only induces a low degree of inflammation. Therefore, the host response to Helicobacter is dependent both on the host genetic background and the bacterial strain. These points should be taken into consideration when evaluating the vaccine-induced immune response in different mouse models.

Taken together, the BabA induced protection was neither associated with an altered Th1/Th2 cytokine expression pattern, nor with post-immunization gastritis. Moreover, we could not identify any gene that was specifically induced by the immunization, as the putative "immunization signature" basically overlapped with the "*H. pylori* infection signature".

4.4 Paper IV

As we discussed previously, *H. pylori* virulence factors have been suggested to be crucial in determining the outcome of infection. The *H. pylori* adhesion protein BabA is thought to play an important role in bacterial colonization and in induction of a severe gastric inflammation, particularly in combination with expression of CagA and VacA (75-77). However, the influence of these virulence factors, especially BabA, on the pathogenesis of *H. pylori* infection, is still poorly understood. Our previous study (paper I) suggested that there was no correlation between the presence of *BabA2*, or *CagA* and the gene expression pattern in the gastric mucosa, although, due to the limited number of samples available, we were not able to make a detailed analysis. In a continuation of the previous study, the inflammatory gene expression profiles from three groups of patients infected with triple-negative strains (lacking *CagA*, *BabA2* and *VacAs1*, but expressing *VacAs2*), double-positive strains (type I strains, expressing *CagA* and *VacAs1*) and triple-positive strains (expressing *CagA*, *VacAs1* and *BabA2*) were investigated. In addition, 8 *H. pylori*-negative samples were included as controls.

Identification of gene expression profile and differentially expressed genes

Hierarchical cluster analysis showed that two main gene expression profiles could be identified (Fig. 1B in paper IV). Group I included 7 of the 8 *H. pylori* negative samples, whereas group II contained one negative and all the *H. pylori* positive samples. Several clusters of genes, which were differentially expressed in biopsies from patients with *H. pylori* infection and uninfected controls, largely overlap with the "*H. pylori* infection signature" identified in our previous study (paper I). However, no subgroup could be assigned based on the *H. pylori* genotypes. To identify a potential effect of BabA2, CagA and VacA on gene expression in the gastric mucosa, three groups of *H. pylori*-positive samples were compared with *H. pylori*-negative samples. Almost all genes that were differentially expressed as compared to the *H. pylori*-negative samples were shared by the three groups of *H. pylori*-positive samples. Moreover, no differentially expressed genes could be identified by pair-wise comparisons when the three groups of *H. pylori*-positive samples were directly compared. Thus, our data suggest a lack of correlation between the host inflammatory responses in the gastric mucosa and *BabA2, CagA* and *VacAs1* status.

Correlation of *BabA* genotype of *H. pylori* with inflammatory gene expression in gastric mucosa

Our results show that the inflammatory gene expression in double-positive and triple-positive samples is similar. Thus, it might indicate that the effect of BabA mediated adherence of *H. pylori* on the host immune response is less important than hitherto suggested. However, due to the diversity of the *BabA2* gene and its complicated regulation of expression, binding activity mediated by BabA might be highly variable (73, 74, 224, 225). Therefore, *BabA* genotyping by PCR might not reflect the "true" adherence properties of bacteria (226) and an *in vitro* Lewis b adhesion assay might be required to address this point.

Correlation of the CagA genotype of *H. pylori* with IL-8 gene expression in gastric mucosa

In our study, expression levels of ENA-78 and IL-8 in triple-negative, double-positive and triple-positive samples were similar. These data thus seem to contradict previous reports, where IL-8 and ENA-78 were expressed at a higher level in *CagA*-positive or *VacAs1*-positive samples than in *CagA*-negative or *VacAs1*-negative samples (209, 227), and more dominant in triple-positive samples than in double-positive samples (77). However, previous studies have shown that the expression of IL-8 is depended on most of the 31 genes of the cag PAI rather than the *CagA* gene itself (52, 61, 228). In addition, it has been reported that the expression of a novel virulence factor encoding gene, *oipA* (outer inflammatory protein), rather than the expression of the *CagA*, *BabA2*, or *VacAs1* gene, was significantly associated with a high level of mucosal IL-8 expression (229, 230), although a recent study shows that the status of *oiA* is not related to IL-8 expression *in vitro* (231).

In summary, our study supports the hypotheses that the presence of virulence factors of *H. pylori,* BabA, CagA and VacA, is not associated with a selective gene expression pattern in the infected human gastric mucosa.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

My thesis work has focused on investigation of the host immune responses in gastric mucosa during *H. pylori* infection and after vaccination by microarray analysis. The main conclusions are:

We found a remarkably consistent pattern of genes differentially expressed in normal and *H. pylori*-infected human gastric mucosa. Moreover, a set of genes encoding Toll-like receptors, adhesion molecules, metalloproteinases, chemokines and interleukins were identified and might serve as a "Helicobacter-infection signature".

We showed that *H. pylori* infection leads to an increase in MMP-9 and MMP-2 activity with no change of TIMP-1 or -2 activity in the antrum mucosa. The net increase in gastric MMP activity is likely to contribute to tissue damage in *H. pylori*-associated gastritis. Moreover, we identified that the increase in MMP-9 activity in the gastric mucosa, was probably associated with tissue-resident macrophages.

We demonstrated that the gene expression pattern in the antrum gastric mucosa from patients infected with different *H. pylori* strains (triple-negative, double-positive and triple-positive) was similar and that no differentially expressed genes could be identified. We thus propose that there is a lack of correlation between the host inflammatory responses in the gastric mucosa and expression of the *BabA2, CagA and VacAs1* genes.

We explored the gene expression profile in Lewis^{b} transgenic mouse gastric mucosa after BabA vaccination, in order to understand the mechanism underlying protective immunity against *H. pylori*. We showed that the protection was neither associated with an altered Th1/Th2 cytokine expression pattern, nor with post-immunization gastritis. Moreover, we could not identify any gene that was specifically induced by the immunization, as the putative "immunization signature" is part of the "*H. pylori* infection signature".

To identify potential markers that may predict the clinical outcome, a large number of biopsies from patients with non-*H. pylori*-infected gastritis, peptic ulcer and gastric cancer will be needed. Moreover, analysis of expression profiles from different cell populations extracted from gastric tissue using laser capture microdissection (LCM), would help to characterize the role of different types of cells in the pathogenesis of *H. pylori* infection. In addition, using *in vitro* explant culture models (232), analysis of the gene expression profiles in gastric mucosa stimulated with different genotypes of *H. pylori* (*BabA-, SabA-, BabA-/SabA-* and wild type), might provide a better understanding of the influence of different virulence factors.

In our current study, intranasal immunization with BabA and CTA1-DD as an adjuvant successfully reduced *H. pylori* colonization in mouse stomachs and induced specific antibody responses both systemically and locally in the gastric mucosa. To pave the way for future human trials, BabA vaccination studies in primates will help us to better understand the protective mechanism induced by the BabA vaccine and to develop an optimized vaccine formula.

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