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ABBREVIATIONS

BSA	bovine serum albumin
CagA	cytotoxin associated gene A
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleoside triphosphate
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetate
ELF-3	E74-like factor-3
ELISA	enzyme linked immunosorbant assay
G3PDH	glyceraldehyde 3-phosphate dehydrogenase
GARG	glucocorticoid attenuated response gene
GAS	(interferon) gamma activated site
GBP	guanylate binding protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
H. felis	*Helicobacter felis*
H. mustelae	*Helicobacter mustelae*
H. pylori	*Helicobacter pylori*
HRP	horseradish peroxidase
IFN	interferon
IFNAR1	interferon-alpha receptor domain 1
IgA	immunoglobulin A

IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
ISGF3	interferon stimulated gene factor 3
ISRE	interferon-stimulated response element
JAK	Janus kinase
LPS	lipopolysaccharide
MALT	mucosa associated lymphatic tissue
MEF	mouse embryonic fibroblasts
MHC	major histocompatibility complex
mIRF	murine interferon regulatory factor
MRP	myeloid-related protein
NapA	neutrophil activating protein A
NFκB	nuclear factor κB
NK cells	natural killer cells
OD	optical density
Oligo(dT)	oligo-deoxythymine
PE	phycoerythrin
PAI	pathogenicity island
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SS1	Sydney strain 1
STAT	signal transducer and activator of transcription
TAE	tris acetate EDTA
TGF-beta	transforming growth factor beta
TH	T-helper
TMB	tetramethylbenzidine
VacA	vacuolating cytotoxin A

1 INTRODUCTION

Helicobacter pylori infects about half of the world's population. The bacterium was first discovered by Warren and Marshall in 1983, who isolated and cultured spiral organisms from human gastric biopsy specimens and demonstrated a strong correlation between the presence of the organism and gastric pathology (1). Two independent investigators satisfied Koch's postulates by creating histologically confirmed gastritis in themselves following the ingestion of viable *Helicobacter* (then named *Campylobacter* like organism) cultures. The bacteria were subsequently recovered from their own stomachs and *H. pylori* was widely accepted as a cause for the development of gastritis (2,3). Today, *H. pylori* is not only considered a causative agent of chronic type B gastritis and peptic ulcer disease, but also an important risk factor for gastric cancer and MALT-lymphoma.

It is estimated that 50% of the world's population is infected with *H. pylori*. Infection rates, like for many other infectious diseases, correlate with poor living conditions, especially in childhood (4,5), close person to person contact (6-8), and low socioeconomic status (9). Thus the prevalence of *H. pylori* infection ranges from approximately 20 to 50% in industrialized countries to over 90% in developing nations (10-13). As determined in a study of the Robert Koch Institute in 2000, the prevalence of *H. pylori* infection for the total population of Germany is 40% (14).

Humans appear to be the only natural host of *H. pylori* identified to date, although some studies suggest that animals also may act as a reservoir, as reviewed by Mitchell (15). The survival of *H. pylori* outside the human host and its transmission has not been completely understood. Direct person to person transmission is considered to be the most likely mode and it is believed that the transmission either follows the faecal-oral or the oral-oral route. Faecal-oral transmission gained importance by the detection of *H. pylori* in the faeces of infected patients (16), while studies comparing the distribution patterns of *H. pylori* with Hepatitis A virus, an organism transmitted by the faecal-oral route, suggest that this transmission mode may be of limited importance (17). There is evidence that acquisition of infection takes place predominantly in early childhood (18), and the host usually remains infected for a lifetime. Transmission of the bacterium among adults seems less frequent although it seems favoured when there is close personal contact, as there are higher prevalences in institutions (7).

H. pylori is a curved or spiral shaped Gram negative bacillus with up to three windings (1). It measures 3-5 μm in length and approximately 0.5 μm in diameter. There are two to six unipolar sheathed flagella (1), which are essential for the bacterium's motility. Under unfavourable conditions *H. pylori* can transform into a coccoid form, which probably reflects degenerative changes (19). The microaerophilic bacterium possesses both oxidase and catalase activities. The complete genome of a *H. pylori* strain was sequenced by Tomb *et al.* in 1997 (20).

Different virulence factors facilitate the bacterium's survival in the unique niche of the gastric mucosa and contribute to host damage.

The urease enzyme, which can represent up to 6% of the bacterium's protein mass (21,22), catalyses the hydrolysis of urea into ammonia and carbon dioxide, thus elevating the pH of the microenvironment and protecting the acid sensitive *H. pylori* from the bactericidal effect of acid (23). Additionally, the metabolite ammonia has cytotoxic effects and may lead to epithelial damage like intracellular vacuolisation (24). Flagella (25) are an essential colonisation factor (26). Motility allows the bacterium to enter or traverse the gastric mucus layer thus facilitating the colonization of the gastric mucosa in close approximation to the gastric epithelium, protected from the bactericidal acidic environment of the stomach lumen.

Another virulence factor, the vacuolating cytotoxin A (VacA) (27), is associated with a pathogenicity island (PAI), a 40 kb DNA insertion that encodes for a multicomponent type IV secretion system, and the cytotoxin associated gene A (CagA), an immunodominant protein of unknown function which has been used as a marker for the presence of the PAI, as well as other proteins of unknown function (28). While VacA has been shown to cause vacuolisation (29) and inhibit proliferation of epithelial cells (30), CagA is translocated into the gastric epithelial cell by the type IV secretion system encoded in the pathogenicity island (31,32). CagA is then tyrosine phosphorylated and can induce changes in the tyrosine phosphorylation state of distinct host cellular proteins, which may influence signal transduction pathways and cytoskeletal plasticity (33-35). The consequences of this modulation of the host cell by *H. pylori* are yet unknown.

The pathogenicity island is not present in all *H. pylori* strains, but the presence of a pathogenicity island in CagA positive strains (type I strains) is associated with increased risk of severe gastritis, peptic ulceration, atrophic gastritis and gastric cancer (36-40). Other virulence factors include catalase (41), protecting the bacterium from phagocytosis, superoxide dismutase (42), which provides protection against toxic oxygen radicals, the

neutrophil activating protein NapA, putative adhesins, such as hemagglutinin, and others (as reviewed in 43,44).

H. pylori does not invade the host tissue but rather is restricted to the mucus overlaying the gastric epithelium. During its colonization of the gastric mucosa *H. pylori* is challenged by the innate and adaptive immune response, and the organism has developed strategies of avoidance and subversion of the immune system. The virulence factors described above support the organism's ability to establish a persistent infection, but mechanisms of immune evasion such as antigenic shedding of urease, antigenic variation and a possible immune suppression by *H. pylori* may contribute as well.

Urease in an important immunodominant protein that makes its way to the cell surface, where it is not covalently attached (21), and free urease or urease adsorbed to bacteria has been detected in the gastric mucosa of *H. pylori* infected patients (45-46). Shed urease could bind to secretory IgA and thereby overcome its antibacterial activity.

After the sequencing of the *H. pylori* genome, sequence similarities among a group of outer membrane proteins have become evident. It seems feasible that recombination events could lead to mosaic organization and thus contribute to antigenic variation (47).

In vitro experiments (48-52) have shown that the proliferation of peripheral blood mononuclear cells is decreased in *H. pylori* infected patients compared to healthy blood donors. Thus some authors have argued that *H. pylori* infection might lead to a suppression of the host cellular immune response. When this study was commenced in the year 2000, the mechanisms of a possible immune suppression by *H. pylori* remained to be elucidated.

Both bacterial virulence factors and damage caused by the host inflammatory immune response contribute to the pathogenesis of *H. pylori* gastritis.

Direct cell damage can be caused by VacA and urease, which both lead to vacuolisation of the gastric epithelial cell *in vitro*.

Indirect damage to the host is caused by a vigorous immune response. *H. pylori* activates the proinflammatory transcription factor NFκB in gastric epithelial and monocytic cells, probably via CagA dependent and independent pathways in different cell types (53,54). This pathway of NFκB activation, together with other pathways, results in epithelial production of IL-8, a chemotactic factor for human neutrophils (54-56). The chemotactic effect of IL-8, together with a chemotactic effect of *H. pylori* surface proteins (45), leads to an infiltration of the gastric mucosa with neutrophils and monocytes, causing inflammation and mucosal damage.

H. pylori adheres to and is phagocytosed by the infiltrating cells. Since it has been shown that *H. pylori* is able to activate a respiratory burst in human neutrophils and monocytes (57,58), mucosal damage may be due to the oxygen radicals produced in this reaction.

Additionally, molecular mimicry between *H. pylori* LPS and host antigens might contribute to mucosal damage through autoimmune reactions. The LPS of 85% of *H. pylori* strains is composed of Lewis x and/or Lewis y antigens, human blood group glycoantigens whose epitopes may also be present on the beta chain of the H^+/K^+-ATPase of the human gastric parietal cell. Thus *H. pylori* LPS is able to stimulate strong anti-Lewis x and anti-Lewis y responses in humans (59-61). These findings suggest that molecular mimicry-induced autoimmune reactions could contribute to the pathogenesis of *H. pylori* infection.

There are data suggesting that *H. pylori* affects serum levels of gastrin, a peptide hormone that is produced by gastric G-cells and stimulates gastric acid secretion. Studies comparing the serum gastrin levels of healthy and *H. pylori*-infected individuals indicate that infection is correlated with increased serum gastrin levels. However, the acid secretion was only elevated in patients presenting with duodenal ulcers compared to healthy control individuals, while it was normal in asymptomatic *H. pylori*-infected patients (62). This increased acid production might also contribute to mucosal damage.

Diseases associated with *H. pylori* include chronic gastritis, peptic ulcer disease, gastric cancer and MALT-lymphoma.

Today the bacterium is considered the main causative agent of chronic type B gastritis (63). The inflammation primarily affects the antrum and is characterized by neutrophilic and lymphocytic infiltrates and distinctive epithelial degenerative changes (64).

H. pylori is an important risk factor for peptic ulcer disease, and infection has been diagnosed in 60%-100% of gastric and 90%-100% of duodenal ulcers cases, although in the duodenum the bacterium has only been detected in areas of gastric metaplasia indicating its tissue specifity (65). Eradication of *H. pylori* from *H. pylori*-positive peptic ulcer patients provides the most effective treatment resulting in ulcer healing and low frequencies of relapse (66,67).

The chronic inflammation caused by *H. pylori* can result in mucosal atrophy, metaplasia and dysplasia, thereby creating the bases for a malignant transformation (68,69). Therefore *Helicobacter* infection is associated with an increased risk of gastric cancer (70,71).

Clinical and epidemiological studies provide data for an association of the bacterium and MALT lymphoma. *H. pylori* infection has been diagnosed in over 90% of MALT lymphoma patients (72), and antimicrobial eradication therapy can result in tumor remission in clinical studies (73). *In vitro*, the growth of isolated lymphoma cells required a T cell activation induced by *H. pylori* (74,75).
A causal link between *H. pylori* and non-ulcer dyspepsia, operationally defined as chronic or recurrent pain or discomfort centered in the upper abdomen in the absence of peptic ulceration, has not been established. However, *H. pylori*-positive patients presenting with non-ulcer dyspepsia have experienced symptomatic benefit from eradication therapy (76).

As a non-invasive bacterium *H. pylori* is not exposed to the effector mechanisms of the immune system which typically eradicate infectious agents from the host tissue. Resistance of the gastric mucosa is provided by the mucosal immune system. The immune response to *H. pylori* consists of the innate immune response mainly represented by a neutrophil and macrophage infiltration as already described above, and the adaptive immune response.
On the humoral level of the adaptive response, infection with *H. pylori* generally results in both a systemic and local antibody response with antibodies specific for a large number of *H. pylori* antigens, including putative virulence factors such as urease, VacA, and CagA.
Early studies of cell mediated adaptive responses to *H. pylori* using human gastric explants or peripheral blood mononuclear cells revealed a propensity for cells from uninfected individuals to respond more strongly in proliferation and release of IFN-γ to *H. pylori* than cells from infected patients (48-52), while others reported that *Helicobacter* positive individuals show a stronger proliferative response than do *Helicobacter* negative individuals (77). *H. pylori* infection is associated with the presence of mainly IFN-γ producing T cells in the gastric mucosa, which are thought to contribute to the disease (78-82).

In an updated version of *The Maastricht Consensus Report* the *European Helicobacter Pylori Study Group* has recommended a 7 day course consisting of a proton pump inhibitor based triple therapy, using a proton pump inhibitor (or ranitidine bismuth citrate) and two of the following antibiotics: clarithromycin, metronidazole and amoxicillin as first choice for the treatment of a symptomatic *H. pylori* infection (83,84). In approximately 30% of patients treatment is accompanied by adverse effects like a metallic taste or oral mucositis, nausea, diarrhoea, reduced appetite, vomiting and heartburn, and oral or genital candidiasis (85). These adverse effects contribute to patient non-compliance and reduce the efficacy of therapy

(86). Additionally, antibiotic resistances are an important backdraw of antimicrobial therapy and it is anticipated that resistance will increase in the future (87). Also, a widespread use of these antibiotics in *H. pylori* eradication would favour the development of resistant strains of other pathogenic microorganisms, further questioning the benefit of antibiotic therapy in this very frequent infection. Antibiotic cure does not prevent subsequent reinfection with *H. pylori*, as demonstrated in a study by Ramirez-Ramos, where infection recurred in 73% of the individuals who returned for follow-up endoscopy within the 8-month follow-up period after eradication therapy (88). Finally, since antibiotic eradication therapy is given only to symptomatic patients, asymptomatic patients still remain at risk of developing severe complications, such as gastritis and gastric cancer. All these factors support a role for vaccination in the control of *Helicobacter* infection.

In addition to the general medical assertion that prevention is always better than therapy there are other benefits a prophylactic vaccine would have over antibiotic therapy.
Adverse effects of antibiotic eradication therapy and development of resistant strains could be prevented while providing protection from reinfection. A vaccine would be especially helpful in developing countries, where the prevalence of *H. pylori* infection reaches up to 90% and antibiotic therapy is prohibitively expensive.

The study of *Helicobacter* immunity and vaccines began in earnest with the development of murine models of *Helicobacter* infection (89-92) and the demonstration that mice could be protectively immunized against *Helicobacter* infection through prophylactic oral immunization. In 1991, Czinn et al. developed the first oral immunization protocol generating a *H. pylori*-specific systemic and mucosal antibody response after immunization with *H. pylori* lysates and cholera toxin (93). The first protective immunizations against *H. felis* were achieved by use of a modified oral vaccination protocol with cholera toxin, which resulted in protection of more than 80% of the experimental animals against challenge with live bacteria (94,95). This vaccination protocol has since been employed in modified forms by several groups. The first subunit vaccines, which rendered protection rates ranging from 25 to 80%, consisted of recombinant *H. pylori* urease B subunit (96) although full protection could only be achieved when a combination of *Helicobacter* antigens (Urease B subunit and heat shock protein Hsp 60) was used (97). Antigens employed in other subunit vaccines included VacA and CagA (98), the neutrophil activating protein NapA (99), and catalase (100).

Because of the toxicity of cholera toxin for humans other mucosal adjuvants were used, such as *E. coli* heat-labile enterotoxin (LT), also in a detoxified form (98). Protection of mice against *H. pylori* infection could also be obtained by immunization with an attenuated strain of *Salmonella typhimurium*, expressing *H. pylori* urease A and B subunit, in one study even with a single dose (101,102). Finally, prophylactic immunity was shown to be long-lived in mice, which had been immunized 15 months prior to challenge (103).
Therapeutic vaccines have been administered to both animals and humans. Immunization of mice with VacA and CagA, which were administered together with a genetically detoxified mutant of the heat-labile enterotoxin of *E. coli* (104) or immunization with recombinant *H. pylori* urease B subunit and cholera toxin (105) resolved *Helicobacter* infection in mice, which were also protected against a subsequent challenge with *H. felis*. A naturally occurring infection of ferrets with *H. mustelae* could be eradicated in 30% of the animals following therapeutic immunization with purified *H. pylori* urease holoenzyme and cholera toxin (106).
In a phase II clinical study a therapeutic vaccination with recombinant *H. pylori* urease and *E. coli* heat labile toxin against a preexisting *H. pylori* infection did not lead to an eradication of the bacterium in any of the volunteers, although there was a modest decline in bacterial infection in those subjects who received the vaccine. However, 66% of the participants experienced significant diarrhoea, indicating the toxicity of *E. coli* heat labile toxin (107).
Since, in many cases of oral immunization, protection was not complete, and to overcome difficulties with adjuvant toxicity, other routes of vaccine delivery including intranasal and rectal administration have also been investigated (108,109). Surprisingly, protective immunity to *Helicobacter* infection could also be achieved by systemic immunization (110-112).

The problem of all present vaccination studies is that they use adjuvants that are not approved for human use and often cause adverse effects when administered to humans. The identification of the mechanisms of protective immunity could promote the development of safe and efficacious vaccination protocols for humans.

Once an individual is infected with *Helicobacter* the bacterium persists in the gastric mucosa of the host despite of a vigorous immune response. However, prophylactic immunization can prevent *Helicobacter* infection. Possible explanations for the success of prophylactic vaccination could be either the difference in the chronology of the immune response in relation to infection, the induction of a qualitatively different host response by immunization, or a quantitative boost to the immune response during infection which drives inflammation

above a certain threshold level necessary to clear the bacteria. The mechanisms by which this distinctive immune response is induced remain to be elucidated.

Many groups have studied the role of antibodies, the typical mucosal defence mechanism against non-invasive pathogens. Mice can be passively immunized against *Helicobacter* infection with *Helicobacter* specific IgA (95), and the incubation of the bacterium with urease-specific monoclonal antibodies prior to challenge protects mice from *H. felis* infection (113). While the quantity of the antibody response is not altered by immunization, there can be a shift in the antigen specifity of the response (114). *H. pylori* has been observed coated by antibodies in the gastric mucosa. However, IgA deficient mice can be protectively immunized against *H. felis*. This is not due to a compensatory increase in IgM production, since protection can also be achieved in μMT mice, which lack any type of antibody response (115). Although antibodies may still play a role in *H. pylori*-immunity, they are not essential for protection.

The role of the adaptive cellular immune response in protective immunity is not completely understood. It is now apparent that protection can be achieved in the absence of MHC Class I restricted T cells (116,117). In fact, the only component of the adaptive immune response that has been shown to be necessary for the induction of protective immunity are MHC class II restricted $CD4^+$ T cells (112,116,117). Whether T cell differentiation into TH_1 or TH_2 type contributes to protection remains controversial. So far, no difference between protective and non-protective *Helicobacter*-specific T cells has been reported and the effector mechanisms induced by these T cells remain unknown. (This introduction reflects the knowledge about *H. pylori* infection and vaccine development in the year 2000, when this study was commenced.)

A broad-spectrum analysis comparing the genes expressed in the gastric mucosa of *H. pylori*-immunized/challenged mice to those expressed in the gastric mucosa of infected mice in a gene array could provide information of a mechanism of protective immunity. The advantages of the gene array technology are that it allows simultaneous expression analysis of thousands of genes to be monitored in parallel and it permits identification of quantitative differences in gene expression. The goal of the present study was to identify the genes that are expressed in the gastric mucosa of mice with a chronic *Helicobacter* infection, or protection from *Helicobacter*, and to identify potentially important differences that could be telling of a mechanism of protective immunity. Identification of the mechanism(s) associated with protective immunity in mice could facilitate the development of an efficacious and safe vaccine for use in humans.

2 MATERIALS AND METHODS

2.1 Stocks and Solutions

2.1.1 Helicobacter cultures

Solid phase culture medium for H. pylori and H. felis: 22 g of Columbia Blood Agar Base (Difco, Detroit, MI) in 500 ml of deionized water were autoclaved for at 121 ^{0}C for 20 min. Media was cooled in a 56 ^{o}C water bath and then sterile horse blood (Cleveland Scientific, Cleveland, OH) and antibiotics were added as specified below. The Blood Agar was poured into sterile petri dishes, 25 ml each, and left at room temperature until solid. All plates were stored at 4 ^{o}C.

Liquid culture medium for H. pylori: 21.5 g of Brucella Broth (Difco) in 500 ml of deionized water were autoclaved at 121 ^{0}C for 20 min and stored at room temperature. Shortly before use, culture medium was supplemented with 10% sterile fetal bovine serum (Gibco BRL, Grand Island, NY) and antibiotics were added as specified below.

Liquid culture medium for H. felis*:* 18.5 g of Brain Heart Infusion Broth (Difco) in 500 ml of deionized water were autoclaved at 121 ^{0}C for 20 min and stored at room temperature. Shortly before use, culture medium was supplemented with 10 % sterile fetal bovine serum (Gibco BRL) and antibiotics were added as specified below.

Antibiotics: Selection media for *H. pylori* contained final concentrations of 20 μg/ml trimethoprim, 6 μg/ml vancomycin, 2.5 μg/ml amphotericin B, and 16 μg/ml cefsulodin (all Sigma Chemical Co., St. Louis, MO). *H. felis* was grown using a similar selection media except that cefsulodin was substituted with 0.125 μg/ml polymyxin B (Sigma Chemical Co.).

2.1.2 Phosphate buffered saline (PBS)

PBS contained 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 and 1.47 mM KH_2PO_4 in deionized water, pH 7.4 (all reagents Fisher Scientific, Fair Lawn, NJ) and was autoclaved at 121 ^{0}C for 20 min.

2.1.3 Lowry assay reagents

Lowry reagent A: 1% $CuSO_4$ x $5H_2O$ (Fisher Scientific) in deionized water

Lowry reagent B: 2% sodium tartrate (Fisher Scientific) in deionized water

Lowry reagent C: 2% Na_2CO_3 (Fisher Scientific) in 0.1 M NaOH (Fisher Scientific) solution

Alkaline Copper Reagent: A:B:C at 1:1:98 respectively

2.1.4 Stuart's urease test broth

0.1 g/l yeast extract (Difco), 0.091 g/l KH_2PO_4 (Fisher Scientific), 0.095 g/l Na_2HPO_4 (Fisher Scientific), 20 g/l urea (Gibco BRL), 0.01g/l phenol red (Sigma Chemical Co.), in deionized water, sterile filtered

2.1.5 Reagents for anti-*Helicobacter* IgG ELISA assay

Carbonate/bicarbonate coating buffer: 0.1 M Na_2CO_3/$NaHCO_3$ (Fisher Scientific), pH 9.6, in deionized water

***Blocking buffer*:** 1% BSA (bovine serum albumin; Sigma Chemical Co.) in PBS

***Reagent diluent*:** 0.1% BSA in PBS

***Wash buffer*:** 0.05% Tween 20 (polyoxyethylenesorbitan monolaurate; Fisher Scientific) in PBS

Alkaline Phosphatase buffer: 0.1 M glycine (Fisher Scientific), 1 mM $MgCl_2$ (Fisher Scientific), pH 9.6, in deionized water

2.1.6 Reagents for RNA extraction

***DEPC-treated water*:** 0.1% of DEPC (Sigma Chemical Co.) were added to deionized water and thoroughly mixed with a sterile pipette tip. After 18 h, the water was autoclaved at 121 ^{0}C for 50 min.

2.1.7 Reagents for RT-PCR

***DNase I reaction buffer*:** 20 mM tris-HCl (pH 8.4), 2 mM $MgCl_2$, 50 mM KCl (provided as a 10x stock solution by Life Technologies, Gibco BRL)

PCR buffer: 20 mM tris-HCl (pH 8.4), 50 mM KCl (provided as a 10x stock solution by Life Technologies, Gibco BRL)

Loading buffer: 0.25% bromophenol blue (Fisher Scientific), 0.75% xylene cyanol (Kodak, Eastman Fine Chemicals, Eastman Kodak Company, Rochester, NY), and 70% glycerol (Fisher Scientific) in deionized water

TAE: 40 mM tris acetate, 2 mM Na_2EDTA x 2 H_2O in deionized water, pH 8.5

Agarose gel: 1% agarose (Fisher Scientific) in TAE, stained with 0.5 μg/ml ethidium bromide (Fisher Scientific) were heated until dissolved and cast into a gel.

2.1.8 Reagents for bulk spleen cell assay

***Complete HL-1 medium*:** serum-free HL-1 media (Biowhittaker, Walkersville, MD) supplemented with 1 mM L-glutamine (Gibco BRL)

2.1.9 Reagents for $CD4^+$ T cell assay

***Complete DMEM media*:** DMEM (Dulbecco's Modified Eagle Medium, Life Technologies, Gibco BRL) containing 10 mM HEPES (Fisher Scientific), 1 mM sodium pyruvate (Fisher Scientific), 50 μM β-mercaptoethanol (Fisher Scientific) and 50 units/ml penicillin base, 50 μg/ml streptomycin (utilizing penicillin G, disodium salt and streptomycin sulfate (Life Technologies, Gibco BRL)) supplemented with 10% FBS.

2.1.10 Reagents for IFN-γ ELISA

Wash buffer and reagent diluent: 0.1% BSA in PBS

Block buffer: 1% BSA in PBS

Alkaline phosphatase substrate buffer: 0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$ in deionized water, pH 10.6

2.2 Mice

Six- to eight-week-old, female, C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). For *in vitro* experiments, some female mice were also purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in microisolator cages under positive air pressure and fed autoclaved laboratory chow and water *ad libitum* at the Case Western Reserve University School of Medicine. Sera taken at the time of harvest from all immune mice included in the microarray analysis were tested for antibodies to 19 different viruses and mycoplasmas by Charles River Diagnostics (Wilmington, MA). No anti-viral antibodies were detected from any of these mice. The Case Western Reserve University animal facility is fully accredited by the American Association for Accreditation of Animal Care.

2.3 Bacteria

The *H. pylori* used was the mouse-adapted *H. pylori* SS1. *H. felis* strain CS1 was a generous gift from Dr. Steven Czinn at the Case Western Reserve University School of Medicine, Cleveland, OH. Both *H. pylori* SS1 and CS1 were grown on Columbia blood agar under microaerobic conditions (5% O_2, 10% CO_2) at 37 ^oC for 96 h. This microaerobic environment was created using the BBL® CampyPak Plus™ system within BBL®GasPak® jars. Prior to use as a challenge inoculum, bacteria were transferred to liquid media. *H. pylori* was grown in stationary cultures of 50 ml in a 37 ^oC incubator with 5% CO_2 for 24 to 48 hours. *H. felis* was transferred to 200 ml liquid culture medium and cultures were placed under microaerobic conditions (5% O_2, 10% CO_2) on a rotary shaker at 100 rpm at 37 ^oC for 24 – 48 hours.

2.4 Preparation of *H. pylori* and *H. felis* whole cell lysates

2.4.1 Sonication of bacteria

Cell lysates were prepared for immunizations and for ELISA antigen. Bacteria from 200 ml saturated liquid cultures of *H. pylori* or *H. felis* were harvested by centrifugation at 4000 g for 20 min. Cell pellets were resuspended in 2 ml PBS. The cells were lysed by 4 x 30 s bursts of power using a probe sonicator (Sonics and Materials Inc., Danbury, CT) set at 5% duty cycle and a power setting of 5. Between sonications, samples were kept on ice to prevent overheating. Unlysed bacteria were removed by centrifugation at 5000 g for 20 min and the supernatant filtered through a 0.45 μM pore filter. Sonicates were stored at –20 ^{o}C until used.

2.4.2 Protein determination assay

Protein concentration of sonicate was determined by the colorimetric method of Lowry (118). Briefly, 100 μl sample in different dilutions, standard (BSA at increasing concentrations of 0.1 mg/ml to 1 mg/ml) or deionized water were incubated with 1 ml Alkaline Copper Reagent each at room temperature for 15 min. 100 μl 1 M Folin and Ciocalteu's Phenol Reagent (Sigma Chemical Co.) were added to each tube and then mixed thoroughly. After 30 minutes all tubes were evaluated for optical density in a Beckman DU 640 spectrophotometer (Beckman instruments, Inc., Schaumburg, IL) at 750 nm. A standard curve was generated by plotting the predetermined concentration of each BSA standard against its corresponding optical density. The equation of the line was used to determine the concentration of the experimental samples using the observed OD_{750nm} readings.

2.5 Immunization and challenge of mice

Mice were immunized intranasally on days 0, 7, 14, and 21 by applying 100 μg *H. pylori* whole cell lysate antigen combined with 5 μg cholera toxin (List Biological Laboratories, Inc. Campbell, CA) directly onto the nares of each mouse. The total volume of inoculum was 20 μl. Mice were challenged with 1 x 10^7 CFU *H. pylori* SS1 on day 28 by oral inoculation.

Challenge was delivered by gastric intubation using sterile flexible tubing on the end of an 18g needle. When challenge was used to determine protection after immunization, always naïve animals were parallely infected as a control of the infectiveness of the challenge dose.

2.6 Experimental infection with *H. pylori* and *H. felis*

Chronic infection by *H. pylori* SS1 and *H. felis* CS1 was accomplished using liquid grown cultures as described above. The viability of both species was determined by microscopic examination for motility and considered sufficient if greater than 90% of the bacteria were motile. For *H. pylori*, six to eight week old mice were inoculated with 1 x 10^7 CFU on two consecutive days. The concentration of bacteria was determined by spectrophotometry based on a previously established growth curve for *H. pylori* at OD_{450nm}, and reconfirmed by quantitative culture. Since with *H. felis* no quantitative cultures are feasible because of its confluent growth, no growth curve could be determined. Therefore when using *H. felis*, mice were inoculated with 0.5 ml of saturated broth culture. Inoculations were accomplished by gastric intubation using flexible tubing on an 18g needle.

2.7 Serum collection

Blood was collected from mice prior to challenge by tail bleed and at necropsy by cardiac puncture. Blood samples were chilled on ice for 30 min in 1.5 ml microfuge tubes then stored overnight at 4 ^{o}C. Serum was then removed by centrifugation and stored at –20 ^{o}C until evaluated.

2.8 Diagnosis of infection

2.8.1 Urease detection assay

The presence of *H. pylori* or *H. felis* was assessed by the presence of urease activity in gastric biopsies. Gastric biopsies were surgically removed from experimentally infected mice by

cutting a narrow strip of tissue along the greater curvature of the stomach encompassing the antrum and fundus. Biopsies were placed in 0.5 ml of Stuart's urease test broth (119), and incubated at room temperature. Colonization by Helicobacter was confirmed by a change in broth color from orange to red within the first 24 hours.

2.8.2 Culture of organisms from biopsies

Cultures were performed on gastric biopsies from *H. pylori* and *H. felis* challenged mice to confirm the presence or absence of bacteria. Two biopsies were surgically removed from the antral/fundic region and homogenized in 200 µl Columbia broth (Difco). Homogenates were plated on solid culture medium in 100 µl aliquots at different dilutions. After 96 hours in microaerobic conditions, bacteria were confirmed as *H. pylori* or *H. felis* based upon colony morphology, Gram stain, and the production of urease, catalase, and oxidase.

2.8.3 Histologic bacterial load determination

Histologic bacterial load determination was performed to confirm infection in the event that *H. pylori* or *H. felis* could not be easily quantified by bacterial culture. A longitudinal strip of stomach wall encompassing all gastric tissue layers was surgically removed from the greater curvature of the stomach from the duodenum to the gastric cardia. Tissues were fixed in 10% buffered formalin (Fisher Scientific) and processed for histologic examination at Histology Consultation Services (Everson, WA). Tissues were embedded in paraffin, cut into 7 µm sections and silver-stained by the Steiner method to facilitate the identification of *H. pylori* and *H. felis* based on bacterial location and morphology. Sections were evaluated by Dr. Raymond W. Redline, Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH, with the examiner blinded to the experimental treatment of each mouse. Extent of infection was estimated by the average number of *H. pylori* positive glands/centimeter observed in histological sections. Both fundus and antrum of the stomach were scored in this manner and averaged to give one value (from 0 to 5) for bacterial load of each mouse.

2.9 Histology

A longitudinal strip of stomach wall encompassing all gastric tissue layers was surgically removed from the greater curvature of the stomach from the duodenum to the gastric cardia. Paraffin embedded 7 μm sections of each gastric strip were stained with Hematoxylin and Eosin for histologic evaluation by Histology Consultation Services. Sections were evaluated by Dr. Raymond W. Redline, Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH, with the examiner blinded to the experimental treatment of each mouse. Inflammation was graded on a scale from 0 – 5 based upon the linear extent (focal, multifocal, patchy, or diffuse), depth (superficial and/or basal, panmucosal, or extending to submucosa or muscular layers), and character of the inflammatory infiltrate as defined by the types of infiltrating cells and tissue architecture changes (Grade 1 = rare inflammatory cells, Grade 2 = multiple clusters of inflammatory cells, Grade 3 = diffuse inflammation of variable intensity, without architectural disruption, Grade 4 = diffuse inflammation, uniformly severe, without architectural disruption, Grade 5 = diffuse inflammation, uniformly severe, with architectural disruption)

2.10 Anti-*Helicobacter* IgG determination

Ninety-six-well microtiter plates (Nalge-Nunc, Roskilde, Denmark) were coated with 50 μl/well *H. pylori* SS1 or *H. felis* CS1 lysate (10μg/ml) in carbonate/bicarbonate coating buffer overnight in a humid chamber at 4 ^{o}C. Plates were emptied by inversion and blocked for 1 h at room temperature with 200 μl/well of blocking buffer. Plates were washed three times between each step with wash buffer. Wells were then incubated with 50 μl of a serum sample, serially diluted with the reagent diluent in half log increments, for one hour at room temperature. One sample with previously known high titers was used as a positive control, serum from a naïve animal as negative control. Two wells were filled with reagent diluent only to serve as a blank and the conjugate control. Helicobacter-specific antibodies were detected by incubating the wells with 50 μl of 0.2 μg/ml alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) in reagent diluent for a further 1 h at room temperature. Again in this step the blank well was filled with reagent diluent only. Plates were developed by the addition of 100 μl/well of 1 mg/ml p-

nitrophenylphosphate (Sigma Diagnostics, Inc., St.Louis, MO) dissolved in alkaline phosphatase buffer, and the optical density at 405 nm was measured and recorded on a Spectra Max plate reader (Molecular Devices, Sunnyvale, CA). End-point titers were defined as the highest dilution of sample that generated a signal of at least 0.05 OD_{405nm} units above the conjugate control.

2.11 RNA extraction

After removing longitudinal strips of the greater curvature for histology, and biopsies for urease detection and culture from each mouse stomach the remaining stomach tissue (both antrum and fundus encompassing all layers of the gastric wall) was frozen at –70 ^{o}C until processed for RNA isolation. Cell suspensions from in vitro assays were transferred to microfuge tubes, pelleted by centrifugation and directly processed after removal of supernatants. Total RNA was isolated using TRIzol reagent according to manufacturer's instructions (Life Technologies, Gibco BRL). Briefly, tissue or cells were homogenized in 1 ml TRIzol Reagent per 50 to 100 mg of tissue or 5-10 x 10^6 cells and centrifuged at 12000 g at 2 to 8 ^{o}C for 10 min to remove extracellular membranes, polysaccharides and high molecular weight DNA. Samples were than incubated for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform (Fisher Scientific) per 1 ml of TRIzol were added, tubes shaken vigorously for 15 s, incubated at room temperature for 2 to 3 min, and centrifuged at 12000 g at 2 to 8 ^{o}C for 15 min. The colorless upper aqueous phase was then transferred to a fresh tube, and the RNA was precipitated by addition of 0.5 ml isopropyl alcohol (Fisher Scientific), and incubation at room temperature for 10 min. Following centrifugation at 12000 g for 15 min at 2 to 8 ^{o}C, the supernatant was removed and the RNA pellet washed with 1 ml 75 % ethanol (Fisher Scientific) per ml TRIzol. Then samples were centrifuged at 7500 g at 2 to 8 ^{o}C for 5 min, supernatants removed and the pellet air-dried for 5 to 10 min. The RNA was then dissolved in DEPC-treated water by incubation at 55 ^{o}C for 10 min.

RNA amounts were determined by spectrophotometry in a Beckman DU 640 spectrophotometer at 260 nm, while complete dissolution of RNA was confirmed by the OD_{260nm}/OD_{280nm} quotient.

2.12 Gene array analysis

For gene array analysis RNA from 5 mice per group was pooled together, 20 μg RNA from each mouse for a total of 100 μg per group. Each pool of total RNA was used to generate cRNA and hybridize Affymetrix Murine Genome U74A array gene chips (Affymetrix, Inc., Santa Clara, CA) by the Comprehensive Cancer Center of Case Western Reserve University and University Hospitals of Cleveland Gene Expression Array Core Facility. Raw data were then analyzed with Affymetrix software (in part with assistance of Patrick Leahy, Gene Expression Array Core Facility), further analysis and grouping of genes was then carried out with Microsoft Excel software.

2.13 RT-PCR

2.13.1 DNase I digestion of RNA preparation

4 μg total RNA was incubated with 2 units of Deoxyribonuclease I, Amplification grade (Life Technologies, Gibco BRL) in DNase I reaction buffer (Life Technologies, Gibco BRL) in a 20 μl total volume for 15 min at room temperature. The reaction was stopped by addition of 2 μl of 25 mM EDTA (Life Technologies, Gibco BRL) and incubation at 65 ^{o}C for 15 min.

2.13.2 First strand cDNA synthesis

Half of the DNase treated RNA (2 μg) was then used to generate cDNA using the Superscript™ Preamplification System for First Strand Synthesis (Life Technologies, Gibco BRL) according to manufacturers instructions, while the other half was used as non reverse transcribed control. All reagents were provided in the kit unless otherwise mentioned.
Briefly, 1μl of 0.5mg/ml Oligo(dT)$_{12\text{-}18}$ were added to 11μl of the DNAse digestion reaction mix (containing 2 μg of RNA) and the mixture was denatured at 70 ^{0}C for 10 min. After addition of 2 μl of 10X PCR buffer, 2 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP mix and 2 μl of 0.1 M DTT, the reaction mix was blended gently and collected by centrifugation, then

annealed at 42 ^{0}C for 5 min. Then, 200 units of SUPERSCRIPT II RT reverse transcriptase were added and, after gently mixing, synthesis of the cDNA carried out at 42 ^{0}C for 50 min. Final concentrations of reagents in this reverse transcription reaction mix were 30 mM Tris-HCl (pH 8.4), 3.5 mM $MgCl_2$, 75 mM KCl, 1.25 mM EDTA, 0.5 mM dNTP mix (containing 0.5 mM of each dATP, dGTP, dCTP and dTTP), 10 mM DTT, 0.025 mg/ml $Oligo(dT)_{12-18}$ and 10 units/μl reverse transcriptase. The reaction was terminated by heating to 70 ^{0}C for 10 min. To remove RNA, 2 units (1 μl) of *E. coli* RNase H were added and tubes were incubated at 37 ^{0}C for 20 min. cDNA was either directly used for PCR or stored at –20 ^{0}C until needed. As a positive control, a parallel reverse transcription of the control RNA provided in the kit was carried out along with samples.

2.13.3 Amplification of the target cDNA

Subsequent PCR on 10% of the generated cDNA for each reaction was then performed as recommended in the instructions of the Superscript™ Preamplification System. Final concentrations of reagents in the reaction mix were 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 0.2 μM of each primer and 50 units/ml (2.5 units total) Platinum® Taq polymerase (Life Technologies, Gibco BRL).

The following primers were used:

Primer	***Sequence***
G3PDH (forward)	5'-CCCTTCATTGACCTCAACTACATGGT-3'
G3PDH (reverse)	5'-GAGGGGCCATCCACAGTCTCCTG-3'
at annealing temperature 55 ^{o}C	
GARG 16 (forward)	5'-CTCAGAGCAGGTCCAGTTCC-3'
GARG 16 (reverse)	5'-CCTCACAGTCCATCTCAGCA-3'
at annealing temperature of 58 ^{o}C	
GARG 49 (forward)	5'-AGAGCTATTGAGTTACTTGGAA-3'
GARG 49 (reverse)	5'-ATTTGCTACTGGAGGTTAAGATCTG-3'
at annealing temperature of 55 ^{o}C	

mIRF7 (forward)	5'-AGCAGCGGCCAGTACGAGGG-3'
mIRF7 (reverse)	5'-GGCTGTACTGCAGAACCTGAA-3'
at annealing temperature of 55 ^{o}C	
Schlafen 4 (forward)	5'-GCTGGCTCTGCCTATATTGC-3'
Schlafen 4 (reverse)	5'-GTCCCATTCCATCTCTGGTG-3'
at annealing temperature 55 ^{o}C	
IRG-47 (forward)	5'-GATCTG GGTGCGTTTGATTTCC-3'
IRG-47 (reverse)	5'-TTAAAGCCTGGAAGGCAGAA-3'
at annealing temperature 55 ^{o}C	

Primers were ordered from Integrated DNA Technologies, Inc., Coralville, IA.

The chain reaction was performed in an Amplitron II thermal cycler (model DB 80235, Barnstead/Thermolyne, Dubuque, IA) with the following steps:

Step	***Temperature***	***Time***
1 - Denaturation	94 ^{o}C	180 s
2 - Denaturation	94 ^{o}C	60 s
3 - Annealing	see primer table	60 s
4 - Extension	72 ^{o}C	90 s

Steps two to four were repeated in 35 cycles, and tubes chilled on ice after the reaction.
As a positive control, PCR on the cDNA generated from the control RNA was carried out with the primers provided in the Superscript™ Preamplification System kit along with samples.

2.13.4 Visualization of amplification products and quantification

9 µl of PCR products were mixed with 1 µl of loading buffer and resolved on 1% agarose slab gels in TAE, stained with ethidium bromide. 1 µg of a 100 bp DNA ladder (Life Technologies, Gibco BRL) was used in one lane to estimate the size of amplified products.

Analysis of gels was performed using a Gel Doc 1000 digital documenting system (BioRad Laboratories, Hercules, CA). Images were analysed with Multi-Analyst software (Bio-Rad Laboratories).

2.14 Bulk spleen cell assay

2.14.1 Isolation of bulk spleen cells

At necropsy, spleens were surgically removed from naïve, *H. pylori*-infected, or *H. pylori*-immunized mice and then homogenized in 5 ml of sterile filtered Hank's balanced salt solution without calcium or magnesium (Gibco BRL). Cell suspension was filtered (70 μm pore size) and cells were collected by centrifugation at 200 g for 8 min. Red blood cells were lysed by resuspension of cells in 1 ml of distilled water and passage through a 5 ml pipette tip. To prevent lysis of leucocytes, 9 ml of Hank´s balanced salt solution without calcium or magnesium were added immediately. White cells were collected by centrifugation at 200 g for 8 min and resuspended in 10 ml of the media to be used in the culture. Cells were counted after staining a diluted sample with equal amounts of 0.4% trypane blue solution (Sigma Chemical Co.) and diluted to the desired concentration.

2.14.2 *In vitro* assay

Cells were resuspended in complete HL-1 media, pooled for each group, and distributed in 48 well tissue culture plates at 5 x 10^6 cells per well in 1 ml complete HL-1 media. Stimulation was provided by the addition of either 5 x10^7 *H. pylori* SS1 per well or 100 μg/ml *H. pylori* SS1 whole cell lysate antigen. Where live *H. pylori* SS1 was added, the bacteria were first washed in sterile PBS and then resuspended in complete HL-1 media. To confirm the bacterial concentration, quantitative cultures were performed on a proportion of this suspension. As a positive control, cells were stimulated with 10 μg/ml Concanavalin A, while negative controls were incubated with media only. Each group consisted of at least three separate wells. After incubation at 37 ^oC at 5% CO_2 for 72 h, RNA was isolated from cell pellets, supernatants were collected and stored at –70 ^oC until assessed for interferon levels.

2.15 $CD4^+$ T cell assay

2.15.1 Isolation of macrophages

Activated peritoneal macrophages were obtained from mice injected intraperitoneally with 100 μg Concanavalin A, four days prior to harvest. At harvest, the peritoneal cavity of mice asphyxiated with CO_2 was exposed and filled with 10 ml sterile PBS by injection through the lower abdominal fat pad. Each mouse was gently rocked to optimize macrophage recovery and the PBS subsequently withdrawn using a syringe. Cells were collected by centrifugation at 500 g at 4 ^{0}C for 7 min, resuspended in 10 ml complete DMEM and a proportion of 50 μl was removed and diluted with 200 μl of acetic acid to lyse red blood cells for counting. Cell suspensions were diluted to the required concentration and then 2.5 x 10^5 cells/well were plated in 96 well tissue culture plates. Cells were incubated for 8 h at 37 ^{o}C in 5% CO_2 and the nonadherent cells were removed by washing three times with complete DMEM.

2.15.2 Purification of $CD4^+$ T cells

$CD4^+$ T cells were purified from spleen cells suspensions of naïve, *H. pylori*-infected, or *H. pylori*-immunized mice using a Mouse T cell $CD4^+$ Subset Column Kit (R&D Systems, Inc., Minneapolis, MN) based on manufacturer's instructions. Briefly, spleen cell suspensions were incubated with a cocktail of monoclonal antibodies specific for surface antigens present on B cells, monocytes and other T cell subsets. Antibody-coated cells were removed by binding to glass beads coated with anti-immunoglobulin antibodies and the $CD4^+$ T cells were eluted.

2.15.3 Determination of $CD4^+$ cell purity

The quality of separation was evaluated by flow cytometry. $CD4^+$ spleen cell preparations from each group were pelleted at 1.25 x 10^6 cells per tube, washed with sterile PBS and stained with 1 μg/ml PE-conjugated anti-mouse CD4 monoclonal antibody L3T4 (Pharmingen, San Diego, CA). Cells were fixed with 4% paraformaldehyde (Fisher Scientific)

and analyzed in a fluorescent activated cell sorter (FACScan, Becton Dickenson, Mt View, CA). An unstained sample of cells was used to set gates and cut-off.

2.15.4 *In vitro* assay

The $CD4^+$ T cells were suspended in complete DMEM and distributed in a 96 well tissue culture dish at 5 x 10^5 cells per well. Prior to the addition of the $CD4^+$ T cells each well had been coated with 2.5 x 10^5 activated peritoneal macrophages as described above. Stimulation was provided by the addition of 3.9 x 10^5 *H. pylori* SS1 per well (as determined by colony count) or 100 μg/ml *H. pylori* SS1 whole cell lysate antigen. Controls consisted of macrophages without further stimulation, macrophages stimulated by *H. pylori* SS1, $CD4^+$ T cells and *H. pylori* SS1 in the absence of macrophages, and $CD4^+$ T cells alone. Each group consisted of at least three separate wells. Cells were cultured for 72 hours at 37 ^oC in 5% CO_2. Supernatants were removed and stored at –70 ^oC until assessed for interferon levels.

2.16 IFN-α ELISA

The supernatants from the bulk spleen cell assay were assessed for IFN-α concentrations using a mouse IFN-α ELISA kit according to manufacturer's instructions (PBL Biomedical Laboratories, New Brunswick, NJ). All reagents were provided in the kit unless otherwise mentioned.

Briefly, 100 μl of undiluted supernatant were tested in duplicate in the wells of a precoated microtiter plate. Two rows of microtiter wells were used for IFN-α standards ranging from 12.5 to 500 pg/ml, while two wells were reserved as blank. The plate was incubated for 1 h , then emptied by inversion and washed once with Final Wash solution. 100 μl of Antibody solution were added and the plate was incubated for 24 h before being emptied and washed for three times. After incubation with 100 μl of HRP Conjugate Solution for another hour, the plate was washed four times and developed by addition of 100 μl TMB Substrate Solution and incubation in the dark for 15 min. The reaction was stopped with 100 μl Stop Solution and the plate was read at 450 nm within 5 min using a Spectra Max plate reader. A standard curve was generated by plotting the predetermined concentration of each IFN-α standard

against its corresponding optical density for each plate. The equation of the line was used to determine the concentration of the experimental samples using the observed OD_{450nm} readings.

2.17 IFN-γ ELISA

The supernatants from the bulk spleen cell and $CD4^+$ T cell assays were assessed for IFN-γ concentrations by ELISA. Microtiter plates (Nalge-Nunc) were coated with 50 µl/well rat anti-mouse IFN-γ capture antibody (clone R4-6A2, Pharmingen) at 2.5 µg/ml in PBS overnight at 4 ^oC. Plates were emptied by inversion, and washed three times with 200 µl/well wash buffer between each subsequent step. Wells were blocked for 2 h with 200 µl/well blocking buffer. Undiluted samples were tested in triplicate at 50 µl/well for 2 h. Two rows of microtiter wells were used for IFN-γ standards (recombinant mouse IFN-γ, Genzyme, Cambridge, MA; dilutions ranging from 10 ng/ml to 0.078125 ng/ml in 2-fold serial dilutions in reagent diluent). Six wells were reserved as blank and conjugate control. IFN-γ was detected using 50 µl/well biotinylated rat anti-mouse IFN-γ monoclonal antibody (clone XMG1.2, Pharmingen) at 2 µg/ml in reagent diluent for 2 h and then incubating with 100 µl/well Streptavidin-alkaline phosphatase (ZyMax™ Streptavidin-Alkaline Phosphatase, Zymed, San Francisco, CA) at 1 µg/ml in reagent diluent for 90 min. Plates were developed by the addition of 100 µl/well p-nitrophenylphosphate at 1 mg/ml in substrate buffer. Wells were read at 405 nm until the 10 ng/ml IFN-γ standard equalled OD_{405nm} 2.1 on a Spectra Max plate reader. The concentration of IFN-γ in each sample was determined by comparison to the standard curve generated from each plate.

3 RESULTS

3.1 Prerequisite: Mice used in gene array analysis are protected from *H. pylori* infection by intranasal immunization with *H. pylori* sonicate and immunized/challenged animals display heightened inflammation and systemic antibody responses compared to chronically infected mice

In order to generate stomachs with the appropriate pathology to perform a molecular analysis of the gastric tissues of chronically infected and immunized/challenged mice, groups of five mice were either chronically infected with *H. felis* or *H. pylori*, or immunized and challenged with live *H. pylori* as described above. The stomachs were assessed on day 56 (28 days post challenge for immunized mice, and 56 days post inoculation of infected mice). As a prerequisite for a comparison between a protective and a non-protective immune response, the protective efficacy of the immunization had to be determined by analysis of the bacterial load in stomach sections of the experimental animals. Gastric inflammation was analysed in histologic sections, and the systemic anti-*Helicobacter* antibody response was measured.

3.1.1 Experimental animals are protected from *H. pylori* infection by intranasal immunization

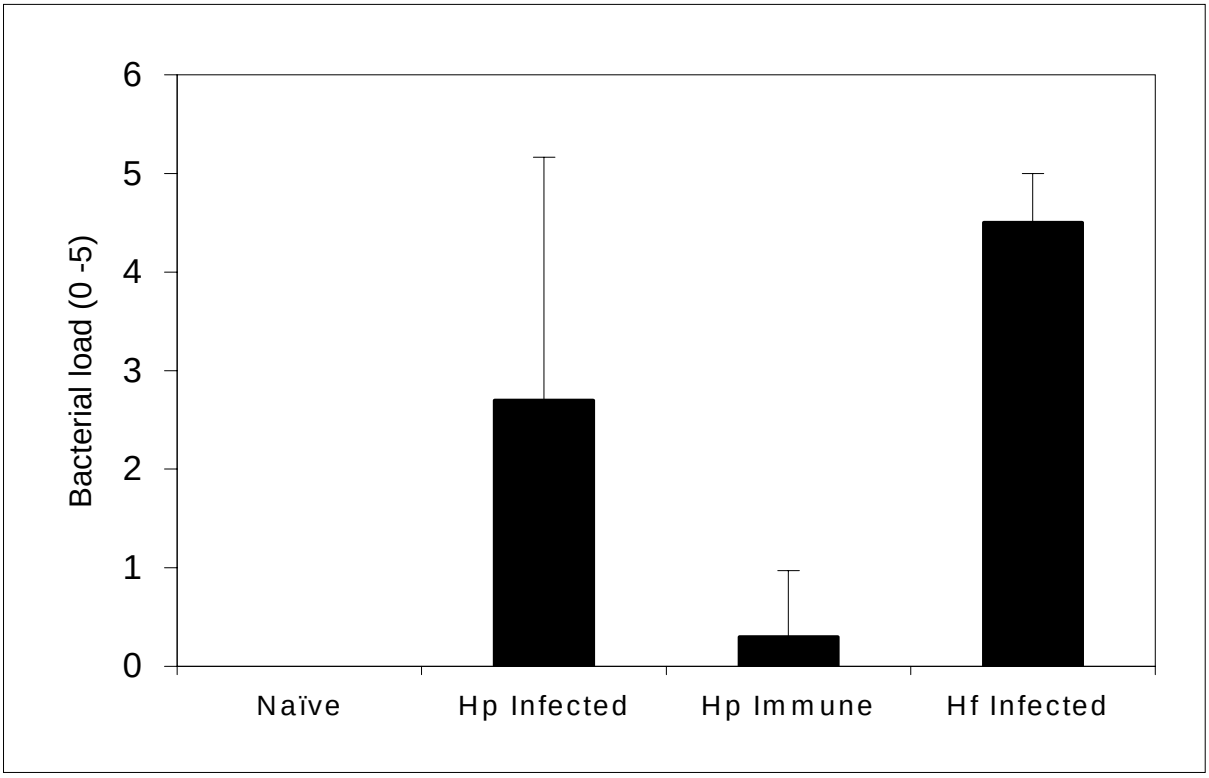

Figure 1. *Helicobacter* load in the gastric glands of naïve, *H. pylori*-infected, *H. pylori*-immunized/challenged and *H. felis*-infected mice analysed by direct visualization of silver stained gastric sections. Mice were assigned a global score ranging from 0 – 5 based upon the number of Helicobacter positive glands/centimeter in longitudinal gastric strips. Each group included five animals whose stomach RNA was later used for microarray analysis.

Since part of the cultures performed from the gastric tissue of experimental animals was contaminated analysis of bacterial loads was carried out by histology. A longitudinal strip from the greater curvature of the stomach was used for histology and bacterial load determination, while gastric tissue from the fundus and from the antrum encompassing the whole stomach wall (including the muscular layer) was used to generate RNA for microarray analysis, as described above.

No *H. pylori* could be identified in gastric biopsies from immunized/challenged mice by either urea broth test or bacterial culture of gastric biopsies, although bacteria could be seen in one mouse on direct inspection of silver-stained histologic sections (**Figure 1**). In contrast, three of five non-immune, *H. pylori*-infected mice generated positive urea test broth reactions and organisms were readily identified in the glands of silver stained tissue. The average global bacterial load determined in histologic sections for these mice was significantly higher than

for immunized mice (P = 0.0113). *H. felis*-infected mice displayed robust colonization of gastric tissue. **Image 1** shows the mucosal layer of two representative sections of silver stained stomach strips used for quantification of the bacterial load.

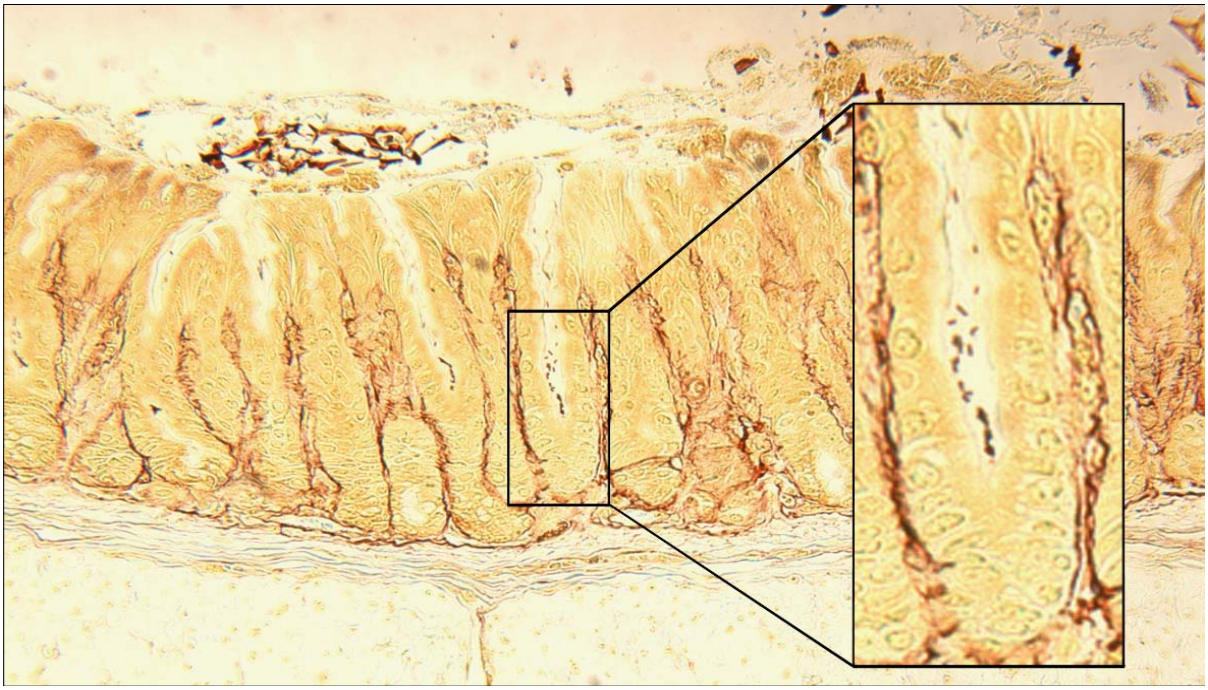

Image 1.a *H. pylori* can be readily detected in the gastric glands of infected mice in silver stained stomach sections (Steiner's stain).

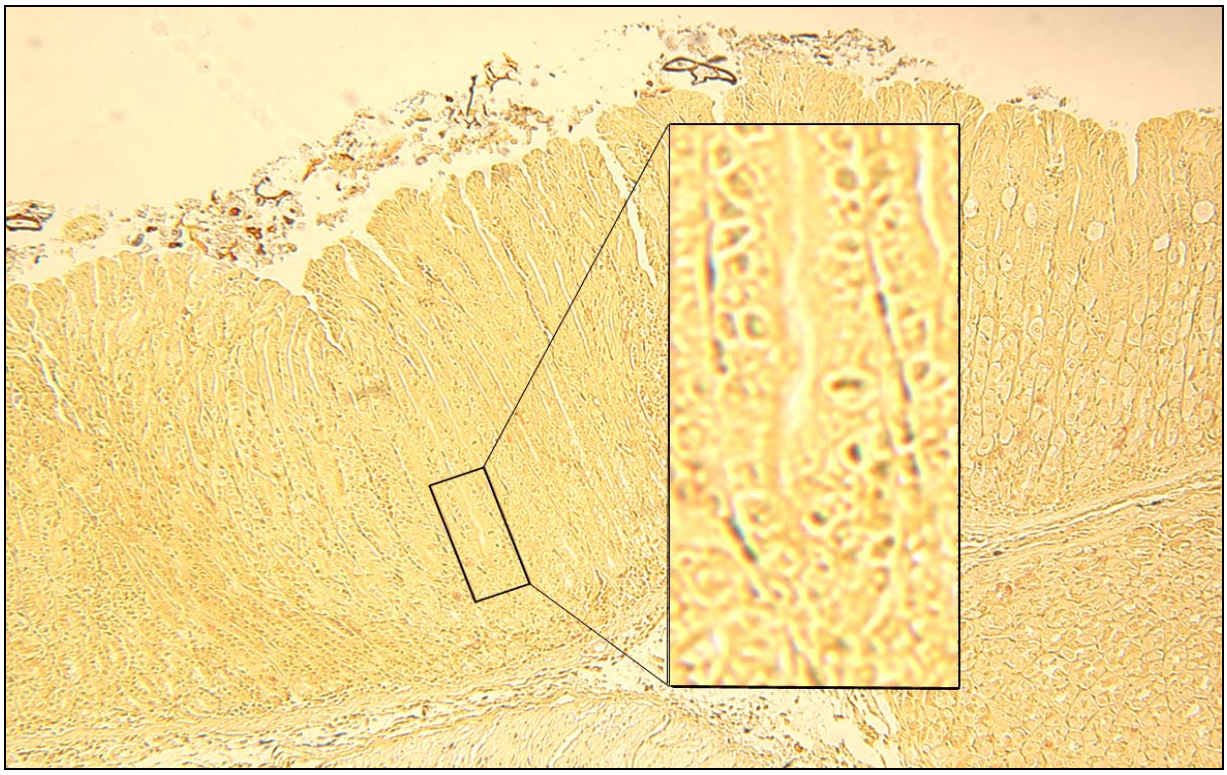

Image 1.b No *H. pylori* can be detected in the gastric glands of immunized/challenged mice in silver stained stomach sections (Steiner's stain).

3.1.2 Gastric inflammation is more intense in *H. pylori*-immunized/challenged mice than in *H. pylori*-infected mice

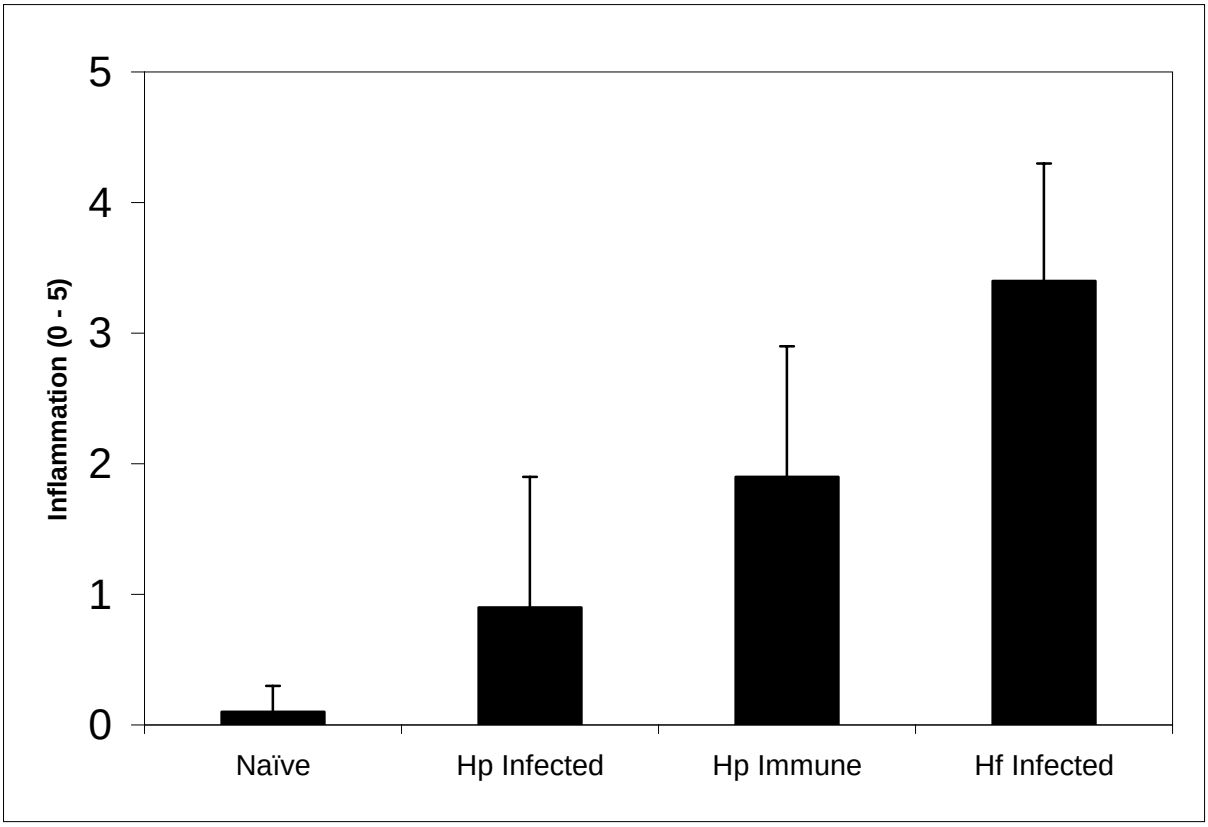

Figure 2. Inflammation of the gastric mucosa following infection or challenge of immunized mice. Inflammation was graded on a scale from 0 – 5 (Grade 1 = rare inflammatory cells, Grade 2 = multiple clusters of inflammatory cells, Grade 3 = diffuse inflammation of variable intensity, without architectural disruption, Grade 4 = diffuse inflammation, uniformly severe, without architectural disruption, Grade 5 = diffuse inflammation, uniformly severe, with architectural disruption). Each group included five animals whose stomach RNA was later used for microarray analysis.

Gastric tissues from the mice used for microarray analysis were also scored for inflammation by assessment of Haematoxylin and Eosin stained gastric sections (**Figure 2**). One longitudinal strip of the stomach was used for histology and bacterial load determination, while gastric tissue from the fundus and from the antrum encompassing the whole stomach wall (including the muscular layer) was used to generate RNA for microarray analysis, as described above.

Images 2.a-c show sections of Haematoxylin and Eosin stained stomach strips used for histologic evaluation. These images are representative histologic pictures of each group of mice included in the microarray analysis.

H. felis-infected mice responded with vigorous inflammation, which was the highest of all groups at an average inflammatory score of 3.4 ± 0.9, compared to *H. pylori*-infected mice which displayed only mild inflammation averaging 0.9 ± 1.0 (P = 0.0002). Mice that had been immunized against *H. pylori* prior to challenge also showed inflammation. This "post-immune gastritis" has been reported previously and similar to other studies its intensity (1.9 ± 1.0) was greater than in non-immunized *H. pylori*-infected mice although this difference did not reach significance.

Due to the differences in the inflammatory infiltrate between groups the material used for RNA preparation did also contain different amounts of inflammatory cells, especially neutrophils, which were most numerous in the *H. felis*-infected and the *H. pylori*-immunized/challenged group. These differences in the cellular composition of the biopsies need to be taken into consideration in the interpretation of the microarray results.

Image 2. Histologic pictures of gastric tissue (including mucosal, submucosal and in some sections muscular layer)

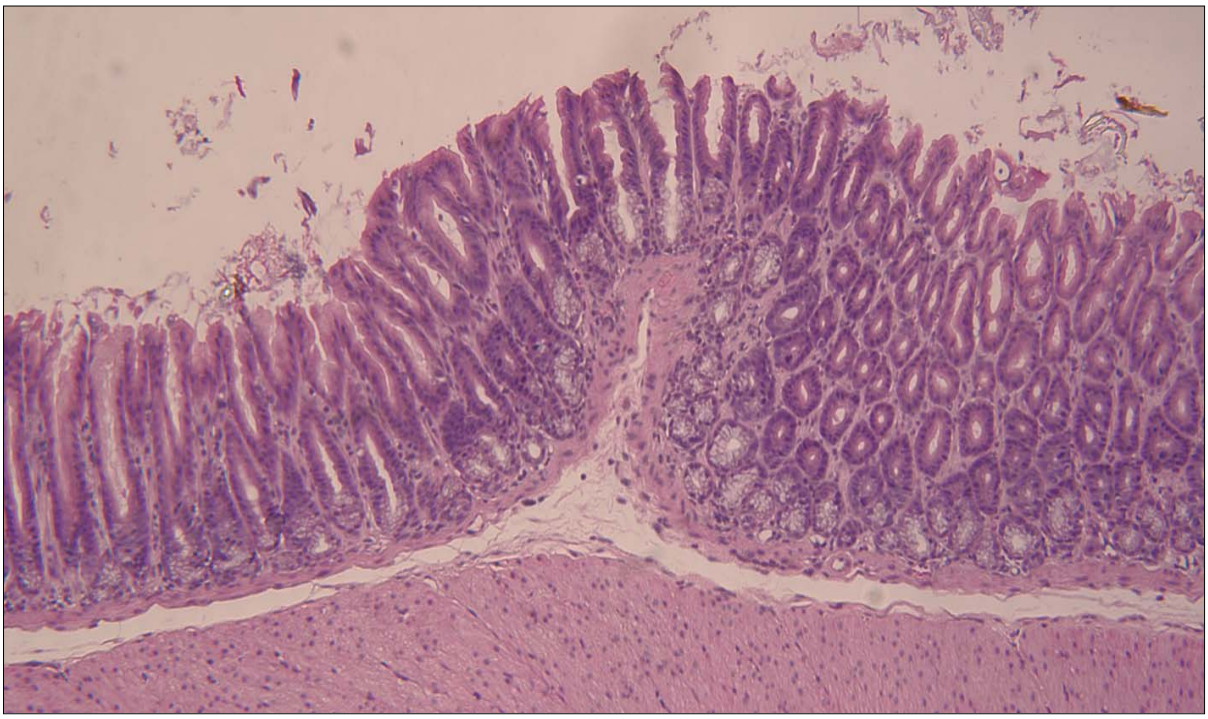

Image 2.a Haematoxylin and Eosin stained gastric section of a naive mouse without signs of inflammation.

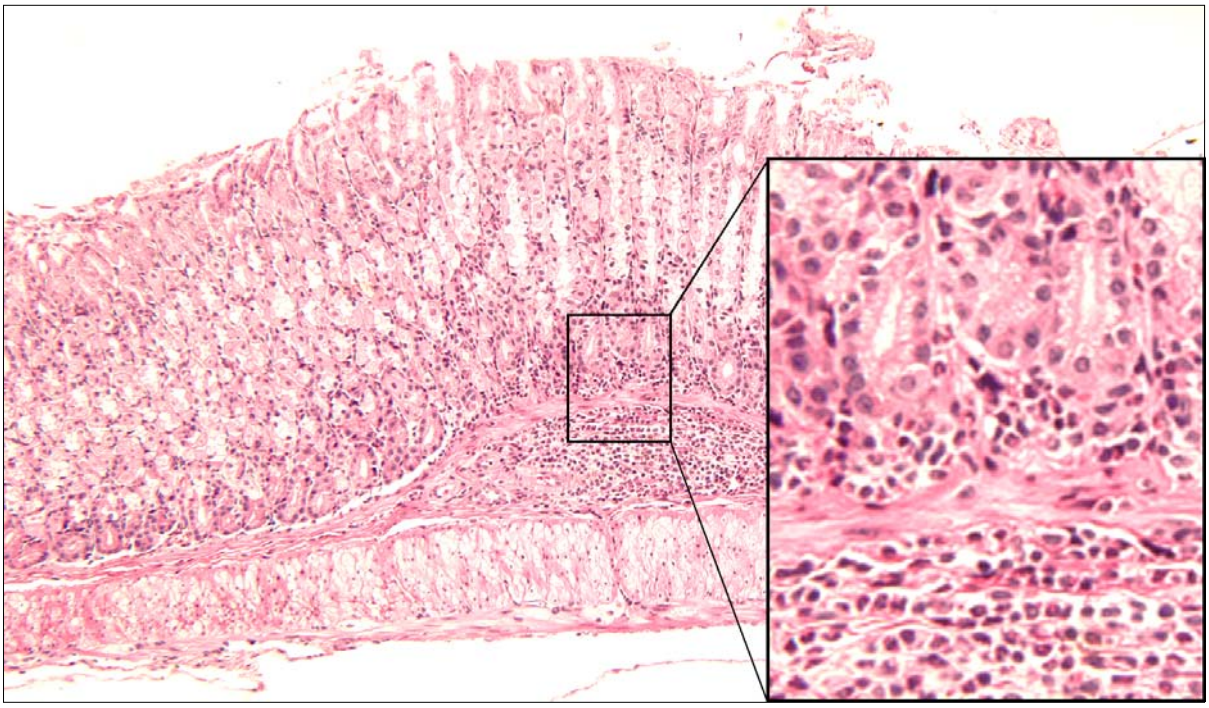

Image 2.b Haematoxylin and Eosin stained gastric section of a mouse with chronic *H. pylori* gastritis. Inflammatory cells mainly consisting of neutrophil granulocytes infiltrate the gastric mucosa.

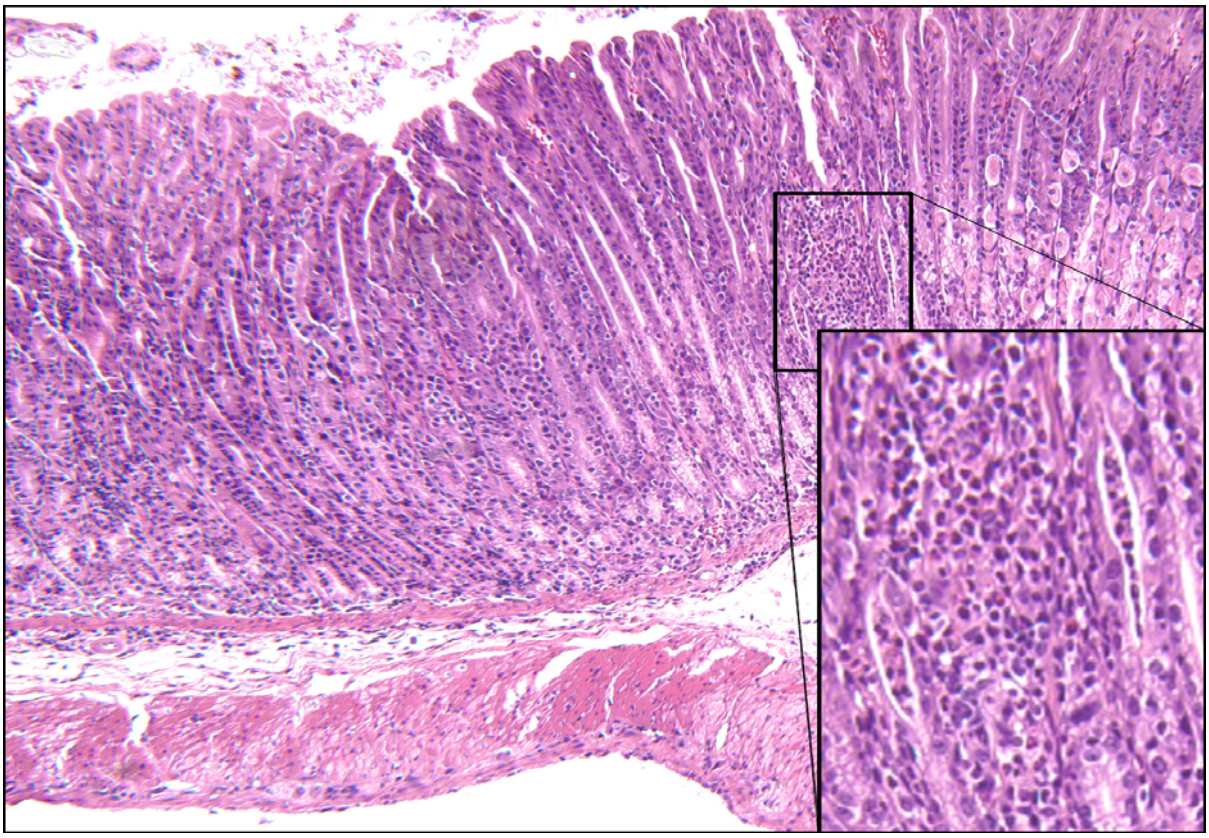

Image 2.c Haematoxylin and Eosin stained gastric section of a *H. pylori*-immunized and challenged mouse. An inflammatory infiltrate mainly consisting of neutrophil granulocytes arises in the gastric mucosa. Crypt abscesses can be detected in gastric glands.

3.1.3 *H. pylori*-immunized/challenged animals display a heightened systemic antibody response compared to chronically *H. pylori*-infected mice

Serum anti-*Helicobacter* titers were determined as a measure of the host adaptive systemic immune response to infection or immunization (**Figure 3**). Serum IgG titers were determined using the bacterial lysate antigen from the relevant bacterial species and compared between groups. Anti-*Helicobacter* IgG titers were compared at the time of harvest, depicting the momentary intensity of the humoral immune response.

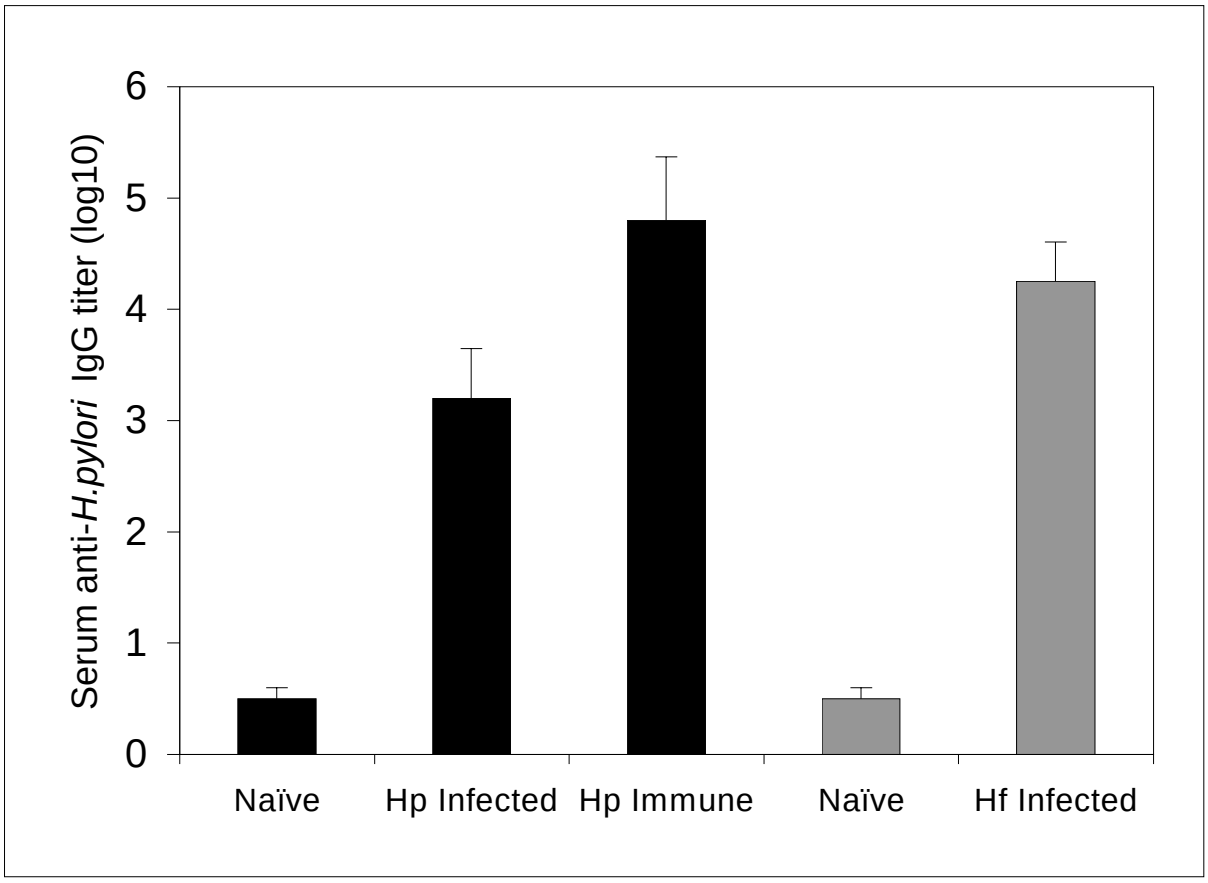

Figure 3. Serum anti-*H. pylori* or anti-*H. felis* IgG titers from naïve, *H. pylori*-infected, *H. pylori*-immunized/challenged and *H. felis*-infected mice at time of harvest. Sera were assessed by ELISA with bacterial lysate antigen, either from *H. pylori* (black bars) or *H. felis* (grey bars). Each group included five animals whose stomach RNA was later used for microarray analysis.

Mice that had been challenged with *H. pylori* subsequent to immunization had the highest titers at necropsy ($\log_{10}$ 4.8 ± 0.57). These titers were significantly greater than in mice with chronic *H. pylori* infection ($P < 0.0001$). Infection with *H. felis* also generated a robust

humoral immune response that nearly reached the level of the *H. pylori*-immunized/challenged animals. All three experimental groups of mice responded with significantly higher anti-*Helicobacter* IgG titers than their corresponding naïve controls ($P < 0.0001$).

In order to detect changes of the humoral immune response over time, and to further characterize the immune response to challenge or infection, ELISAs were performed on the different groups of animals at several time points (**Figure 4**).

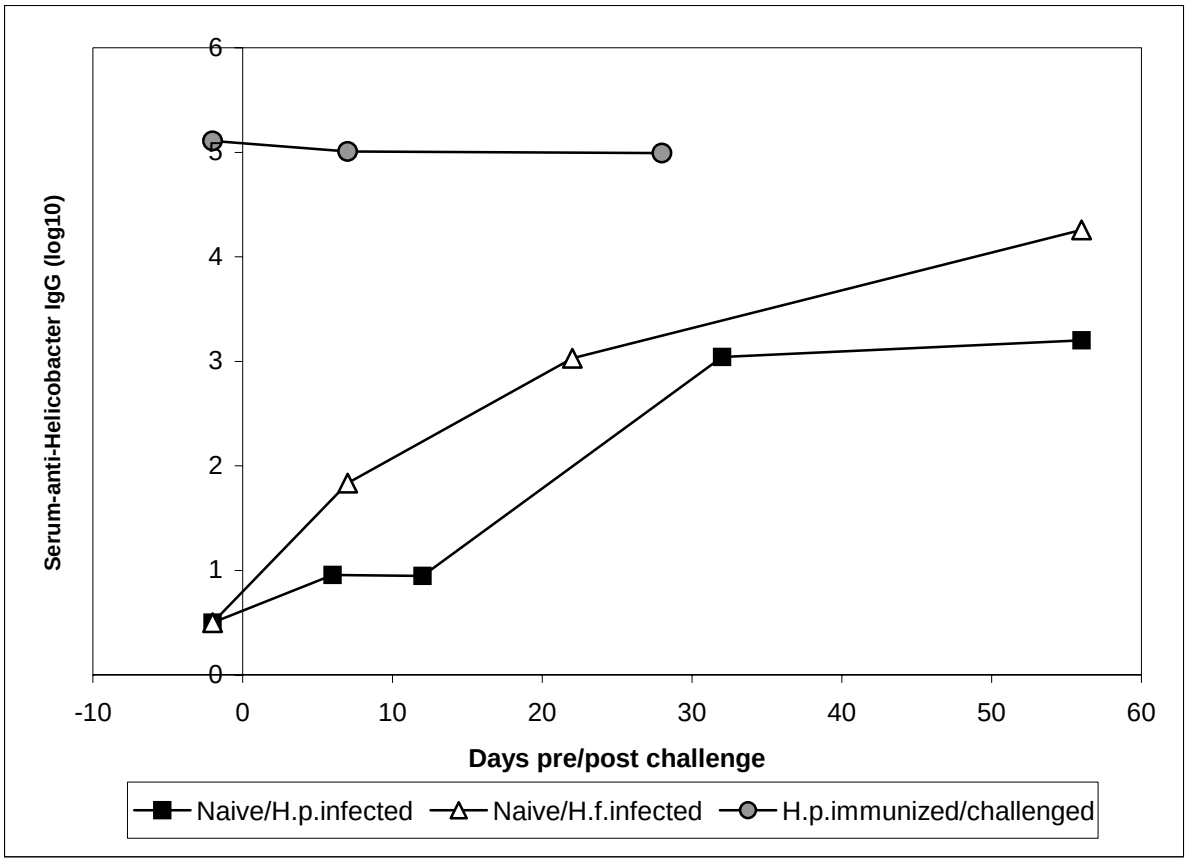

Figure 4. Serum anti-*Helicobacter* IgG titers of experimental animals at several time points. Either naïve or *H. pylori* immunized animals were inoculated with *H. pylori* or *H. felis* respectively on day 0 (and 1). ELISAs with *H. pylori* and *H. felis* bacterial lysate were performed at several time points before and after challenge. Each data point reflects the average of a group of five mice.

While pre- and post-challenge titers from immunized animals were not appreciably different, and did not change over time after challenge, naïve animals responded to a *H. pylori* or *H. felis* infection with titers that gradually increased over time.

3.2 Gene expression patterns in gastric tissue of immunized/challenged mice differ from those in chronically infected mice

The RNA from mice in each group was pooled in equal proportions and used to generate labeled cRNA for binding to gene array chips containing sequences representing over 10,000 different genes. RNA from the gastric mucosa of five naïve control mice was pooled and used to establish background levels of expression. A 2-fold increase relative to tissue from naïve mice was used as the threshold for a significant change in expression of each gene.

There were 314 genes of known and unknown function upregulated in *H. pylori*-immunized/challenged mice compared to naïve mice. For *H. felis*-infected mice and *H. pylori*-infected mice there were 327 upregulated genes and 89 upregulated genes compared to naïve respectively.

3.2.1 Differential expression of MHC genes in *H. pylori*-immunized/challenged, *H. pylori*-infected and *H. felis*-infected mice

Gene or gene region name	*Access. Number*	*H.p. Imm.*	*H.p. Inf.*	*H.f. Inf.*
MHC Class I				
Macropain (proteasome subunit)	Y10875	12.6	-	4.9
Transporter 1, ATP binding cassette	U60020	9.5	3.0	8.6
Large multifunctional protease 7	U22033	8.5	5.0	9.0
Transporter 2, ATP binding cassette	U60091	7.3	3.0	6.3
Set 1 repetitive element for MHC Class I	X00246	5.5	2.0	4.4
MHC (Qa) Q2-k gene for class I antigen	X58609	5.3	2.0	4.6
MHC I I Q4 beta-2-microglobulin (Qb-1)	M18837	4.2	-	3.0
Q4 class I MHC gene (exon 5)	X16202	3.3	-	2.7
MHC I D-region cell surface antigen (D2d)	M27034	3.1	-	-
MHC (A.CA/J(H-2K-f) class I antigen	M58156	2.8	-	2.1
Beta-2 microglobulin	X01838	2.6	-	2.0
H-2K gene, MHC class I H-2K (allele b)	V00746	2.5	-	-
H-2, T region locus 10	M35244	5.0	2.0	3.6
H-2, K region locus 2	M27134	4.7	-	4.4
H-2, T region locus 17	M35247	4.1	2.0	2.9
H-2, D region locus 1	M69069	3.0	-	2.6
H-2, T region locus 23	Y00629	2.9	-	2.6
MHC Class II				
H-2, antigen A	X52643	19.3	18.0	45.9
H-2 I-A beta (k haplotype)	M21932	14.5	19.0	41.0
H-2 Mb1	U35330	12.5	17.0	31.9
H-2 Ma	U35323	9.4	18.0	30.8
Invariant chain (Ii)	X00496	6.2	4.0	8.8
H-2 I-E beta-b	X00958	2.0	-	2.1

MHC Class III				
MHC class III region RD gene	AF109906	2.9	2.0	-

Table 1. Differential expression of MHC genes in the gastric mucosa of *H. pylori*-immunized/challenged (H.p. Imm.), *H. pylori*-infected (H.p. Inf.), or *H. felis*-infected (H.f. Inf.) mice. Values in the tables are noted as a fold increase of gene expression compared to naïve animals.

Both immunization/challenge and *Helicobacter* infection induced MHC gene expression (**Table 1**). Several genes involved in MHC Class I expression were prevalent including a proteasome subunit. MHC I genes were most prominently upregulated in *H. pylori*-immunized/challenged mice, although expression in the *H. felis*-infected group reached nearly the same level. In *H. pylori*-infected mice there was a less pronounced increase in expression for some of the MHC I genes compared to *H. felis*-infected mice.

In contrast, MHC II genes were most strongly (up to 45.9-fold) expressed in *H. felis*-infected mice, whereas in *H. pylori*-immunized/challenged and *H. pylori*-infected mice, expression levels were lower but still reached up to a 19.3-fold increase. Overall, MHC Class II messages were two to three fold higher in *H. felis*-infected mice than for either *H. pylori*-infected or *H. pylori*-immunized/challenged mice.

Only one member of the MHC III gene group was present among upregulated genes, with the strongest expression (2.9-fold) in *H. pylori*-immunized/challenged mice, slightly lower in *H. pylori*-infected and absent in *H. felis*-infected mice.

3.2.2 Differential expression of innate immune defense and inflammatory genes in the gastric mucosa of *H. pylori*-immunized/challenged, *H. pylori*-infected and *H. felis*-infected mice

Gene or gene region name	*Access. Number*	*H.p. Imm.*	*H.p. Inf.*	*H.f. Inf.*
Lactotransferrin	J03298	12.2	-	38.0
MRP8	M83218	7.6	-	67.7
Serum amyloid A	X03505	3.2	-	53.1
MRP14	M83219	-	-	49.6
CD14 antigen	X13333	-	-	3.4

Table 2. Differential expression of innate immune defence and inflammatory genes in the gastric mucosa of *H. pylori*-immunized/challenged (H.p. Imm.), *H. pylori*-infected (H.p. Inf.), or *H. felis*-infected (H.f. Inf.) mice. Values in the tables are noted as a fold increase of gene expression compared to naïve animals.

More striking differences were observed for genes associated with innate immunity and macrophage activation such as the MRP8 and MRP14 calcium binding proteins, serum amyloid A and lactotransferrin, which were most strongly upregulated (up to 67.7-fold) in *H. felis*-infected mice (**Table 2**), while an increase in expression was only present for some of the genes and less pronounced in *H. pylori*-immunized/challenged animals. No increase in gene expression was noted in *H. pylori*-infected animals. Expression of these markers correlates with the intensity of gastric inflammation as shown in **Figure 2.**

3.2.3 Differential expression of T lymphocyte genes in *H. pylori*-immunized/challenged, *H. pylori*-infected and *H. felis*-infected mice

Gene or gene region name	*Access. Number*	*H.p. Imm.*	*H.p. Inf.*	*H.f. Inf.*
Schlafen 2	AF099973	43.3	4.0	35.1
T cell specific protein	L38444	14.8	10.0	30.8
Schlafen 4	AF099977	9.4	2.0	-
Ly-6 alloantigen	X04653	7.1	-	5.6
Regulatory protein, T lymphocyte (Rpt-1)	J03776	4.7	-	-
Schlafen 3	AF099974	2.4	-	-

Table 3. Differential expression of T lymphocyte genes in the gastric mucosa of *H. pylori* immunized/challenged (H.p. Imm.), *H. pylori*-infected (H.p. Inf.), or *H. felis*-infected mice. Values in the tables are noted as a fold increase of gene expression compared to naïve animals.

Immunized/challenged mice also displayed increases of gene expression for several T cell specific genes including Schlafen 3 and 4, and a T cell regulatory protein (**Table 3**). A T cell-specific protein of unknown function was preferentially expressed in *H. felis*-infected mice.

3.2.4 Differential expression of cytokine and cytokine receptor associated genes in *H. pylori*-immunized/challenged, *H. pylori*-infected and *H. felis*-infected mice

Gene or gene region name	*Access. Number*	*H.p. Imm.*	*H.p. Inf.*	*H.f. Inf.*
Germline IL-1 receptor antagonist (IL-1rn)	L32838	5.0	-	10.6
Small inducible cytokine B subfamily member	U27267	4.3	-	26.5
IL-1-beta converting enzyme	L28095	3.2	3.0	4.8
Tumor necrosis factor super family 3 like	AF076482	3.0	-	6.9
Mouse IL-4 receptor (secreted form)	M27960	-	-	6.2
Small inducible cytokine A6	M58004	-	-	2.2
IL-18	D49949	-	-	-3.0
IL-11 receptor, alpha chain	U69491	-	-	-3.0
Transforming growth factor, beta 2	X57413	-	-	-3.2
Stromal cell derived factor 1	L12030	-	-	-3.2
I-TRAF	U59864	-3.1	-	-

Table 4. Differential expression of cytokine and cytokine receptor associated genes in the gastric mucosa of *H. pylori*-immunized/challenged (H.p. Imm.), *H. pylori*-infected (H.p. Inf.), or *H. felis*-infected (H.f. Inf.) mice. Values in the tables are noted as a fold increase of gene expression compared to naïve animals.

Another distinct family of genes shown to be differentially regulated between groups of mice included cytokine related genes other than IFN-γ (**Table 4**). Several cytokine and cytokine receptor related genes such as IL-1 receptor antagonist, a small inducible cytokine B subfamily member, IL-1-beta converting enzyme and a tumor necrosis factor super family 3 like cytokine had strong expression in *H. felis*-infected mice. These genes were present only in lower levels in *H. pylori*-immunized/challenged mice, and nearly absent in *H. pylori*-infected mice. Again, these levels correlate somewhat with the gastric inflammation present in the respective group. An increase in messenger RNA of IL-4 receptor in its secreted form and small inducible cytokine A6, as well as a downregulation of other cytokines or cytokine receptors, such as IL-18, the alpha chain of IL-11-receptor and TGF-β2, were exclusively noted in *H. felis*-infected mice.

3.2.5 Differential expression of interferon associated genes in *H. pylori*-immunized/challenged, *H. pylori*-infected and *H. felis*-infected mice

Gene or gene region	*Access. Number*	*H.p. Imm.*	*H.p. Inf.*	*H.f. Inf.*
Transcription factors				
mIRF-1	M21065	5.0	2.0	5.3
mIRF-7	U73037	21.6	2.0	-
ISGF3 (IFN-dependent transcription factor 3)	U51992	4.4	-	-
eLF-3	AF016294	2.2	-	-
GTP-binding proteins (GBP)				
65-kDa GBP family				
mGBP-2	AJ007970	29.2	24.0	58.8
47-kDa GBP family				
IIGP	AJ007971	17.8	6.0	17.5
IRG-47	M63630	15.6	11.0	20.4
GTPI	AJ007972	5.0	2.0	5.3
LRG-47	U19119	2.9	-	2.7
GTPase IGTP	U53219	-	-2.0	-
Others				
Interferon-gamma receptor	M28233	-2.5	-	-
Type 1 interferon receptor, IFNaR2b	Y09864	2.3	-	-
IFN-γ induced Mg11	U15635	20.2	6.0	18.5
15-Kda protein	X56602	19.5	3.0	-
GARG-49/IRG2	U43086	15.7	-	-
GARG-16 (IFN induced protein)	U43084	13.4	4.0	
Monokine induced by gamma interferon	M34815	2.6	-	6.6

Table 5. Differential expression of interferon associated genes in the gastric mucosa of *H. pylori*-immunized/challenged (H.p. Imm.), *H. pylori*-infected (H.p. Inf.), or *H. felis*-infected (H.f. Inf.) mice. Values in the tables are noted as a fold increase of gene expression compared to naïve animals.

In immunized/challenged mice there was an increase in type I IFN receptor not seen in mice infected with either *H. pylori* or *H. felis* (**Table 5**). The most marked increases in expression compared to chronically infected mice were seen in many IFN associated genes such as mIRF-7, GARG-16 and GARG-49, a 15 kDa protein of unknown function and ISGF3. These genes were absent in *H. felis*-infected animals, indicating their association with the protective immune response but not for an inflammatory response in general. Interferon induced GTPases were upregulated at a similar level in both *H. pylori*-immunized/challenged and in *H. felis*-infected animals, while less expressed in *H. pylori*-infected mice. Just a single guanylate binding protein of the 65-kDa GBP family was preferentially expressed in *H. felis*-infected mice.

3.3 RT-PCR analysis confirms gene array results

To further corroborate the gene array results several genes that were strongly upregulated in *H. pylori*-immunized/challenged mice were examined in a second set of mice at a single animal level by RT-PCR.

Five to seven mice per group were infected or immunized/challenged with *H. pylori*. The animals were sacrificed on day 28 after challenge or infection respectively. Stomachs were assessed for bacterial load by urease test, culture and microscopic visualization in silver stained stomach sections. Six of the seven infected mice had positive tests for urease, bacteria were readily detected in silver stained stomach sections and cultures from the stomachs were all positive at an average of 395333,3 CFU ± 412543,0 CFU per gram stomach tissue. Protection was complete in the immunized/challenged group, with no positive urease tests and no bacteria detected in silver stained sections or cultured from the stomach of any animal after challenge.

3.3.1 Prerequisite: RNA samples used were free of genomic DNA contamination

RNA was isolated from stomach tissue and treated with DNase to eliminate genomic DNA. To determine the success of the DNAse treatment, PCR analysis was performed on non reverse transcribed controls of each sample to exclude genomic DNA contamination. As shown in **Image 3** no amplification products were observed when PCR analysis was performed on non reverse transcribed controls confirming the absence of genomic DNA, while DNA was apparent in samples prior to DNase treatment. The DNase treatment left the RNA intact, as subsequent RT-PCR on the samples was successful.

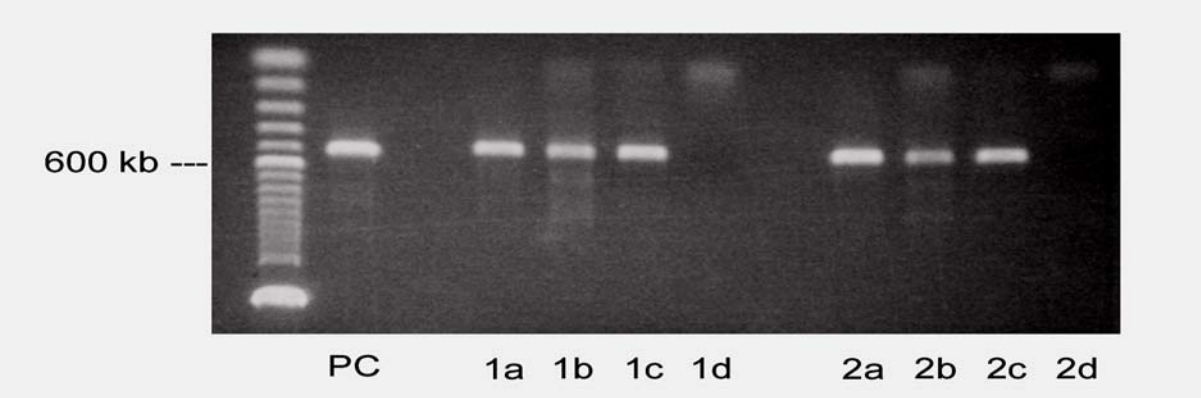

Image 3 . Gel electrophoresis of amplification products from two samples (1 and 2) of RNA amplified with primers specific for G3PDH. Samples were either reverse transcribed without previous DNase treatment (a), non reverse transcribed without DNase treatment (b) DNase treated and reverse transcribed (c), or DNase treated and non reverse transcribed (d) before PCR. Therefore, band b represents genomic DNA contamination, while band c is amplified from RNA only. PC is the positive control product amplified from the control RNA.

3.3.2 Results for Schlafen 4, mIRF-7, GARG 16, GARG 49 and IRG-47

Primers specific for Schlafen 4, mIRF-7, GARG 16, GARG 49 and IRG-47 were used to amplify products from the cDNA generated from naïve, *H. pylori*-immunized/challenged, or *H. pylori*-infected mice. At the same time, primers specific for the housekeeping gene G3PDH were used with all samples as a control for the amount of RNA used in each reaction. PCR products were quantified digitally. To compare expression between groups, the ratio of PCR product amplified from the gene of interest to internal G3PDH product was calculated and plotted. An example of an ethidium bromide stained gel with amplification products is shown below (**Image 4**).

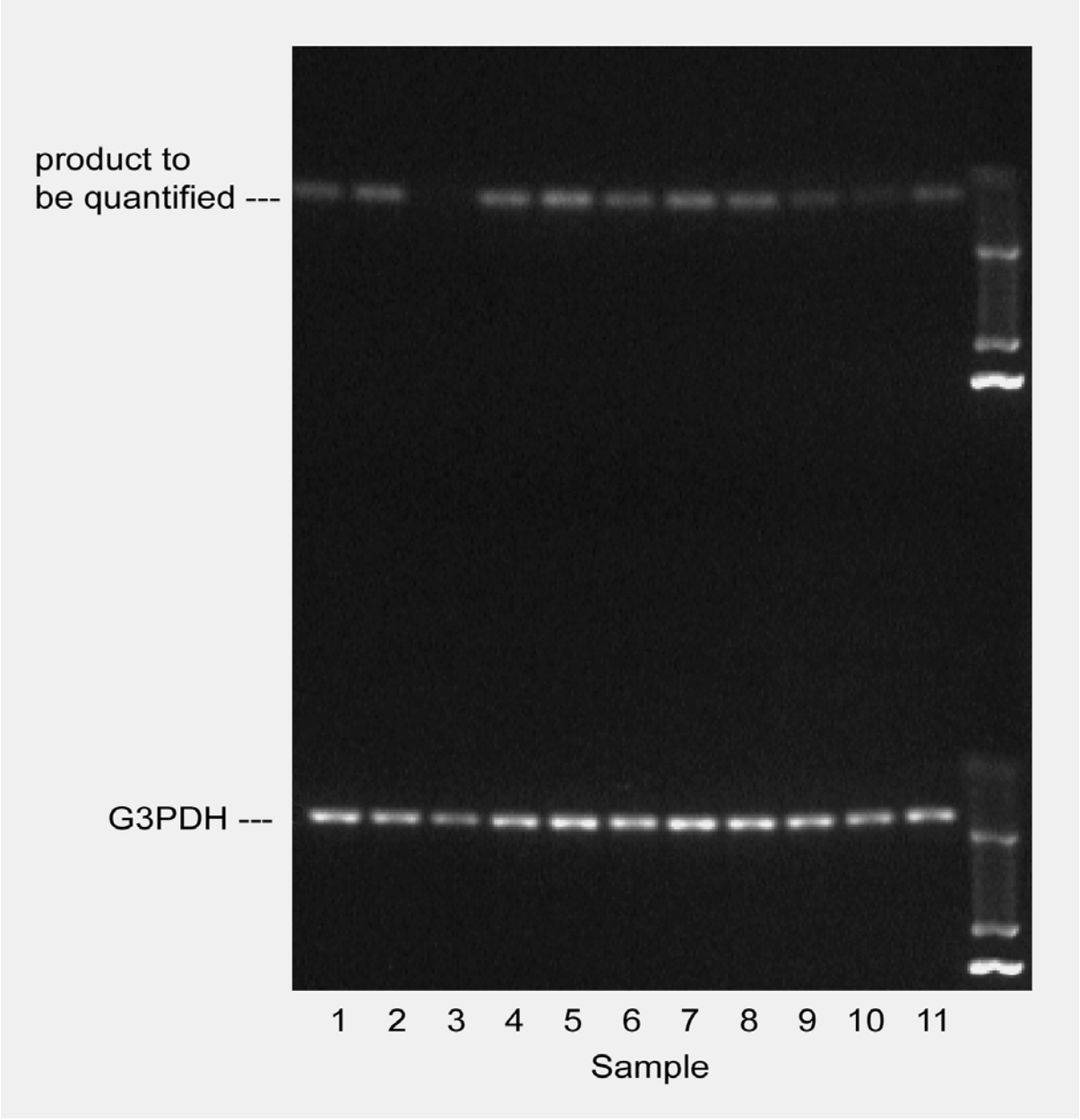

Image 4. Gel electrophoresis of amplification products from PCR. PCR was performed in parallel on the same samples with primers specific for the gene of interest and G3PDH. The products were resolved on the same gel.

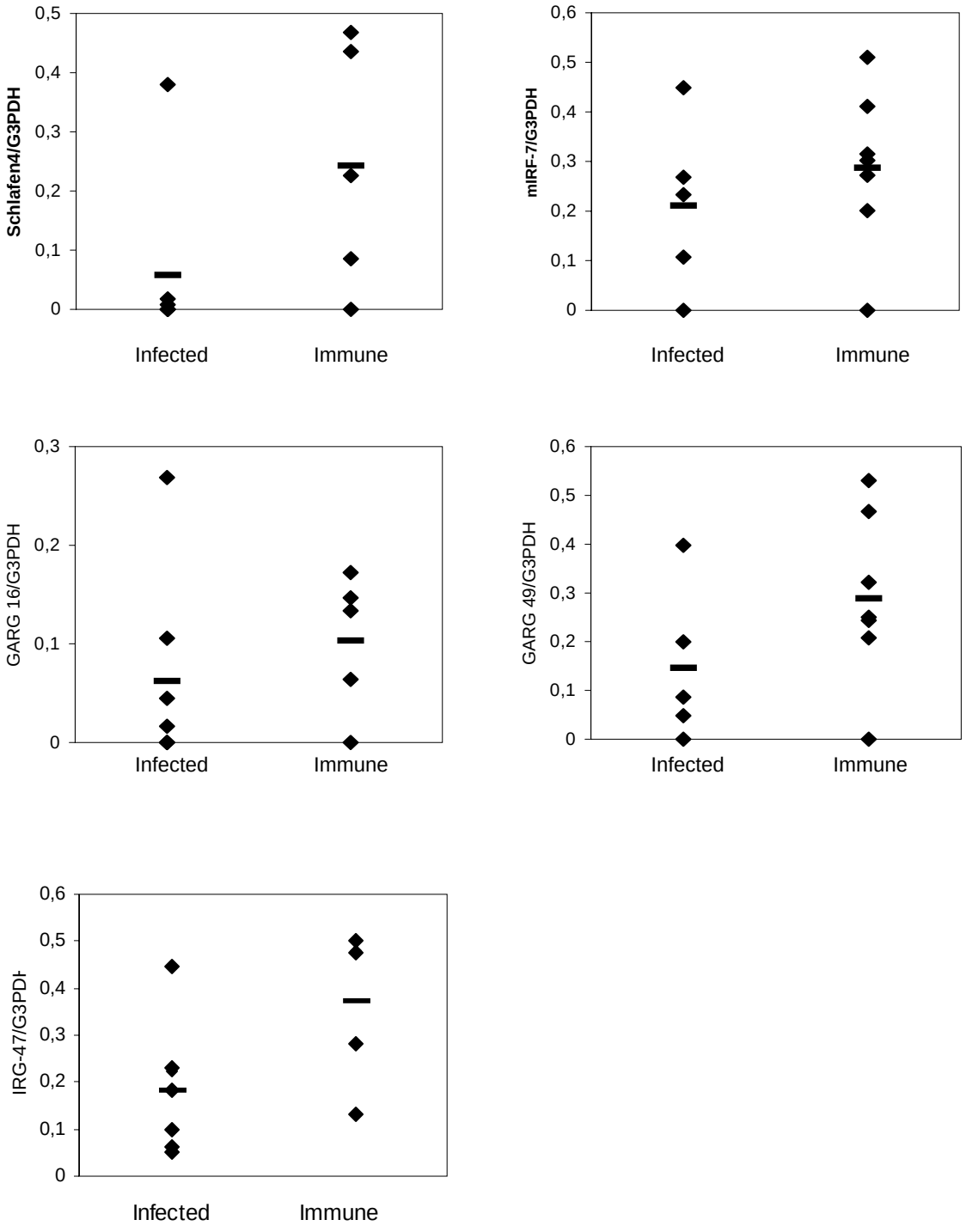

Figure 5. Polymerase chain reaction amplification of genes associated with protective immunity. RT-PCR was performed on RNA from gastric tissue of *H. pylori*-infected and *H. pylori*-immunized/challenged mice. Amplified products were quantified digitally and the data expressed as the ratio of the candidate gene to the internal control G3PDH.

The four genes associated with IFN production (mIRF-7, GARG 16, GARG 49, IRG-47) were moderately higher in *H. pylori*-immunized/challenged mice compared to *H. pylori*-infected mice. The higher levels in immununized/challenged mice compared to infected mice were consistent with DNA array analysis. A more striking difference between the groups was observed for the T cell gene Schlafen 4 which displayed an approximately 3-fold difference.

3.4 *In vitro* assays with bulk spleen cells show an increased production of IFN-γ in *H. pylori*-immunized mice compared to infected mice, while only traces of IFN-α were detected

The gene array analysis was performed on stomach tissue since the stomach is the site of *Helicobacter*-induced inflammation. However, the systemic immune response to *Helicobacter* as demonstrated by the presence of serum antibody suggests the potential to assess *Helicobacter* immunity in other tissues. Several *in vitro* assays were performed using spleen cells to determine whether the differences in gene expression observed in the gene array on a local level could also be observed in *vitro*. Therefore, bulk spleen cells from naïve, *H. pylori*-infected, or *H. pylori* immunized mice were exposed to live *H. pylori* or *H. pylori* sonicate for 72 hours as described above. Since in the previous analyses immunized/challenged animals were used, gene expression after the first contact with the bacterium after immunization was determined. In order to recreate a first contact *in vitro,* spleen cells from immunized but not challenged animals were used. The success of the immunization was determined by anti-*Helicobacter* IgG ELISA, and all animals used showed titers comparable to those used in the microarray. Infection was retrospectively determined by cultures from the stomachs of infected animals, and cultures were positive for all animals used.

3.4.1 Gene expression analysis is difficult in *in vitro* assays – switch to the protein level

RNA was isolated from cell pellets, DNase treated and used for RT-PCR. Again, no amplification products were observed when PCR analysis was performed on non reverse transcribed controls confirming the absence of genomic DNA contamination. Because the cell number in the pellets was very low, a uniform amount of RNA in the samples was not achieved before RT-PCR was performed, as shown in the wide variation of staining intensity of G3PDH bands in **Image 5**.

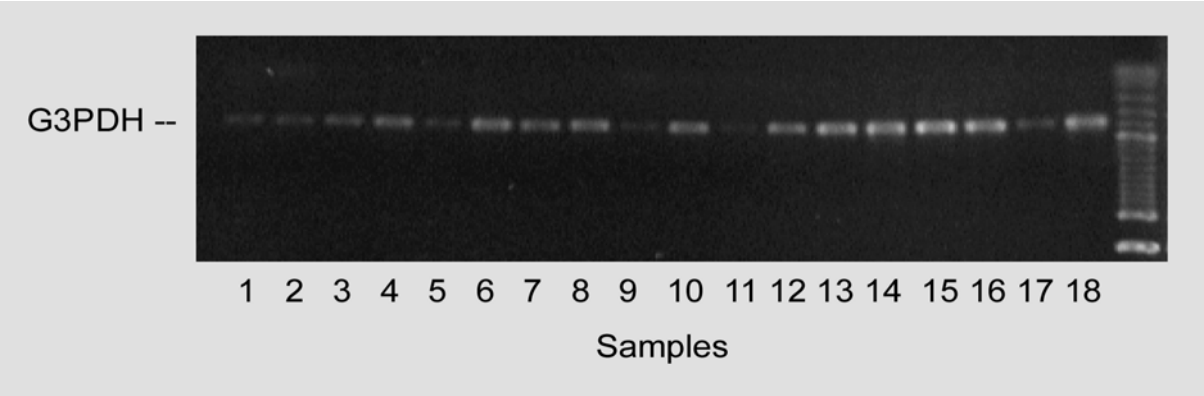

Image 5. PCR Amplification products of the G3PDH gene from RNA isolated from cell pellets of in vitro assay.

Because of the difficulties in analyzing gene expression in the cell pellets of in vitro assays, the supernatants were then assessed at the protein level. Since there were potentially important differences between immunized/challenged and infected mice in the expression of IFN-regulated genes, supernatants of *in vitro* assays were assessed for IFN levels to determine if immunization predisposes the anti-*Helicobacter* response to generate greater levels of either type I or type II IFN.

3.4.2 Bulk spleen cells from mice produce only traces of IFN-α *in vitro* regardless of their stimulation with *H. pylori* antigens, and there is no difference between naive, immunized and chronically infected mice

Cell supernatants were first tested for IFN-α, which is the type I IFN produced by leukocytes. Levels of IFN-β, the other type I IFN, were not determined as cell cultures did not contain fibroblasts.

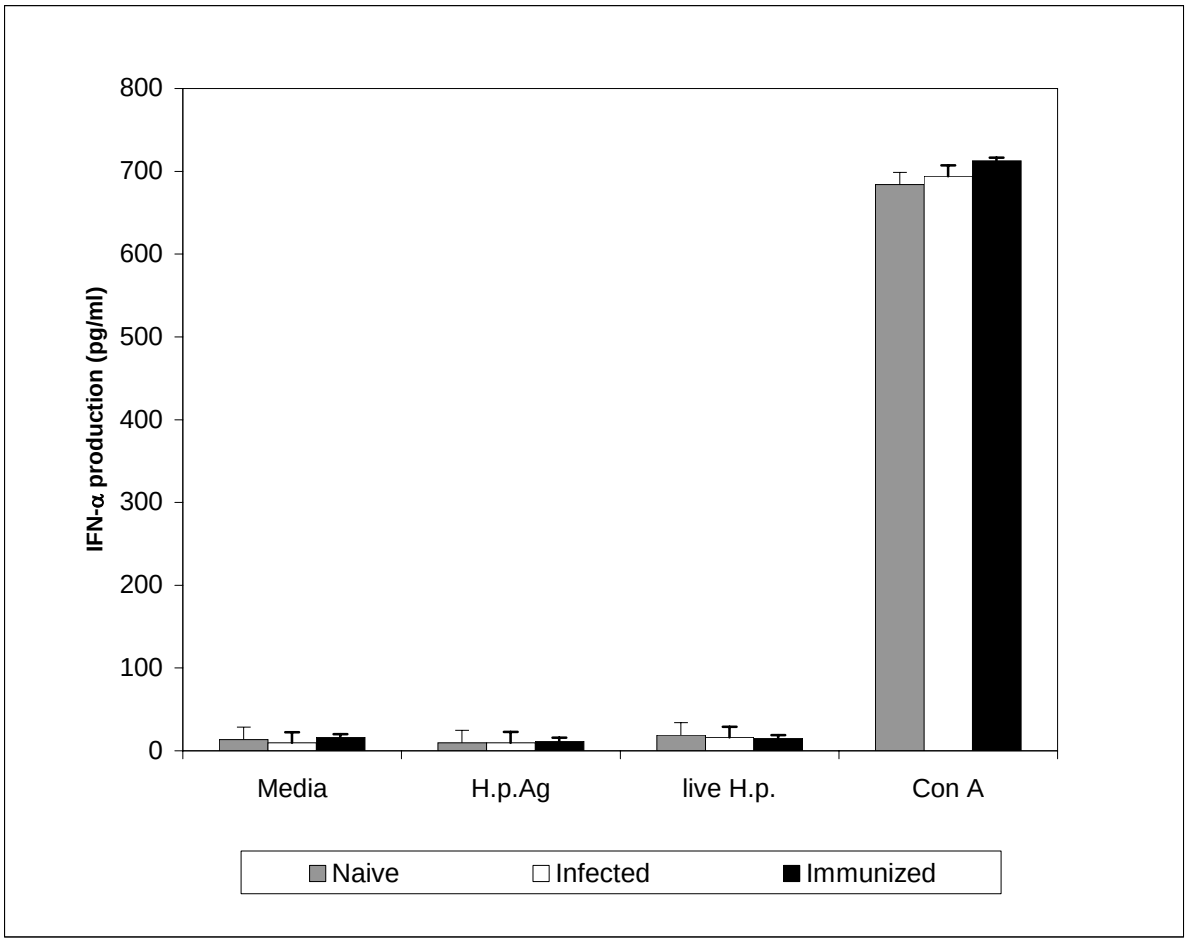

Figure 9. *In vitro* assay for the production of IFN-α by bulk spleen cells following antigenic stimulation. Cells from naïve, *H. pylori*-infected, or *H. pylori*-immunized mice (three mice per group) were pooled, and restimulated *in vitro* with media, *H. pylori* whole cell lysate (H.p.Ag), live *H. pylori* (live H.p.), or Concanavalin A (Con A) in triplicate and the supernatants tested for IFN-α by ELISA. This data set is representative of two consecutive experiments.

As described above, bulk spleen cells from naïve, *H. pylori*-infected, or *H. pylori*-immunized mice were stimulated with live *H. pylori* or *H. pylori* sonicate, while media only or

stimulation with Concanavalin A was used as negative or positive control, respectively. Supernatants were then analyzed for IFN-α production.

In all groups only traces of IFN-α were present regardless of the stimulation provided. The general ability of the cell populations to produce high levels of IFN-α was demonstrated by stimulation with the mitogen Concanavalin A.

3.4.3 Bulk spleen cells from immunized mice produce more IFN-γ than cells from chronically infected mice upon *in vitro* stimulation with *Helicobacter pylori* sonicate or live bacteria

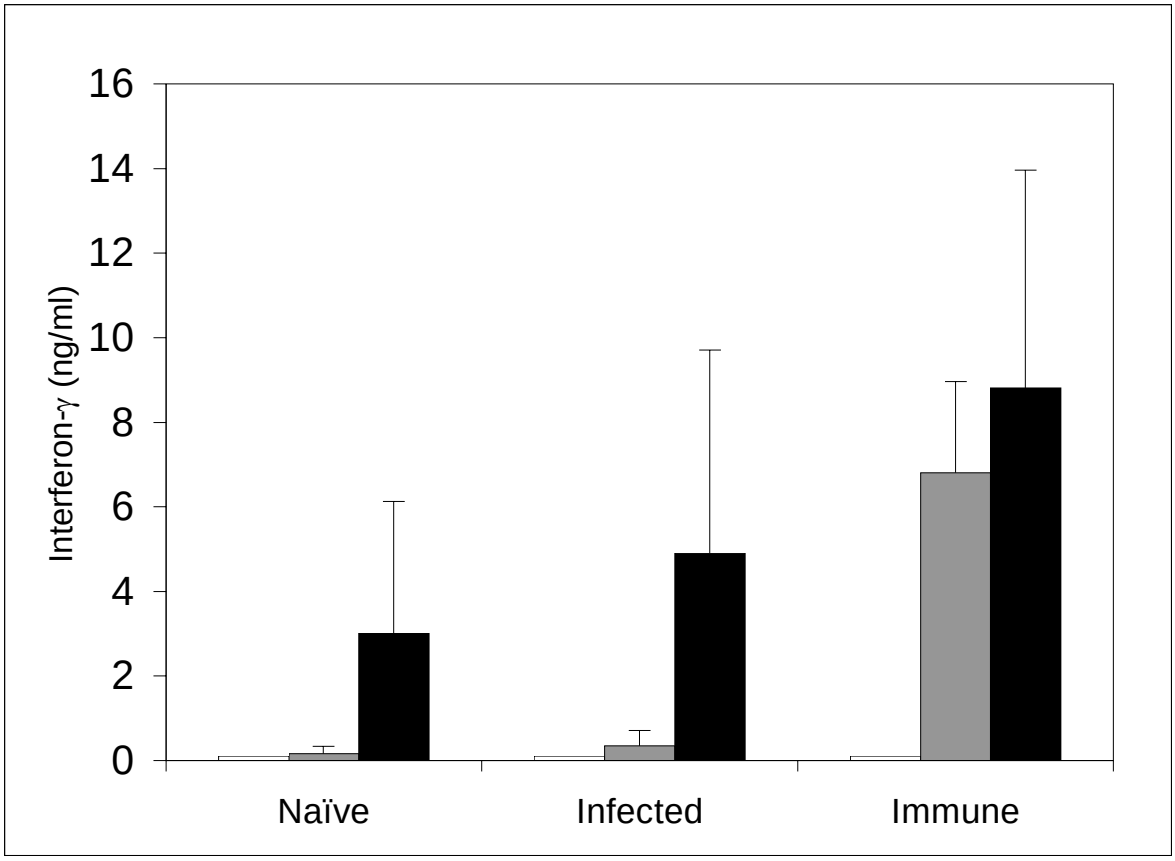

Figure 10. *In vitro* assay for the production of IFN-γ by bulk spleen cells following antigenic stimulation. Cells from naïve, *H. pylori*-infected, or *H. pylori*-immunized mice (three mice per group) were pooled and restimulated *in vitro* with media (white bars), *H. pylori* whole cell lysate (grey bars), or live *H. pylori* (black bars), and the supernatants tested for IFN-γ by ELISA. This data set is representative of two consecutive experiments.

The supernatants from the same *in vitro* assay were then assessed for IFN-γ levels. When bulk spleen cells from these mice were exposed to live *H. pylori* for 72 hours IFN-γ was readily detected in cell culture supernatants from all three groups. However, IFN-γ produced by cells from immunized mice (8.82 ± 5.15 ng/ml) was significantly higher than the naïve group (3.01 ± 3.11 ng/ml; $P = 0.0217$). The difference was more pronounced when whole cell *H. pylori* lysate was used as the stimulating antigen, although overall levels of interferon production were lower than with live bacteria. Spleen cells from naïve and infected animals failed to generate appreciable levels of IFN-γ upon stimulation with *H. pylori* lysate, while spleen cells from immunized mice generated levels of 6.81 ± 2.15 ng/ml ($P = 0.01$ and $P = 0.012$ for immune *vs* naïve and infected mice respectively).

3.5 $CD4^+$ T cells from immunized mice produce more IFN-γ than cells from chronically infected mice upon *in vitro* stimulation with live *H. pylori* in the presence of macrophages

Since bulk spleen cells contain various IFN-producing cell populations, purified $CD4^+$ cells, the population that has been shown to be essential for protection, were then further analyzed in another *in vitro* assay. Again, cells from naïve, *H. pylori*-immunized, and *H. pylori*-infected animals were used. The success of the immunization was determined by anti-*Helicobacter* IgG ELISA, and all animals used showed titers comparable to those used in the microarray. Infection was retrospectively determined by cultures from the stomachs of infected animals, and cultures were positive for all animals used.

3.5.1 Isolated $CD4^+$ T cells were over 90% pure

$CD4^+$ cells were isolated from the spleens of naïve, *H. pyori*-immunized and *H. pyori*-infected mice by negative selection as described above, and their purity was determined by fluorescent-activated cell analysis using an anti-CD4 antibody (**Image 6 and 7**)

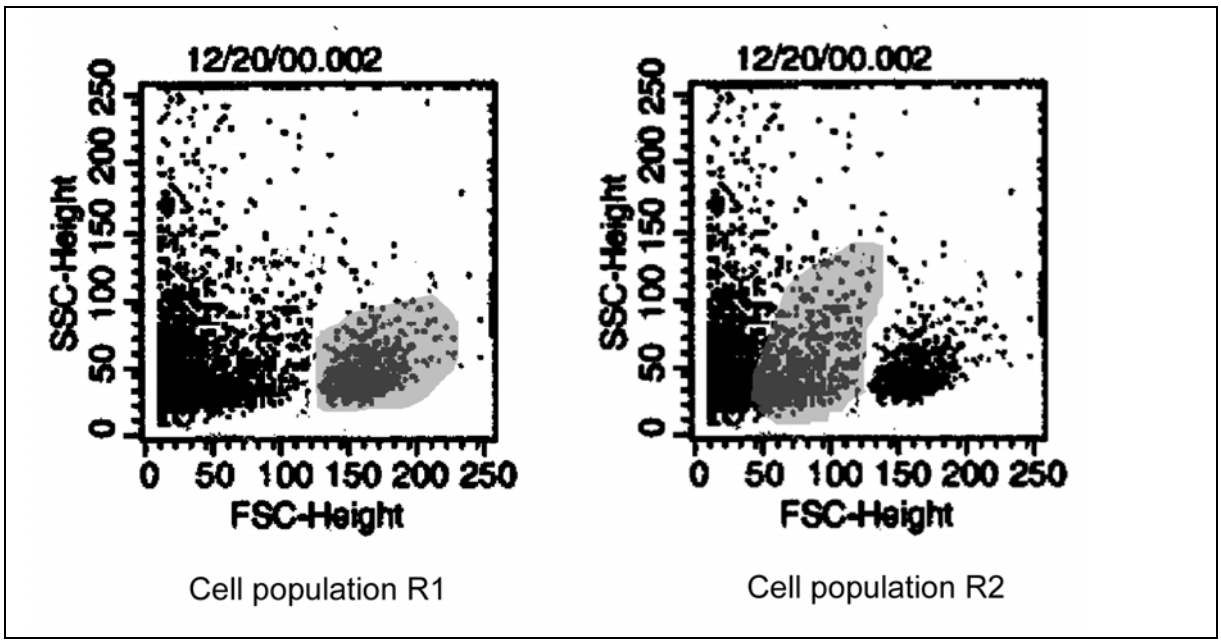

Image 6. Light scatter histograms of unstained spleen cells of naïve mice after purification. Two distinct populations of cells were identified based on clustering.

Purified populations of unstained cells were assessed for uniformity by light scatter histogram. Two distinct populations of cells were identified based on clustering (**Image 6**). Analysis was performed separately on these two populations of cells by setting morphologic gates and analysing them as cell population R1 and cell population R2.

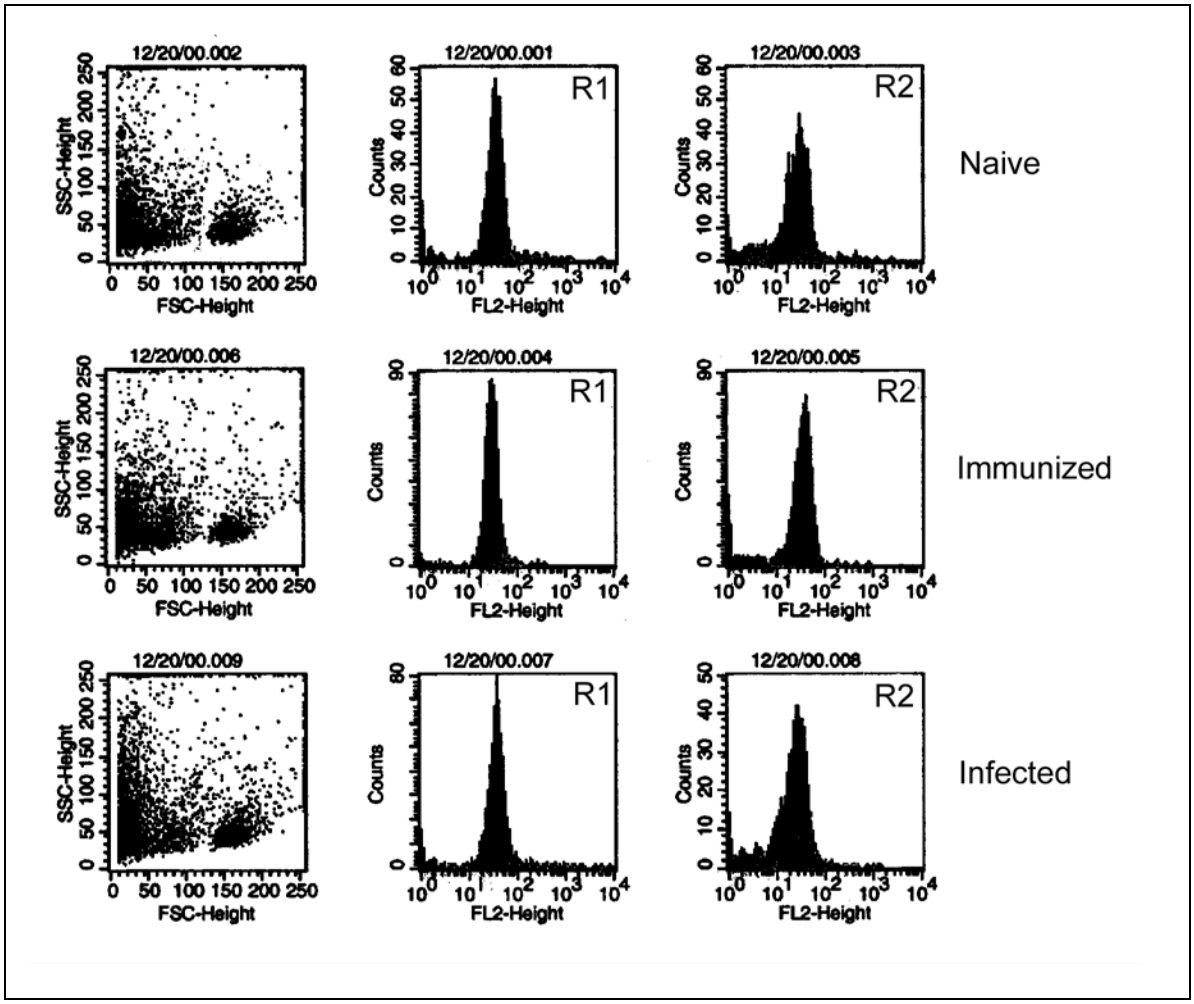

Image 7. FACS-analysis was performed separately on cell population R1 and cell population R2 after staining with PE-labelled anti-CD4 antibody.

Staining of both R1 and R2 populations with anti-CD4 antibody gave identical results (**Image 7**). Both the number of positive cells and the intensity of cell staining were equivalent and did not differ between naive, immune, or chronically infected mice. Based on these histograms, cells were determined to be greater than 90% CD4 positive, verifying the effectiveness of the negative selection purification procedure.

3.5.2 IFN-γ production in *in vitro* assay

Purified $CD4^+$ T cells were incubated with either freshly isolated peritoneal macrophages, live *H. pylori*, a combination of both macrophages and bacteria, or with both macrophages and *H. pylori* lysate, and the supernatants examined for IFN-γ levels. Macrophages were used as antigen presenting cells in this assay because they do not produce IFN-γ and thus IFN-γ production of $CD4^+$ cells could be exclusively analyzed. This was confirmed with control cultures of macrophages alone and in co-culture with live *H. pylori*, which did not produce any IFN-γ (data not shown).

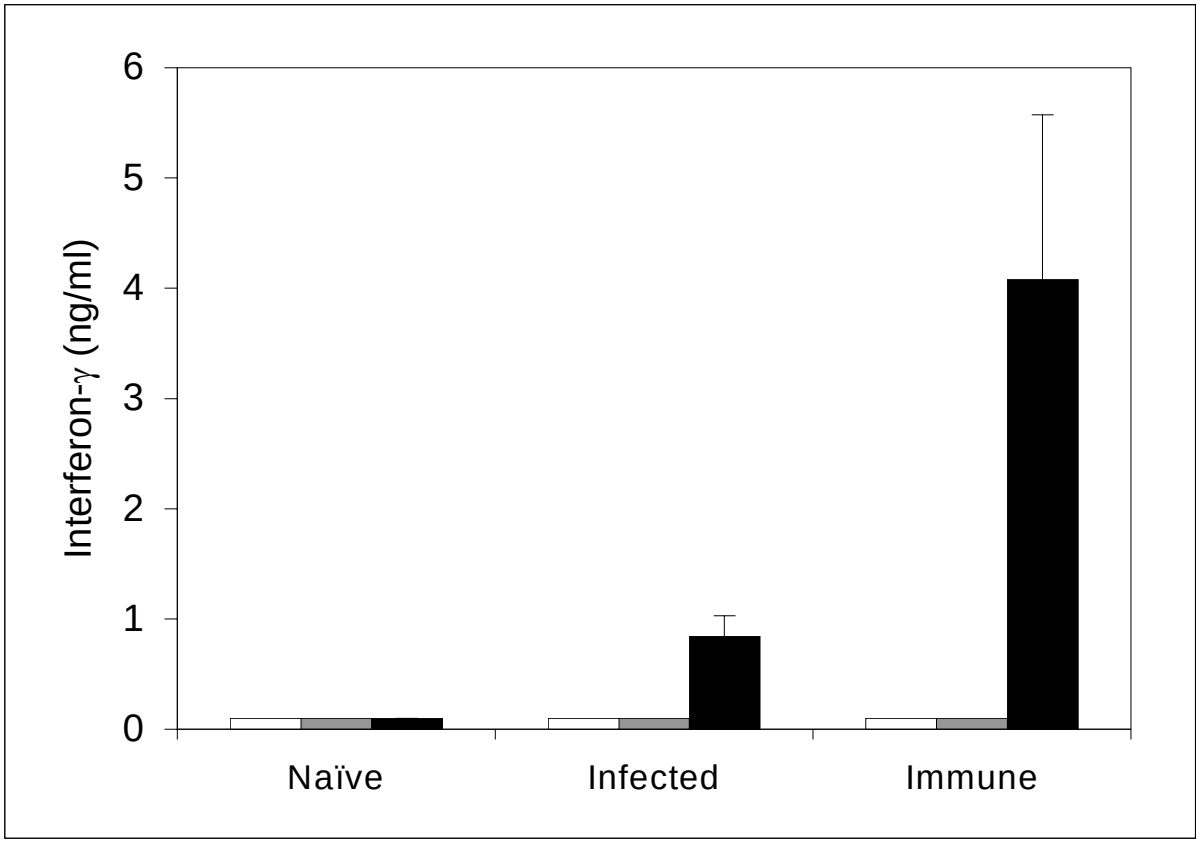

Figure 11. *In vitro* assay for the production of IFN-γ by $CD4^+$ spleen cells following antigenic stimulation. Cells from naïve, *H. pylori*-infected, or *H. pylori*-immunized mice (two mice per group) were pooled and co-cultured with macrophages (white bars), live *H. pylori* (grey bars), or both macrophages and live *H. pylori* (black bars), and the supernatants tested for IFN-γ by ELISA. This data set is representative of two consecutive experiments.

No production of IFN-γ was observed when macrophages and $CD4^+$ T cells were stimulated with *H. pylori* lysate (data not shown). Live *H. pylori* did not induce the production of IFN-γ unless both macrophages and $CD4^+$ T cells were present. However, whereas $CD4^+$ T cells

from infected mice produced 0.84 ± 0.19 ng/ml IFN-γ, cells from immune mice produced 4.08 ±1.49 ng/ml IFN-γ (P = 0.0204). Therefore, prior exposure to *Helicobacter* antigens via immunization results in a significantly stronger IFN-γ response upon challenge compared to prior exposure via infection. Such a response might activate alternative or additional pathways of immune activation that could be important for protection.

4 DISCUSSION

4.1 Open questions in protective immunity against *H. pylori* and working hypotheses

Although *H. pylori* is considered a non-invasive mucosal pathogen none of the classical mechanisms of mucosal immunity such as secretory antibody production have been shown to be involved in the protective immune response in immunized mice. Prophylactic immunization does not prevent colonization by *H. pylori* but rather enables mice to rapidly clear the (temporary) infection or significantly reduce the number of colonizing bacteria. The reduction in bacterial load is associated with gastric inflammation that subsides over time after the bacteria have been eliminated (120). The present study was designed to identify genes associated with the protective, post-immune gastritis observed when immunized mice are challenged with live *H. pylori*.

There are several possible explanations for the increased efficacy of post-immune gastritis in clearing a (transient) *Helicobacter* colonization compared to the immune response and inflammation induced by chronic infection.

It is possible that immunization activates a distinct effector mechanism that is capable of providing protection, but which is not activated in the inflammatory response that accompanies chronic infection. This distinct mechanism could include the recognition of previously unrecognized antigens by both antibodies and T cells, or a shift in the cytokine pattern to previously absent cytokines, e.g. a switch from a TH_1 to a TH_2 pattern of cytokines.

Alternatively, immunization may provide a quantitative boost to the inflammatory response, pushing it above some threshold level necessary to clear *Helicobacter* organisms from the gastric mucosa. The heightened inflammation in post-immune gastritis might be contributing to the protective mechanism itself. An increased inflammatory response could lead to increased antigen presentation by MHC upregulation, more abundant infiltration by

phagocytes and other cells of the unspecific immune system, and an increased efficacy of the oxidative burst and of phagocytic activities.

As mentioned above there is evidence that suggests that *Helicobacter* infection might put the host at a disadvantage in mounting an effective cellular immune response against the pathogen. An immunization might help the host to overcome this impaired immune responsiveness possibly induced by *H. pylori*.

Previous reports on cellular immunity induced by *H. pylori* infection yielded contradictory results. Early studies using human gastric explants or peripheral blood mononuclear cells revealed a propensity for cells from uninfected individuals to respond more strongly to *H. pylori* in both proliferation and release of IFN-γ than cells from infected patients (48-52). Other studies reported stronger proliferative responses in *H. pylori*-positive individuals (77).

These conflicting results may be partly attributed to the large number of variables involved in human studies, including different strains of infecting organisms, variable durations of infection, different antigen preparations used for *in vitro* stimulation, and different host factors such as genetic backgrounds, medications, diet, and stress. Still, the majority of these experiments provide evidence for an impaired immune response towards *H. pylori* infection.

The results of more recent studies now suggest several possible mechanisms of immune suppression by *H. pylori*.

CagA positive strains of *H. pylori* lead to a negative selection of T cells through the Fas/FasLigand pathway *in vitro* (121). This pathway has been described as being responsible for depleting immature, autoimmune T cells in the thymus and mature T cells in the periphery (122,123). Another study implicates the presence of a protein factor in *H. pylori* that inhibits epithelial and also T cell proliferation *in vitro* (124).

Regulatory T cells might also play a role in *H. pylori* infection. Recent studies suggest that these T cells can be induced against viral, bacterial and parasite antigens *in vivo* and might either prevent infection-induced immunopathology or prolong pathogen persistence by suppressing protective TH_1 responses (as reviewed by McGuirk,125).

The presence of *H. pylori*-specific $CD4^+CD25^+$ regulatory T cells during *H. pylori* infection was recently detected by Lundgren *et al.* (126). Their study could also demonstrate that the unresponsiveness of $CD4^+$ T cells towards *H. pylori* antigens could be abolished by depletion of $CD4^+CD25^+$ T cells from the cultures. In a series of adoptive transfer experiments by Raghavan *et al.* (127) athymic C57BL/6 nu/nu mice were reconstituted with lymph node cells containing or depleted of $CD25^+$ T cells before challenge with *H. pylori*. The absence of

$CD25^+$ T cells correlated with high levels of IFN-γ production upon stimulation and low bacterial loads. Thus, these regulatory cells do play a role in the establishment of a chronic *H. pylori* infection by suppression of a possibly protective inflammatory TH_1 immune response against *H. pylori in vivo*. This could also be an explanation for the low inflammation in *H. pylori*-infected, but not immunized mice compared to the post-immune gastritis that occurs after vaccination.
A vaccine could help to overcome this immune suppression by inhibiting the induction of regulatory T cells within the appropriate stimulation conditions, or might help prevent *H. pylori*-induced apoptosis of immune cells.

Animals and humans cured of *Helicobacter* infection by antibiotic eradication therapy could be readily reinfected (88,128), thus demonstrating a difference between the non-protective immune response generated by infection and protective immunity induced by vaccination. The success of therapeutic vaccinations, which changes a non-protective inflammatory response into a protective response provides further evidence for different host responses following immunization. Thus immunization would seem to induce a class of memory cells that function differently than lymphocytes induced by chronic infection.
Recent reports using gene-deficient, transgenic mice have demonstrated the necessity of MHC class II restricted $CD4^+$ T cells for protective *Helicobacter*-immunity (116,117). Additionally, in a previous study, an increase in $CD4^+$ T cells in the lamina propria correlated with increasing grades of gastritis (129). Since protective immunity requires MHC II restricted responses the observed upregulation of MHC II genes in post immune gastritis could be expected in the present analysis. To date, MHC class II restricted $CD4^+$ T cells are the only part of the adaptive immune system proven to be necessary to achieve protection from *H. pylori* infection. Evidence was provided by Gottwein *et al.* who were able to transfer protective immunity to *rag2*$^{-/-}$ mice, which lack an adaptive immune system, by adoptive transfer of $CD4^+$ T cells from *H. felis*-immunized mice (112). However, no difference between protective and non-protective T-cells has been reported and the effector mechanisms induced by these T cells remain unknown. Since no direct anti-microbial activity has been described for $CD4^+$ T cells, the study by Gottwein *et. al.* suggests that the immune effector mechanism, while receiving help signals from primed T cells, is non-lymphocytic in nature.

A direct comparison between mice chronically infected with *H. pylori* and mice that had been immunized prior to challenge was performed in order to identify potentially important

differences between non-protective chronic gastritis and the protective nature of post-immune gastritis. While *H. pylori*-inoculated mice harbored organisms in the gastric mucosa and generated anti-*H. pylori* antibody titers, they developed only a mild inflammatory response. It has been previously described that the gastritis in the *H. pylori* mouse model is commonly mild relative to the post-immune gastritis following challenge of immunized mice (82,115,130-132). This was consistent with the results of this study. Therefore, *H. felis*-infected mice were also included in the analysis. *H. felis* induces severe inflammation in C57BL/6 mice (133), which is very similar to the human gastritis in response to *H. pylori*. Histologic analysis in this study confirmed the different natures of the inflammatory response between *H. pylori* and *H. felis*. By including *H. felis*-infected mice it was possible to distinguish between genes upregulated uniquely in immune mice from genes upregulated during severe but nonprotective chronic inflammation. On the other hand assuming that the intensity of the inflammatory response could reflect part of its protective potential, it might still be difficult to distinguish genes specific for inflammation from genes specific for protection.

4.2 Genes associated with the inflammatory response

Genes consistent with inflammation such as lactotransferrin, MRP8 and serum amyloid A were predominantly upregulated in *H. felis*-infected mice, and only slightly upregulated in *H. pylori*-immunized/challenged mice. Expression levels remained at a baseline level in *H. pylori*-infected mice. Both lactotransferrin and serum amyloid A are acute-phase proteins (134) and MRP8 is a calcium binding inflammation-related protein expressed in neutrophils and monocytes recruited to a site of inflammation (135). As expected, the expression of these genes correlated with the levels of gastric inflammation present in the different groups of animals. Thus these genes are probably a direct measure of inflammation rather than being characteristic of a protective immune response. Similarly, the high inflammation *H. felis*-infected mice displayed heightened levels of several cytokine related genes relative to immune mice including a member of the small inducible cytokine B family and small inducible cytokine A6.

4.3 T cell genes associated with protection

In the past, several laboratories have attempted to characterize the T cells associated with protective immunity in mice by both surface marker analysis (131) and cytokine secretion profiles (136). Michetti *et. al.* demonstrated that α4β7-integrin-positive $CD4^+$ T lymphocytes might be one cell subset that contributes to protective immunity, since its *in vivo* depletion blocks protection (137).

Yet no phenotype has been reported that might distinguish protective T cells from the T cells present in chronic gastritis. In the gene array results several T cell genes were identified that may help distinguish protective T cells from non-protective T cells. These proteins seem to be uniquely associated with immunized/challenged mice. One protein, Rpt-1, may regulate expression of the IL-2 receptor (138). And there were two members of the newly described Schlafen family, Schlafen 3 and Schlafen 4 that may also be important. The function of the Schlafen proteins has not yet been ascertained although there is some evidence that they may influence the cell cycle. Consistent with their German name, this gene family is thought to play a role in actively keeping T cells in their resting stage (139). These three genes might possibly play a role to distinguish T cells associated with protection from those present in chronic infection.

4.4 Possible role of type I IFN as a novel mechanism of protective immunity

The search for differences in the cytokine profile between infected and immune mice has been a primary emphasis of many research groups. Despite the use of various *ex vivo, in vitro*, and molecular assays, no difference in cytokine expression has been described to explain why immunized mice are able to clear *Helicobacter* organisms. The present analysis revealed only minor differences in cytokine expression levels but these differences may be telling of a protective mechanism. It is intriguing that one gene, an IFN-α receptor subunit, was observed to be upregulated in immunized/challenged mice 2.3-fold while not increased at all in either group of infected mice.

When assessing IFN associated genes further evidence could be found for a role of type I IFN in protective immunity. ISGF3 (interferon dependent transcription factor 3) and IRF-7

(interferon regulatory factor 7) were nearly exclusively upregulated in immunized/challenged mice. These genes play an important role in the auto-amplification of an IFN-α/β response, usually initiated by a viral infection through activation of the transcription factor IRF-3 (**Image 8**). After the binding of the type I IFN to its receptor, STAT 1 and STAT 2 (collectively termed ISGF3-α) are phosphorylated by the IFN-α/β receptor associated Janus kinases and then form a trimolecular complex with ISGF3-γ. This complex, termed ISGF3, acts as a transcription factor on the IRF-7 gene, while IRF-7 induces IFN α and β gene transcription. Thus ISGF3 and IRF-7 are key factors in the positive feedback regulation of IFN-α/β production, as reviewed by Taniguchi *et. al*. and Mamane *et. al.* (140-142).

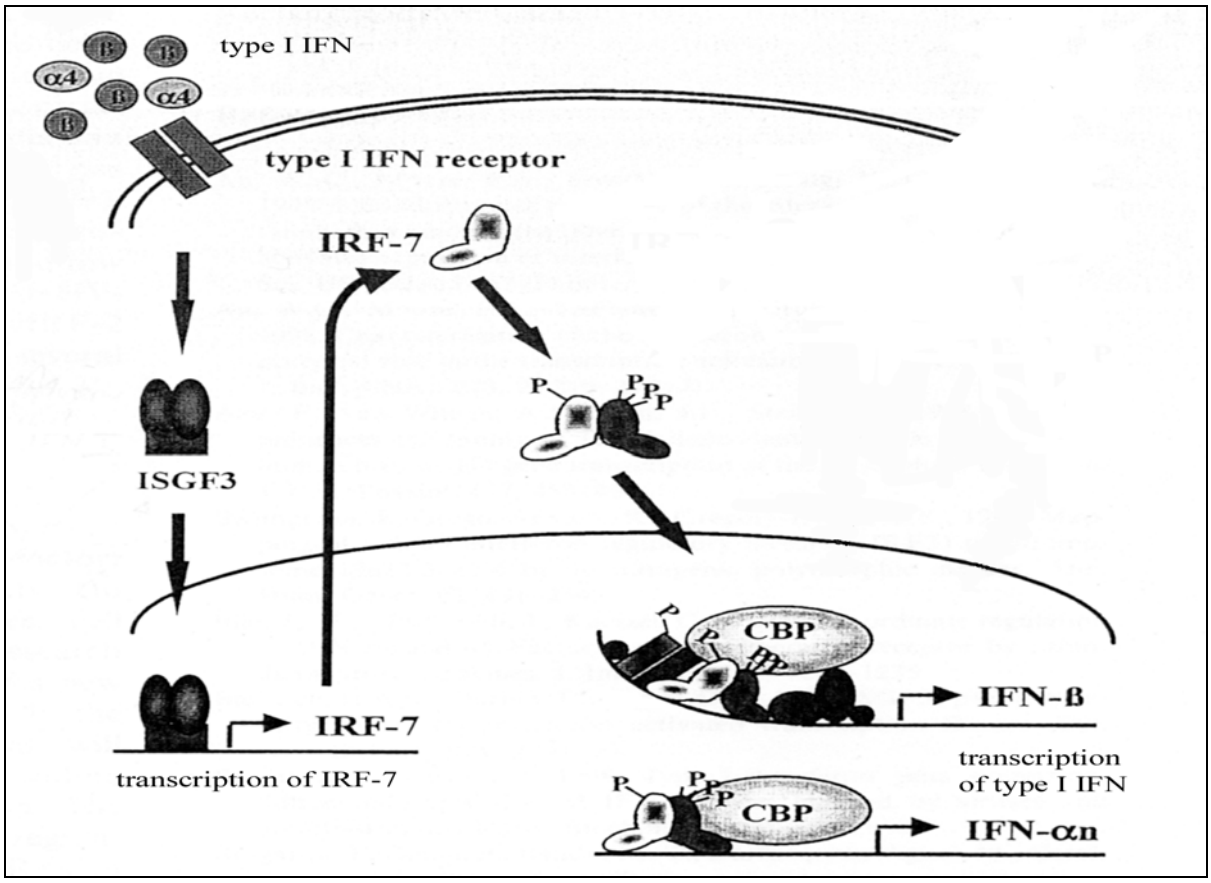

Image 8. Autoamplification of the IFN-α/β response via IRF-7 and ISGF3 (simplified from 142)

Other type I IFN induced genes upregulated in immune mice include the transcription factor IRF-1, thoroughly described in section 4.5, and glucocorticoid attenuated response genes GARG-16 and GARG-49, immediate early/primary response genes whose induction by an inflammatory stimulus is attenuated by glucocorticoids. These genes can be induced in

macrophages upon stimulation with IFN-α/β (143). Thus many type I IFN associated genes are upregulated predominantly in *H. pylori* immunized/challenged mice.

These results may be surprising given that type I IFN is typically associated with viral infections but its expression has also been documented to be induced in macrophages by intracellular bacteria (144). And there are now several reports of *H. pylori* surviving within phagocytes *in vitro* (145,146). Additionally, a recent gene array analysis of Kato 3 gastric epithelial cells exposed to *H. pylori* also registered an increase in IFN-α/βR (147). And, Jiang *et al*. have recently reported that co-infection of *H. felis*-infected mice with a replication-defective adenovirus results in a reduced bacterial load in the gastric mucosa (148). Although type I IFN levels were not determined in that study, type I IFN is normally involved in anti-viral immune response. It may be that the virus associated IFN-α/β played a role in reducing the *H. felis* load.

The *in vitro* assays performed in the present study did not provide further evidence for increased levels of type I IFN in immunized mice, since only traces of IFN-α were detected with no difference between groups or stimulation conditions. However, this does not rule out the production of type I IFN under other conditions of stimulation, or IFN-β production by fibroblasts. Still, these results suggest a novel pathway of an anti-bacterial immune mechanism against *H. pylori.*

4.5 Correlations between heightened inflammation, protection and IFN-γ

As already mentioned immunized/challenged mice displayed an expression profile distinct from infected mice, predominated by IFN associated genes. These genes included transcription factors such as IRF-1, IRF-7, ISGF3 and eLF-3, several GTPases known to be induced upon IFN-γ stimulation, and other genes of poorly characterized function.

IRF-1 can be induced by both type I and type II IFN, although it is thought to be more important in mediating the antiviral effects of IFN-γ than IFN-α (as reviewed by Nguyen, 149). Gene transcription activated by this transcription factor includes iNOS, an enzyme necessary for nitrogen radical production of polymorphonuclear cells, type I IFN (150), and others. IRF-1 also plays a critical role in multiple stages of TH_1 differentiation (141,149). Immune cells from IRF-1 gene deficient mice exhibit defective TH_1 responses, impaired macrophage production of IL-12, deficient $CD4^+$ T cell responses to IL-12, ablated NK cell

development, and exclusive TH_2 differentiation of macrophages and $CD4^+$ T cells in vitro (151). IRF-1 is also required for a TH_1 immune response in vivo (152).

Interestingly, other IRF-1 induced genes such as IL-1-β converting enzyme were also upregulated in immunized/challenged mice. Additionally, the 65-kDa family of the IFN-γ induced GTPases present in the immunized/challenged and infected animals is under transcriptional control of IRF-1 (153). Members of the IFN-γ induced 47-kDa GTPase family, whose expression does not depend on IRF-1, were also upregulated predominantly in immunized/challenged compared to *H. pylori*-infected mice.

ISGF-3 not only plays a role in IFN-γ signalling, but can also be induced by IFN-γ. This trimolecular complex of STAT 1 and STAT 2 and an interferon regulatory factor (ISGF3-γ) participates in the transcriptional activation of a large number of IFN-inducible genes (140-142).

Other IFN-γ associated genes upregulated in the immunized/challenged mice included eLF-3, an IFN-inducible transcription factor that regulates the type II TGF-beta receptor gene (154), glucocorticoid attenuated response genes GARG-16 and GARG-49/IRG2, and a large number of other genes.

The strong presence of MHC genes, especially in immunized/challenged mice is consistent with previous reports documenting IFN-γ production in stomachs of both infected and immunized/challenged mice which is known to induce the expression of MHC antigens. Additionally it is more likely to see differences in the expression of MHC I genes in the context of an inflammatory IFN-γ response, rather than in the context of protection, since it has been previously shown that a protective immune response is not dependent on MHC I.

The increased expression of all these genes in immunized/challenged mice compared to naïve, and in some cases compared to infected mice suggests a strong presence of IFN-γ in this group.

Consistent with this expression of IFN-γ induced genes *in vivo*, the *in vitro* assays showed that bulk spleen cells of immune mice produce higher levels of IFN-γ than chronically infected mice upon stimulation with live *H. pylori* in culture. When purified $CD4^+$ T cells, the cell type known to confer protection, were stimulated with live *H. pylori* this difference was even more pronounced.

Bulk spleen cells from immunized animals also produced IFN-γ in reaction towards *H. pylori* lysate. This IFN-γ production can probably not be totally attributed to $CD4^+$ T-cells, since

IFN-γ production upon stimulation with lysate was abolished when purified $CD4^+$ T-cells were used. In the bulk spleen cell *in vitro* assay also other IFN-γ producing cells like $CD8^+$ T cells or NK cells were present. It is likely that IFN-γ was spontaneously released by NK cells, while an activation of $CD8^+$ T cells seems less likely in the context of MHC II antigen presentation. An activation of NK cells in immunized/challenged animals seems likely in the context of the genes that are upregulated, as NK activation can be stimulated by IL-12, a TH_1 cytokine signalling through IRF-1, and by type I IFN through ISGF3 (141). Both IRF-1 and ISGF3 are selectively upregulated in immunized/challenged mice compared to infected mice. Whether NK activation occurs *in vivo* and if it contributes to protection remains to be elucidated.

It has previously been reported that IFN-γ producing T cells accompany both *H. pylori*-induced inflammation in mice and humans as well as challenge of immunized mice (78-82,112). The results of the *in vitro* assays in this study in which lymphocytes from infected mice produce less IFN-γ than immune mice are consistent with evidence provided by Mohammadi *et. al.* when spleen cells from *H. felis*-infected and immune mice were compared for their ability to proliferate and produce IFN-γ in an *in vitro* recall assay (82).

Early studies have documented that IFN-γ contributes to the *H. pylori*-associated inflammatory response. In many studies on humans and mice, IFN-γ producing cells were the predominant T cell type present in the stomach of hosts with *H. pylori* gastritis (78-82), and more intense disease correlated with a TH_1 phenotype of T cells (155). Additionally, the severity of gastric inflammation was significantly reduced by neutralization of IFN-γ in nonimmunized/infected as well as in immunized/challenged mice (82). Even more convincing, IFN-$\gamma^{-/-}$ mice showed no gastric inflammation when infected with *H. pylori*, whereas their wild type controls displayed a strong inflammatory response.

In one study, *Helicobacter*-induced mucosal inflammation was increased in mice deficient of the main TH_2 gene IL-4, but not in IFN-γ gene deficient mice (156), and Mohammadi *et. al.* could show that the adoptive transfer of TH_2 cells before challenge with *H. pylori* reduced the bacterial load in the challenged animals, while transfer of IFN-γ producing TH_1 cells increased gastric inflammation (157). Additionally, the lack of genes that are important for IFN-γ signalling affects the outcome of gastritis. In a study by Sommer *et. al.*, IRF-1 gene

deficient mice were incapable of generating an inflammatory response towards *H. pylori* infection (158), although the bacterial load was comparable to that from wild type controls. Therefore, an IFN-γ mediated TH_1 response has been thought to contribute to gastric inflammation and host damage, while a TH_2 response has been considered protective by many groups.
However, in the study by Mohammadi *et al*. (157) the TH_2 cell line used for adoptive transfer was derived from a *H. pylori*-specific TH_1 cell line that previously produced only very low levels of IL-5 and no IL-4. Thus it remains unclear whether this cell line acted as TH_2 *in vivo*, as it also slightly exacerbated gastric inflammation.

Recent findings and a closer look to vaccination studies provide evidence that not a TH_2 polarization, but an intense inflammatory response, predominantly mediated by TH_1 cytokines like IFN-γ and IL-12, may be required to achieve protective immunity.

Most of the groups investigating the immune response after vaccination have measured either TH_1 or mixed TH_1/ TH_2 responses with different adjuvants, even after immunization with CT, a TH_2 polarizing adjuvant (82, 101, 110, 159). Thus at least part of the cellular immune response after immunization was directed towards TH_1. When the vaccination was carried out with CT this polarization may have been in part due to LPS contamination of the bacterial sonicate used as antigen in most studies, as LPS is known to induce TH_1 type responses (160, 161).

IFN-γ gene deficient mice are more readily infected with different strains of *H. pylori* than are their wild type counterparts and the inflammatory response towards the bacterium is much milder (162). In another study mice were systemically immunized with *Helicobacter* antigen with either a TH_1 polarising adjuvant (complete Freund´s adjuvant) or a TH_2 polarising adjuvant (alum). Immune polarisation was confirmed by ELISPOT assays of cytokine production. Both of these polarised immune responses resulted in post-immune gastritis upon challenge with *H. pylori* and both immunizations protected mice against challenge with *H. pylori* (112). Jiang *et. al.* demonstrated that the protective effect of intramuscular injection of replication defective adenovirus, as described above, did not occur in mice deficient in the TH_1 cytokines IL-12 and IFN-γ (148).
Another study documents the inability to induce protective immunity in mice lacking IL-12 activity (163). These mice totally lacked an inflammatory response towards challenge with

H. pylori. IL-12 is usually produced by antigen presenting cells and stimulates IFN-γ production by T cells, thus directing the immune response towards TH_1. IL-12 deficient mice are impaired in the ability to produce IFN-γ following endotoxin administration (164), and in humans, the production of IFN-γ by peripheral blood mononuclear cells from *H. pylori*-infected donors upon stimulation with different *H. pylori* preparations is inhibited by addition of anti-IL-12 antibody to cultures (165).

Studies with IFN-$\gamma^{-/-}$ mice have yielded contradictory results. Akhiani *et al.* could demonstrate the requirement of a functional IFN-γ response for protection, as IFN-$\gamma^{-/-}$ mice were not protected against challenge with *H. pylori* (163). However, protection could be achieved in IFN-γ gene deficient mice in another study (162). Although the vaccination protocols were very similar using orally delivered bacterial sonicate and CT, differences in the amount of antigen might have lead to different results.

Additionally, Garhart *et al.* could show that a TH_2 response is not required for protection from *H. pylori* infection as both IL-4 and IL-5 deficient mice could be protected by immunization (166).

However, although IFN-γ may not be required for protection, there is evidence that it can play a role in protective immunity by heightening the inflammatory TH_1 response. The results of this study are actually consistent with an evolving theory regarding the role of inflammation in anti-*Helicobacter* immunity. Thus, while possibly not essential for protection, it may still be important to employ signalling events that are typically associated with IFN-γ but that may be activated by alternative pathways in its absence. This would allow IFN-γ deficient mice to be protected by immunization. However, deletion of genes such as IRF-1 or IL-12 that occur upstream in the inflammatory cascade, prior to some potential branch points would prevent the ability to develop inflammation and thus prevent protective immunity. Thus, a strong inflammatory response predominantly mediated by TH_1 cells plays an important role in protection from *H. pylori* infection.

The mechanisms by which TH_1 cytokines induce protective immunity or reduce *Helicobacter* colonization remain poorly understood. Since no direct anti-microbial activity has been described for $CD4^+$ T cells, the immune effector mechanism, while receiving help signals from primed T cells, is most probably non-lymphocytic in nature.

TH_1 cells in the gastric mucosa could regulate other mechanisms that would affect bacterial survival. The ability of macrophages to phagocytose and control bacterial infection is under

strong influence of IFN-γ and IL-12 and may play a role in the control of gastric *Helicobacter* infection. The efficacy of the oxidative burst of polymorphonuclear cells could be enhanced through IFN-γ by IRF-1 mediated upregulation of iNOS (150), an enzyme necessary for nitrogen radical production.

Moreover, IFN-γ could enhance APC functions of gastric epithelial cells by upregulation of MHC class II antigens (167), thereby focusing specific T cells to the epithelium. Such a mechanism coupled with increased apoptosis of epithelial cells, induced by *H. pylori* (167), could provide unfavourable growth conditions for the bacteria to survive in the gastric mucosa.

The inability of an unimmunized host to clear the infection might also be a consequence of regulatory T cells that actively suppress T cell responses. A vaccine could help to overcome this immune suppression by inhibiting the induction of regulatory T cells within the appropriate stimulation conditions. On the other hand, a strong functional TH_1 response induced by vaccination might be able to counteract immune suppressive effects mediated by regulatory T cells.

4.6 Crosstalk: Overlaps of type I and II IFN pathways

It is likely that the type I and type II IFN pathways are not mutually exclusive, but rather coexist and possibly interact.

Although the majority of the genes listed in **Table 5** are preferentially related to either IFN-α/β or IFN-γ, most of them can be induced by both types of IFN.

As shown in **Image 9**, type I and II interferon can employ common intracellular signalling pathways. For instance, ISGF-3 can be activated by both IFN-α/β and γ. While its expression is only induced by IFN-γ, target genes of this transcription factor include type I IFNs. Both type I and type II IFNs can induce IRF-1 expression, which itself works as a transcriptional activator of IFN-α and β. However, it remains unclear whether these pathways are equally expressed and employed in different cell types.

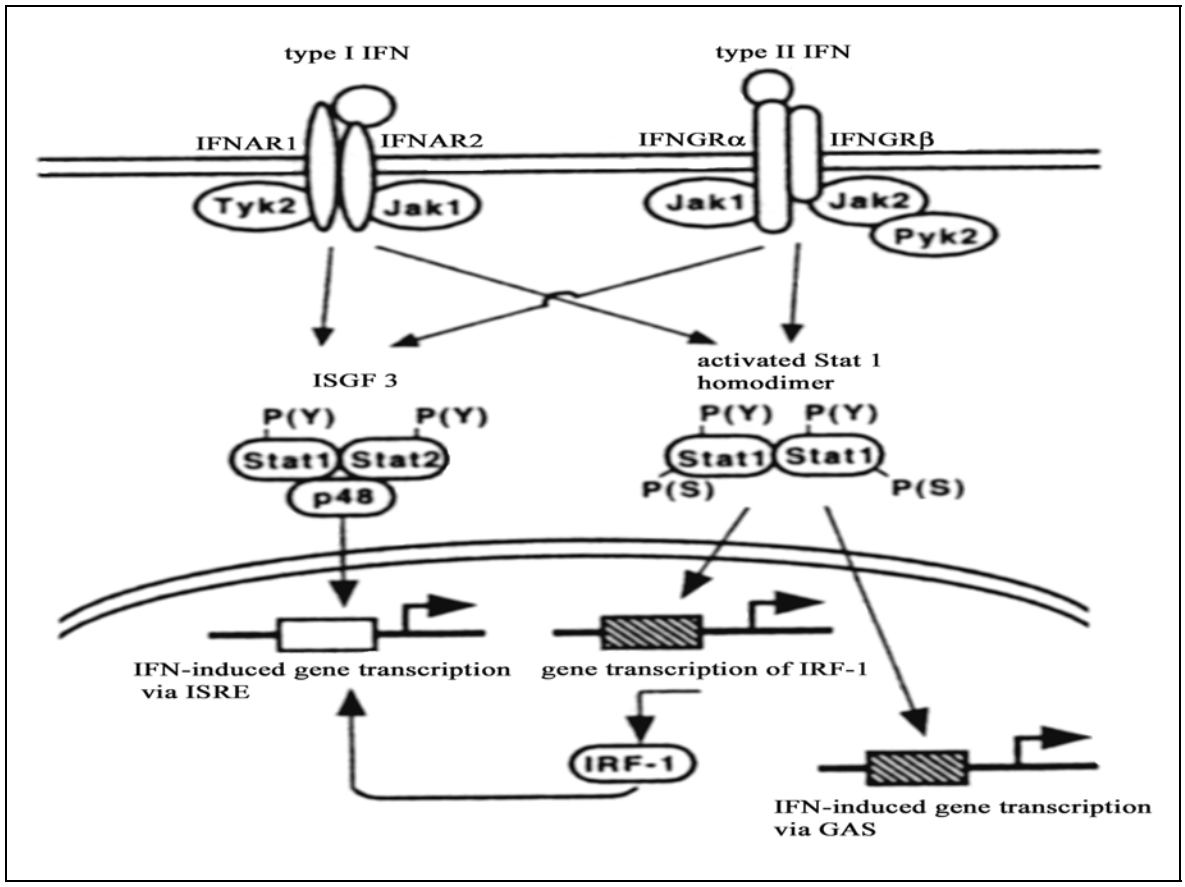

Image 9. Common intracellular signalling pathways of type I and II IFN (adapted from Taniguchi *et. al.*, 141)

Results of the *in vitro* stimulation assay make it seem more likely that expression of these genes is due to type II rather than type I IFN, since only baseline levels of IFN-α were detected. However, even subthreshold levels of IFN-α/β could play a role in the development of the full IFN-γ response because of a crosstalk between IFN-γ and IFN-α/β signalling components, as proposed by Takaoka *et. al.* (168). Their experiments showed an impairment of IFN-γ induced pathways in mouse embryonic fibroblasts (MEF) from mice lacking IFNAR1, a domain of the IFN-α/β receptor. In these cells, IFN-γ induced activation of STAT 1 and ISGF-3 were reduced compared to wild type MEFs. Similar results for STAT 1 were obtained in splenocytes of these mutant mice. Additionally, a deficiency in IFN-γ induced STAT 1 activation was observed in MEF from IFN-β deficient mice, which do not produce either IFN-α or β, based on the dependence of IFN-α production on IFN-β production in MEF. This deficiency was rescued by exogenously added IFN-β in a concentration too low to activate STAT 1 itself. This crosstalk appeared unidirectional in that IFN-γ signalling was dependent on IFN-α/β signalling, but not vice versa.

The present study provides evidence that the inflammatory infiltrate that arises in the gastric mucosa when immunized mice are challenged with *H. pylori* is associated with specific T cells sets and protein families that are distinct from those present in Helicobacter-associated chronic inflammation. Gene array profiles and *in vitro* assays all indicate that immunized mice are more readily poised than infected mice to promote IFN-γ production and IFN related events. These events lead to severe inflammation. However, this inflammation is distinct from the nonprotective inflammatory response observed in both *H. felis* and *H. pylori* infections. Whether these immune T cells and IFN associated proteins promote a specific protective effector mechanism or simply promote a more intense inflammatory response will be the subject of future research. Understanding the fundamental difference between the protective immune response after vaccination and the non-protective response during chronic infection is necessary for the development of effective prevention and therapy against diseases caused by *H. pylori* infection.

5 SUMMARY

Challenge of immunized mice with *H. pylori* induces protective gastric inflammation that is histologically indistinguishable from chronic *H. pylori*-associated gastritis in non-immune mice. To identify mechanisms of protective immunity gene expression in the gastric tissue from infected mice and mice vaccinated prior to challenge was compared by DNA array analysis. Message RNA was used to screen over 10,000 murine genes. Major Histocompatibility Complex antigens and IFN-γ dependent GTP binding proteins were strongly upregulated in both infected and immunized/challenged mice compared to naïve controls. Differences in gene expression were also observed in novel T cell genes, which were exclusively upregulated in immunized/challenged mice. Both IFN I and II associated genes like the IFN-α/β receptor or IFN dependent transcription factors mIRF-1 and ISGF3 were also predominantly expressed in this group. These results were confirmed for several candidate genes by semi-quantitative RT-PCR. Additionally, *H. pylori*-stimulation of $CD4^+$ T cells from immune mice induced significantly more IFN-γ production than stimulation of cells from infected mice. The present study provides evidence that the inflammatory infiltrate that arises in the gastric mucosa when immunized mice are challenged with *H. pylori* is associated with specific T cells sets and protein families that are distinct from those present in *Helicobacter*-associated chronic inflammation. Gene array profiles and *in vitro* assays indicate that immunized mice are more readily poised than infected mice to promote IFN-γ production and IFN related events and thus promote a strong proinflammatory TH_1 response. This study supports recent findings that an immune response dominated by TH_1 cytokines is essential for protection from *H. pylori* infection. This insight could facilitate the choice of the appropriate adjuvants for the development of vaccines against *H. pylori*, which are efficient and safe for use in humans. The mechanisms by which TH_1 cells induce protective immunity or reduce *Helicobacter* colonization remain poorly understood and will be subject of future research.

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbständig verfaßt und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät vorgelegt worden.

Ich erkläre, daß ich bisher kein Promotionsverfahren erfolglos beendet habe und daß eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

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