Virulence factors of *Helicobacter suis* with emphasis on γ-glutamyl transpeptidase

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List of Abbreviations

AA	Amino acids
AP1	Activator protein 1
Arg	Arginine
ATP	Adenosine triphosphate
BabA	Blood group antigen-binding adhesion
CagA	Cytotoxin-associated gene A
*cag*PAI	*cag* pathogenicity island
cAMP	Adenosine 3',5'-cyclic monophosphate
CCK	Cholecystokinin
CLRs	C-type lectin receptors
CaSRs	Calcium-sensing receptor
DCs	Dendritic cells
EAA	Essential amino acid
ECL	Enterochromaffin-like
Foxp3	Forkhead/winged helix transcription factor 3
GGT	γ-glutamyl transpeptidase
GI	Gastrointestinal
Gln	Glutamine
Glu	Glutamate
GSH	Glutathione
GPCRs	G protein-coupled receptors
HCl	Hydrochloric acid
H+/K+ ATPase	Hydrogen potassium ATPase
HpaA	*H. pylori* adhesion A
IHC	Immunohistochemistry
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide

MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinases
mGluRs	Metabotropic glutamate receptors
MyD88	Myeloid differentiation primary response gene 88
NEAA	Non-essential amino acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHPH	Non-*Helicobacter pylori* helicobacters
NLRs	NOD-like receptors
NOD	Nucleotide binding oligomerization domain containing protein
NO	Nitric oxide
OMP	Outer member protein
OMV	Outer membrane vesicles
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
RLRs	RIG-like receptors
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SabA	Sialic acid–binding adhesion
Th	T helper
TIRAP	TIR-containing adaptor protein
TGF-β	Transforming growth factor-β
TLRs	Toll-like receptors
TRAM	TRIF-related adaptor molecule
Tregs	Regulatory T cells
T4SS	Type IV secretion system
VacA	Vacuolating cytotoxin

Chapter 1
General Introduction

1. *Helicobacter suis:* the bacterium

1.1 Characterization of *Helicobacter suis*

In 1990, the first description of large, spiral-shaped bacteria in the mucus layer of lumen and antral pits of pig stomachs was made (Mendes *et al*., 1990; Queiroz *et al*., 1990). Initially, "*Gastrospirillum suis"* was proposed as a name, but further characterization showed that this organism in fact belonged to the genus *Helicobacter* (De Groote *et al*., 1999). A new name, *'Candidatus* Helicobacter suis' was then proposed. Despite numerous attempts worldwide, the first successful *in vitro Helicobacter* (*H*.) *suis* isolate was only obtained in 2008 from the stomach of slaughterhouse pigs (Baele *et al*., 2008).

H. suis is a Gram-negative bacterium, and has a typical spiral-shaped morphology with up to six turns. This bacterium is ~2.3-6.7 µm long and ~0.9-1.2 µm wide and it is highly motile by means of 4-10 flagella at both ends of the cells (Baele *et al*., 2008) (Figure 1).

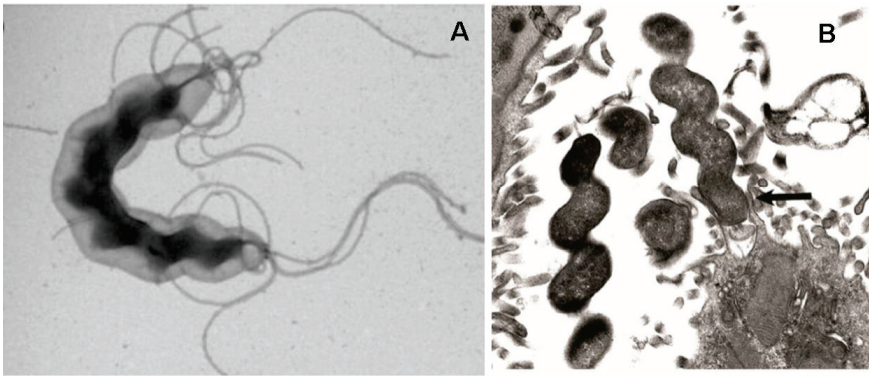

Figure 1. Transmission electron microscopy images of *H. suis in vitro* and *in vivo* (Adapted from Baele *et al*., 2008 and Flahou *et al*., 2012). (A) An *in vitro* cultured *H. suis* bacterium exhibiting bipolar flagellae. (B) Spiral-shaped *H. suis* bacteria adhering to the gastric epithelium in the stomach of an experimentally infected BALB/c mouse.

For primary isolation and culture, specific conditions are required (Baele *et al*., 2008). In general, the bacteria are grown on biphasic *Brucella* agar plates with a pH of 5 and supplemented with 20% fetal calf serum, 5 mg/L amphotericin B, and Vitox supplement under microaerobic conditions (37°C; 85% N_2, 10% CO_2, 5% O_2). Plates should be checked every 2 days. If growth is unsufficient, as assessed by light microscopic examination, *Brucella* broth supplemented with 20% fetal calf serum is added to keep the plates moist (Flahou *et al*., 2012). If sufficient bacteria are present (at least 2 x 10^7 bacteria/ml, but preferably more), the *H. suis* containing broth can be divided onto 2-4 fresh agar plates. Very recently, a new method was established to acquire pure cultures of *H. suis* by growing

bacteria as individual colonies on 1% *brucella* agar plates, after which they are purified and enriched by conventional biphasic subculture as described above (Liang *et al*., 2014).

1.2 Phylogeny and genome of *H. suis*

The differentiation of *H. suis* from other gastric *Helicobacter* species has been done in previous studies by means of sequencing of part of the 16S and 23S ribosomal RNA encoding genes, genes encoding for the urease A and B subunits, the *gyrB* and *hsp60* genes. This resulted in the valid description of this bacterium as a distinct *Helicobacter* species (Baele *et al*., 2008). Its relationship to closely related gastric *Helicobacter* species is depicted in figure 2.

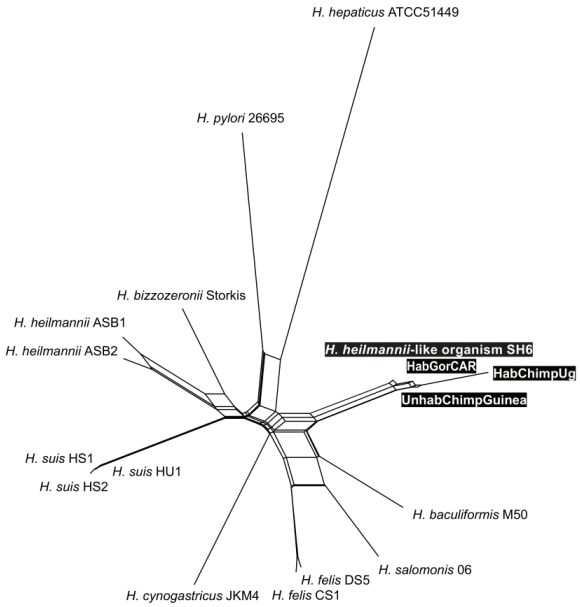

Figure 2. Phylogenetic network of *Helicobacter* species based on partial *ureAB* gene sequences. The network was built with SplitsTree4 (version 4.11.3) by the Neighbor-Net method. A clear distinction can be made between *H. suis, H. heilmannii, H. bizzozeronii, H. salomonis, H. felis, H. cynogastricus, H. baculiformis, H. pylori* and a putative new *Helicobacter* taxon/species present in great apes and humans (shown in black), putatively named '*Candidatus H. homininae'*. HabGorCAR: clone from a habituated gorilla, Central African Republic; HabChimpUg: clone from a habituated chimpanzee, Uganda; UnhabChimpGuinea: clone from an unhabituated chimpanzee, Guinea-Bissau (Flahou *et al*., 2014).

In 2011, a draft whole-genome sequence was published for *H. suis* strains HS1 and HS5 (Vermoote *et al*., 2011). Genome analysis showed that homologs of genes essential for gastric colonization of *H. pylori* were present in the *H. suis* genome, e.g. *H. pylori* adhesin A (HpaA) and *H. pylori* porins related gene B (HorB). In addition, other genes associated with

colonization and virulence of *H. pylori* and other bacteria were also shown to be present in the *H. suis* genome, including γ-glutamyl transpeptidase (GGT), urease encoding genes, neutrophil-activating protein (napA) and flavodoxin. However, homologs of some important virulent genes for *H. pylori*, such as the *vacA* gene, encoding the vacuolating cytotoxin A and most of the genes present in the *cag* pathogenicity island, were shown to be absent or non-functional in *H. suis* (Vermoote *et al*., 2011).

2 Virulence factors of gastric helicobacters involved in colonization, induction of cell death and immune regulation

Complex mechanisms, through the intervention of a large scale of bacterial factors, promote persistent colonization by gastric helicobacters, induce cell death of epithelial cells and other gastric cell types, regulate or evade the host immune response, and finally establish a long-term chronic infection in the host. A brief review of these mechanisms involving virulence factors of gastric helicobacters, will be given below.

Flagellar mobility and acid neutralization

The stomachs of animals and humans are characterized by a low pH as well as a constant secretion and shedding of the mucous layer, which is a challenging environment for most bacteria (Spohn *et al.*, 2001). *Helicobacter* possesses the ability to swim to the epithelial surface of the stomach with the assistance of highly mobile flagella and the presence of urease activity, neutralizing the acidity and decreasing the viscoelastic properties of gastric mucins (Salama *et al.*, 2013). In addition, the extraordinary mobility of gastric *Helicobacter* species in viscous substances is hypothesized to be due to the helical cell shape and the polarity (Lertsethtakarn *et al.*, 2011). Urease is a cytoplasmic protein, which is also found on the surface of stationary-phase bacteria probably due to lysis of a subpopulation (De Reuse *et al*., 2005, Marcus *et al*., 2011). The enzyme hydrolyzes urea to produce ammonia and carbon dioxide to neutralize the secreted acid. A sustained urease production is necessary to establish a pH-neutral microenvironment around the bacteria (Yoshiyama *et al.*, 2000). The urease enzyme complex consists of 2 subunits, Ure A and Ure B, as well as accessory proteins including Ure E, F, G, H and urea channel UreI (Mobley *et al.*, 1995, Akada *et al.*, 2000).

Both flagella and urease activity are indispensable for *Helicobacter* colonization, and *Helicobacter* strains lacking genes of the urease complex or flagellar apparatus are in general not capable of persistently colonizing the host stomach (Eaton *et al.*, 1991, Tsuda *et al.*, 1994,

Andrutis *et al.*, 1997). Whole-genome sequencing of *H. suis* has revealed that most known virulence factors from *H. pylori* involved in acid acclimation and bacterial mobility are also present in *H. suis* (Vermoote *et al.*, 2011). Thus, similar effects of these virulence determinants can be assumed to play a role in bacterial colonization of the stomach.

Adhesion to the gastric epithelial surface

Under the drive of flagella and protection of a pH-neutral microenvironment conferred by urease activity, *H. pylori* can move through and penetrate the viscous mucous layer and adhere to gastric epithelial cells by several specific adhesins (Testerman *et al.*, 2001, Andersen, 2007). Most of the adhesins are members of the large outer member protein family (OMP) of *H. pylori* (Odenbreit, 2005), and the blood group antigen-binding adhesin (BabA) and the sialic acid–binding adhesin (SabA) are the best-characterized adhesins associated with the adherence of *H. pylori* to gastric mucosa (Ilver *et al.*, 1998, Mahdavi *et al.*, 2002). These factors facilitate adherence to the fucosylated ABH/ Lewis b (Leb) blood group antigens and to the inflammation-associated sialyl-Lewis x (sLex) of gastric epithelium, respectively, and also favour bacterial colonization in *H. pylori*-infected patients (Mahdavi *et al.*, 2002, Rad *et al.*, 2002, Aspholm-Hurtig *et al.*, 2004). Moreover, it has been shown that adherence-associated lipoprotein A (alpA) and alpB are required for the adherence of *H. pylori* to human gastric tissue as well as for successful colonization of the guinea pig stomach (Odenbreit *et al.*, 1999, de Jonge *et al.*, 2004). HpaA, another identified adhesin, is a surface-located lipoprotein expressed by almost all *H. pylori* strains, and it was demonstrated that HpaA is essential for colonization in a mouse model based on proteomic analysis and an *in vivo* study using a HpaA mutant strain (Otoole *et al.*, 1995, Carlsohn *et al.*, 2006). Additionally, HorB has been described as an adhesin, and insertional mutagenesis of the *horB* gene in *H. pylori* SS1 exhibits a threefold reduced bacterial colonization capacity in the stomach of mice (Alm *et al.*, 2000, Snelling *et al.*, 2007). Some *H. pylori* genes involved in bacterial adherence have been detected in the *H. suis* genome, such as HorB and HpaA homologs (Vermoote *et al.*, 2011). Possibly, these proteins also contribute to the adherence of *H. suis* to the gastric epithelium.

Oxidative stress detoxification

H. pylori persists in the host stomach and bacterial infection induces a strong inflammatory response. Oxidative stress resistance is considered to be a crucial property that enables pathogenic bacteria to survive the toxic reactive oxygen species (ROS) released by the host (Wang *et al.*, 2004). Inflammation-induced oxidative stress is often not capable to

efficiently eliminate *H. pylori*, which possesses antioxidant enzymes, biological repair systems, and other antioxidant factors (Wang *et al.*, 2004, Wang *et al.*, 2006). Two well-studied bacterial oxidative stress resistance factors are catalase and superoxide dismutase (Wang *et al.*, 2006), but several others have thus far been identified, including methionine sulphoxide reductase (Alamuri *et al.*, 2004), NADPH quinone reductase (Wang *et al.*, 2004), and proline utilization A flavoenzyme (Krishnan *et al.*, 2006). Interestingly, it has been shown that *H. pylori* outer membrane vesicles (OMV) are also involved in bacterial oxidative stress resistance (Chitcholtan *et al.*, 2008). Notably, homologs of genes from gastric helicobacters encoding factors responsible for oxidative stress resistance can be detected in the *H. suis* genome as well, including catalase, superoxide dismutase, and NADPH quinone reductase (Vermoote *et al.*, 2011), indicating that *H. suis* possesses the necessary components to counteract oxidative stress.

Virulence factors involved in induction of cell death and immune regulation

An important mechanism through which *H. pylori* causes mucosal damage is the induction of cell death, including apoptosis and necrosis of the epithelial cell. Gastric epithelial cell death is considered to contribute to gastric ulcer formation, gastric atrophy and gastric cancer (Shirin *et al.*, 1998, Xia *et al.*, 2001). A series of virulence factors of *H. pylori* and NHPH involved in cell death have been identified since the isolation of *H. pylori* by Marshall and Warren in 1984 (Marshall *et al.*, 1984, Covacci *et al.*, 1999a, Radin *et al.*, 2011, Salama *et al.*, 2013).

GGT

GGT is one of the major virulence factors of gastric *Helicbobacter* species. Besides its presence in various bacterial species, membrane-bound GGT is also found in the eukaryotic cells, where it is mainly involved in catalyzation of GSH, as part of the GSH cycle (Yokoyama, H, 2007). GGT is expressed at relatively high levels in kidney, liver and brain tissue in humans. In addition, GGT in the serum serves as a marker of hepatic and biliary tract-associated diseases (Betro *et al*., 1973; Betro *et al*., 1973; Corti *et al*., 2010).

H. pylori GGT, a secreted periplamic deamidase, is the first identified bacterial GGT enzyme playing a role in the establishment of infection in its host (Chevalier *et al.*, 1999, Leduc *et al*., 2010). A number of studies have revealed that *H. pylori* GGT can induce apoptosis of gastric epithelial cells via a mitochondria-mediated pathway (Shibayama *et al.*, 2003, Kim *et al.*, 2007a). In addition, it has been shown to be a pathogenic factor in the

development of peptic ulcer disease (Gerhard *et al.*, 2005, Gong *et al.*, 2010b). *H. pylori* GGT has been shown to exhibit hydrolysis activity with very high affinities for Gln and GSH. These two substrates can be hydrolysed to glutamate by the action of GGT, which can be further transported into *H. pylori* cells by a Na^+-dependent reaction (Figure 3) (Shibayama *et al.*, 2007, Rossi *et al.*, 2012). Leduc and colleagues identified the transporter (GltS) responsible for the transportation of GSH- or Gln-derived glutamate into *H. pylori* (Leduc *et al*., 2010). Recent studies showed that GGT from *H. suis*, *H. bilis*, and *H. pylori* is in part responsible for inducing cell death including apoptosis or necrosis in human gastric epithelial cells or colon epithelial cells, and supplementation of gastric epithelial cells with GSH was shown to enhance the cell death-inducing effect of the enzyme, through the formation of pro-oxidant glutathione degradation products (Flahou *et al.*, 2011, Rossi *et al.*, 2012, Javed *et al.*, 2013).

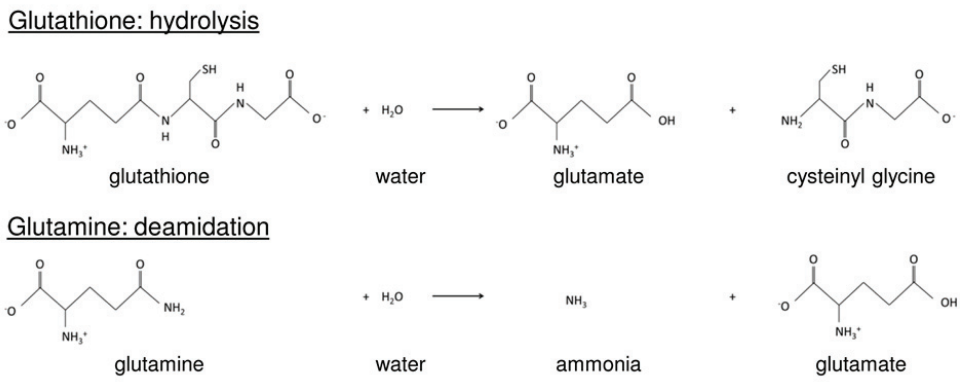

Figure 3. Actions of GGT on glutathione and glutamine.
Shown are GGT-mediated hydrolysis of glutathione and glutamine, with the formation of glutamate.

Besides the cell death-inducing ability, *H. pylori* GGT has also been shown to play a role in extensive immune regulation. Several studies have revealed that *H. pylori* GGT is a vital immunosuppressive factor mediating the inhibition of T cell proliferation by induction of a cell cycle arrest in the G1 phase (Gerhard *et al.*, 2005, Schmees *et al.*, 2007). In addition, microRNA profiling of non-infected and infected human T cells revealed that *H. pylori* infection triggers miR-155 expression *in vitro* and *in vivo*, and the bacterial GGT was identified as a factor regulating this miR-155 expression in human lymphocytes, providing an evidence for the direct link between this enzyme and regulation of microRNA (Fassi Fehri *et al.*, 2010). Recently, Oertli et al. demonstrated that *H. pylori* infection efficiently reprograms

murine DCs toward a tolerogenic phenotype and induces programming of regulatory T cells *in vitro* and *in vivo* with the critical contribution of two major virulence factors, including GGT (Oertli *et al.*, 2013). This suggests that GGT can affect or impair the functional immune response both in a direct and indirect manner.

VacA

VacA is another key virulence factor secreted by *H. pylori*. It was first described by Leunk et al. for its cytotoxic activity (Leunk *et al.*, 1988), and the first purification and characterization of this factor was done in 1992 (Cover *et al.*, 1992). After secretion through a type V autotransport secretion system, the VacA toxin binds to host cells. Subsequently, it is internalized, leading to the induction of severe cytoplasmic vacuolation characterized by accumulation of large vesicles consisting of endosomes and lysosomes (Palframan *et al.*, 2012). The induction of vacuolation in host cells has been observed in primary gastric epithelial cells (Garner *et al.*, 1996, Smoot *et al.*, 1996) and gastric epithelial cell lines (de Bernard *et al.*, 1997, Palframan *et al.*, 2012). Besides its vacuole-inducing ability, it has been shown that this virulence factor shares a series of pathogenic features with GGT, such as conferring advantage for bacterial colonization in mice (McGovern *et al.*, 2001, Salama *et al.*, 2001), inhibiting T cell proliferation (Sundrud *et al.*, 2004, Schmees *et al.*, 2007), inducing cell death of gastric epithelial cells (Kuck *et al.*, 2001, Kim *et al.*, 2007a), and facilitating induction of Tregs in naïve T cells by *H. pylori* (Oertli *et al.*, 2013). Studies showed that purified VacA can impair mitochondrial membrane potential of gastric epithelial cells followed by a decrease in energy metabolism and glutathione metabolism (Kimura *et al*., 1999, Kimura *et al*., 2001). It was further shown that the small *H. pylori* VacA subunit acts as a small pore-forming toxin, targeting to the mitochondrial inner membrane (Domańska *et al*., 2010), and endoplasmic reticulum stress may contribute to *H. pylori* VacA-induced apoptosis of gastric epithelial cells (Akazawa *et al*., 2013). Genome analysis showed that a functional VacA is not present in *H. suis* (Vermoote *et al.*, 2011).

Cytotoxin-associated genes pathogenicity island (*Cag*PAI)

The *Cag*PAI of *H. pylori*, a 40-kb genomic stretch, comprises about 27-31 genes depending on the clinical strain (Akopyants *et al.*, 1998), and was most likely acquired by a horizontal DNA transfer event from an unknown non-*Helicobacter* source during the course of evolution (Censini *et al.*, 1996b). Virulent *H. pylori* isolates often carry the *Cag*PAI, encoding a Type IV secretion system (T4SS) that can translocate the CagA oncoprotein (but

also for instance peptidoglycan) into host cells (Censini *et al.*, 1996a, Odenbreit *et al.*, 2000, Viala *et al.*, 2004). The CagA subsequently interacts with a large group of host proteins involved in host signal transduction pathways (Bourzac *et al.*, 2005), which is associated with the development of peptic ulcer disease, gastric cancer, and other severe *H. pylori*-associated pathologies (Segal *et al.*, 1997, Bourzac *et al.*, 2005, Salama *et al.*, 2013). Several components of *Cag*PAI, such as CagF (Seydel *et al.*, 2002), CagI (Wang *et al.*, 2012), CagL (Shaffer *et al.*, 2011), CagM (Ling *et al.*, 2013), CagY (Wiedemann *et al.*, 2009) have been characterized and shown to be required for the secretion and translocation of CagA.

CagA and other components of the *Cag*PAI have been implied in the regulation of the cell cycle and cell apoptosis. It has been shown that apoptosis of human monocytes induced by *H. pylori* strains is dependent on the functional expression of *Cag*PAI (Galgani *et al.*, 2004). By contrast, the CagA has been described to enhance the ability of B lymphocytes to prevent apoptosis through phosphorylation of the pro-apoptotic protein Bad, which may contribute to the development of MALT lymphoma (Zhu *et al.*, 2007, Lin *et al.*, 2010). Furthermore, miRNA-155 also exerts anti-apoptotic effects on macrophages in a T4SS-dependent manner to increase their resistance to apoptosis induced by DNA damage during *H. pylori* infection (Koch *et al.*, 2012). In an experimental study in Mongolian gerbils, a $CagA^+$ *H. pylori* strain was shown to facilitate host cell survival and to activate anti-apoptotic pathways to conquer self-renewal of the gastric epithelium (Mimuro *et al.*, 2007). With regards to *H. suis*, genome analysis has shown that most members of the T4SS, including the CagA, are not present in the *H. suis* genome, except for homologs of CagE and CagX, indicating that this microorganism lacks a functional cag protein transporter secretion system (Vermoote *et al.*, 2011).

3. *Helicobacter suis* infection in humans and pigs

3.1 Nomenclature of gastric non-*Helicobacter pylori* helicobacters (NHPH)

In the first half of the 20th century, it was generally assumed that the stomach could not be inhabited by microbes, due to the acidity of the gastric environment. However, in 1984, it was reported by Marshall and Warren for the first time that human gastritis and gastric ulceration were caused by a thus far unidentified slightly curved bacterium (Marshall *et al.*, 1984). In 1989 this curved bacterium was named *Helicobacter* (*H.) pylori* (Goodwin *et al.*, 1989), and in subsequent years, it was shown that this bacterium could lead to the development of gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated

lymphoid tissue (MALT) lymphoma in humans (Axon, 1999, Ernst *et al.*, 2000). *H. pylori,* however, is not the only gastric *Helicobacter* species capable of colonizing the human gastric mucosa. Indeed, large spiral shaped non-*H. pylori Helicobacter* (NHPH) species have been detected on numerous occasions in human gastric biopsy samples (Lee *et al.*, 1989, Heilmann *et al.*, 1991, Svec *et al.*, 2000, Trebesius *et al.*, 2001, Van den Bulck *et al.*, 2005, Haesebrouck *et al.*, 2009).

The nomenclature of this challenging group of bacteria has always been very confusing, in part because of the inability to cultivate these bacteria *in vitro*. They were originally named *Gastrospirillum hominis* (McNulty *et al.*, 1989) and soon renamed as *H. heilmannii* (Heilmann *et al.*, 1991). Further analysis of the 16S ribosomal RNA (rRNA) gene identified two different types of bacteria belonging to this "*H. heilmannii*" group: "*H. heilmannii*" type 1 and "*H. heilmannii*" type 2. Subsequent morphological and genetic analysis proved that "*H. heilmannii*" type 1 was identical to *Helicobacter suis*, a bacterium colonizing the stomach of pigs and isolated for the first time *in vitro* in 2008 (De Groote *et al.*, 1999, O'Rourke *et al.*, 2004c, Baele *et al.*, 2008). This micro-organism was first designated as '*Gastrospirillum suis*', then described as '*Candidatus* Helicobacter suis' for almost 10 years until it was isolated successfully *in vitro* in 2008, resulting in the final valid description of *Helicobacter suis* as a species (Mendes *et al.*, 1991, De Groote *et al.*, 1999, Baele *et al.*, 2008). "*H. heilmannii*" type 2 does not represent one single *Helicobacter* species, but it represents various dog- and cat-associated *Helicobacter* species including *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis* and the recently isolated *H. heilmannii* sensu stricto, which has been described to colonize the stomach of cats and dogs with a prevalence ranging from 20% - 100%, depending on the study (Haesebrouck *et al.*, 2009, Haesebrouck *et al.*, 2011).

Due to the very fastidious nature of the organism, *in vitro* culture of *H. suis* has remained unsuccessful, until in 2008, pure cultures of *H. suis* were obtained in the laboratory for the first time (Baele *et al.*, 2008). To date, *in vitro* isolated strains of *H. suis* are only available in the research group of the candidate.

3.2 *H. suis* infection in pigs

3.2.1 The anatomy and physiology of the stomach

The ancient Egyptians already recognized and described the gross anatomy of the stomach, and proposed that the stomach is responsible for the breakdown of food (Baron, 1979). In 1823, hydrochloric acid (HCl) was identified as the acid responsible for digestion of

food in the stomach (Rosenfeld, 2003). The stomach linking the esophagus and small intestine, is the dilated part of the digestive tract, and its main functions are acidification and maceration of the food to the liquid state-chyme through the actions of proteolytic enzymes and HCl, and temporary storage until the contents are passed into the intestine (Dyce *et al*., 2002). Both pigs and humans are monogastric species, and pig stomachs have a gastric diverticulum surmounting the fundus distinguishing them from other simple stomachs (Figure 4) (Dyce *et al*., 2002).

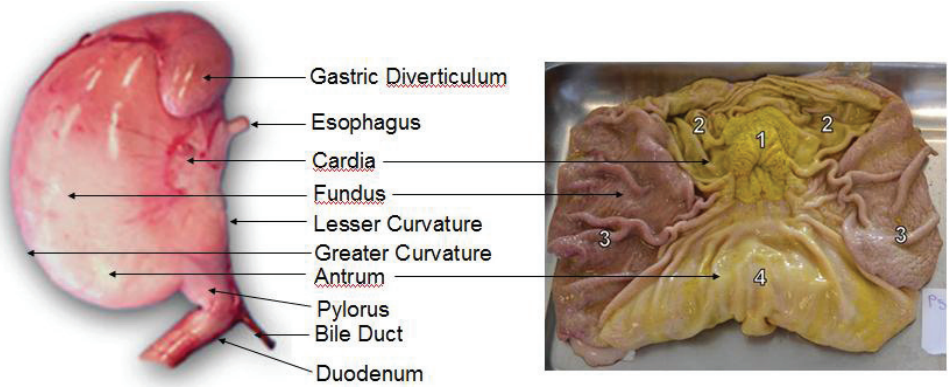

Figure 4. Anatomy of the porcine stomach. In the left panel, a closed pig stomach is depicted. When opened along the curvatura major, the 4 main stomach regions can be seen, each characterized by the presence of a typical epithelial layer: 1) pars oesophagea 2) cardiac gland zone 3) fundic gland zone/corpus 4) antrum/pyloric region. (Adapted partly from http://www.onemedicine.tuskegee.edu/DigestiveSystem/Stomach/Porcine_Shape.html)

In general, the stomach consists of four regions: the cardiac gland zone, the fundic gland zone (corpus or body), the pyloric gland zone (antrum), and the pyloric region (Dyce *et al*., 2002). The corpus and antrum can be distinguished by the transition from oblique rugae to a relatively flat mucosa, and the pylorus can be easily palpated, with a ring of muscle separating the stomach and the duodenum (Soybel, 2005). In the stomach of pigs, a characteristic small rectangular region around the oesophageal opening is composed of non-glandular keratinized epithelium resembling that of the oesophagus. This region is called the pars oesophagea (Krakowka *et al.*, 2006) (Figure 4).

As is typical for the digestive tract, the stomach comprises four main layers. From the innermost to the outermost, these four layers are the tunica mucosa, the tunica submucosa, the tunica muscularis, and the tunica serosa. The mucosal layer can be subdivided into 3 layers from the inside outwards: the lining epithelium, the underlying lamina propria, and the lamina muscularis mucosae (Figure 5). The epithelium of the stomach lining the gastric lumen is simple columnar epithelium, and its main function is secreting products essential for the

digestive process. Gastric glands can be found invaginating the lamina propria, which is a supporting layer of loose connective tissue. The lamina muscularis mucosae is a smooth muscle layer that separates the mucosa from the submucosa. The latter harbours blood vessels, nerve fibers, lymphatic structures and exocrine glands. Contractions of the outer muscle layer (tunica muscularis) cause a physical dissociation of the food bolus and are responsible for the emptying of the stomach.

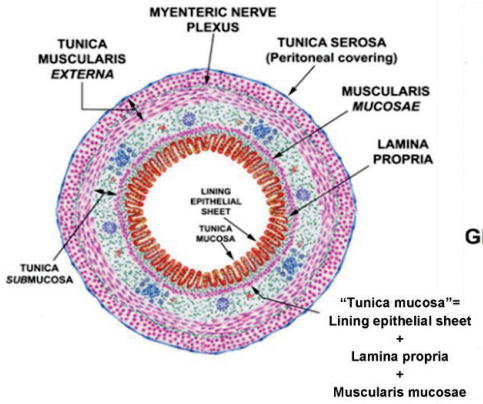

Figure 5. Structure of tubular organs. In general, the digestive tract includes 4 main layers, some of which can be subdivided: the tunica mucosa, tunica submucosa, tunica muscularis and tunica serosa. (Adapted from http://www.vetmed.vt.edu/education/curriculum/vm8054/Labs/Lab18/Lab18.htm##)

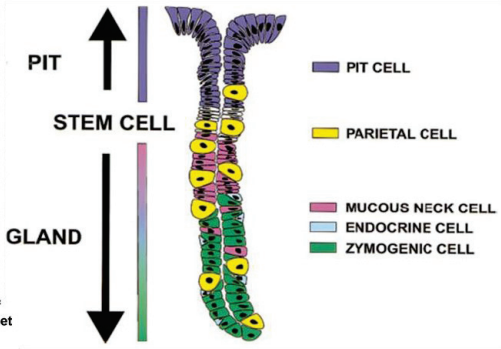

Figure 6. Different cell types in a gastric unit. Shown is a schematic diagram of a gastric gland in the corpus region of the stomach. Different cell types can be distinguished by different colours. (Adapted from van den brink et al., 2001)

Functionally, the glandular gastric mucosa is divided into acid-secreting and non-acid secreting regions based on the ability to secrete acid. The region around the cardia contains cardiac glands secreting mucus and bicarbonate. The corpus harbors acid-secreting glands/units, and the antrum is composed of alkaline-secreting surface epithelium and endocrine gastrin-secreting G-cells and somatostatin-producing D-cells (Soybel, 2005). The gastric unit includes the gastric pit and gland, and the gland consists of a base and a neck (van den Brink *et al.*, 2001).

Zymogen (pepsinogen)-secreting chief cells lining the base of the gland unit can be found in the corpus region. The middle of the gastric gland is largely populated with the HCl-secreting parietal cells, and parietal cells are still present at the neck, but give way to mucus neck cells. Near the opening of the gland into the gastric lumen, the mucosa is largely populated with lining epithelial cells. Enterochromaffin-like (ECL) cells located in the gastric gland beneath the epithelium express histidine decarboxylase, the enzyme that in turn leads to the conversion of histidine into histamine (Chen *et al.*, 1994a, Schubert, 2002) (Figure 6).

3.2.2 Prevalence of *H. suis* infection in pigs

Domesticated pigs are naturally prone to be colonized by *H. suis*, and this bacterium seems to have a worldwide distribution (Grasso *et al.*, 1996, Foss *et al.*, 2013). This tightly curved microorganism was first described to be present in the stomach of slaughtered pigs by Queiroz et al. (Queiroz *et al.*, 1990). *H. suis* infection in pigs has been reported with varying prevalence rates, depending on diagnostic tools used, the geographic region, the age of the animals, etc. In general, reported prevalence rates range from ~9% to ~95%, and the incidence of *H. suis* infection increases with age. Indeed, very low incidences have been described before weaning, whereas it can be as high as 90% in slaughter pigs (Barbosa *et al.*, 1995, Grasso *et al.*, 1996, Park *et al.*, 2004, Hellemans *et al.*, 2007a). A recent report in the United States has shown that the incidence of *H. suis* exceeds 50% in several major pig-producing areas (Foss *et al.*, 2013).

3.2.3 Clinical role of *H. suis* infection in porcine gastric pathology

H. suis infection has been reported to cause gastritis, which is mainly composed of infiltration with lymphocytes, plasmacytes and, to a lesser extent, granulocytes in the tunica mucosa or submucosa. Although this can be observed in all regions of the glandular stomach, the link between *H. suis* infection and the development of gastritis is the most obvious in the antrum. In addition to inflammation, infection with *H. suis* in pigs has also been described to cause gastric ulceration as well as other gastric pathological changes (Mendes *et al.*, 1991, Barbosa *et al.*, 1995, Hellemans *et al.*, 2007b, De Bruyne *et al.*, 2012). In 2012, De Bruyne et al. (2012) demonstrated for the first time under experimental conditions that pure *in vitro* cultures of *H. suis* do not only cause gastritis but also a notable reduction of the daily weight gain, which no doubt leads to substantial economic losses, although no exact data are available.

In the pig stomach, the pars oesophagea, surrounding the esophageal opening, is covered by stratified squamous epithelium. Due to the lack of mucus-producing glands and the sodium bicarbonate buffering system, which are general features of the gastric glandular mucosa, the pars oesophagea is more prone to damage inflicted by the acidic content of the stomach and other risk factors (Embaye *et al.*, 1990, Argenzio *et al.*, 1996, Casagrande Proietti *et al.*, 2010). Studies performed by several research groups have shown that pigs with lesions in the gastric pars oesophagea of the stomach are frequently colonized by *H. suis* (Barbosa *et al.*, 1995, Queiroz *et al.*, 1996, Cantet *et al.*, 1999). Interestingly, Sapierzynski et al. showed that *H. suis* infection can increase the number of gastrin-producing G cells,

decrease the number of somatostatin-producing D cells and increase the ratio of G cells/D cells, thus possibly leading to the secretion of excessive amounts of HCl, which may be responsible for gastroesophageal ulceration in swine (Sapierzynski *et al.*, 2007). In some studies, however, no obvious correlation was observed between the occurrence of ulcers and the presence of *H. suis* infection in the pig stomach (Cantet *et al.*, 1999, Hellemans *et al.*, 2007c), although conflicting results have clearly been reported (Queiroz *et al.*, 1996, Bedel *et al.*, 1997, Roosendaal *et al.*, 2000, Casagrande Proietti *et al.*, 2010). Similar to *H. pylori* infection in humans, most likely only a small portion of individual animals with *H. suis* infection will develop gastric ulcers (Megraud *et al.*, 1992, Cohen, 2000). The most plausible explanation may be that besides *H. suis* infection, a series of other factors may play a role, including nutritional conditions, stress, housing, management, changes in the environment, the presence of other bacteria, etc. (Queiroz *et al.*, 1996, Choi *et al.*, 2001, Appino *et al.*, 2006). It has been demonstrated that ensuring the stomach contains feed at all times (Friendship, 2003) and less fine grinding of the feed can avoid the occurrence and aggravation of lesions of the non-glandular pars oesophagea (Friendship *et al*., 2003).

Infection of pigs with stomach homogenate from *H. suis* infected mice (Hellemans *et al.*, 2007c) or a pure *in vitro* cultured strain of *H. suis* (De Bruyne *et al.*, 2012) can result in a decreased body weight gain ranging from 5% to 10%. In view of the high prevalence of *H. suis* infection in pigs along with its observed effects on animal health and welfare as well as production parameters, further research on the underlying mechanisms is needed.

H. suis is the most predominant *Helicobacter* infection species in the pig stomach, although on rare occasions, other *Helicobacter* species have been reported in the pig stomach, including *H. bilis*, *H. trogontum*, *H. pullorum*, and *H. pylori*-like bacteria (Roosendaal *et al.*, 2000, Hanninen *et al.*, 2003, Hanninen *et al.*, 2005, Krakowka *et al.*, 2005, Szeredi *et al.*, 2005).

3.3 The prevalence and clinical significance of *H. suis* infection in humans

H. pylori, colonizing more than half of the world's population, is considered to be the most prevalent *Helicobacter* species persistently residing in the human stomach, and infection may cause gastritis, peptic ulcer disease, as well as the development of gastric adenocarcinoma and MALT lymphoma (Axon, 1999, Ernst *et al.*, 2000, Wroblewski *et al.*, 2010, Salama *et al.*, 2013). Besides *H. pylori*, lower incidences of gastric NHPH infection have been reported in human patients, with prevalences ranging in general from 0.08% to 2%.

Especially in developing countries, such as China and Thailand, higher prevalences up to 6% have been reported (Oliva *et al.*, 1993, Stolte *et al.*, 1997, Yali *et al.*, 1998, Yang *et al.*, 1998, Andersen, 2001, Singhal *et al.*, 2005). Among all the NHPH species reported so far, *H. suis* is the most prevalent one in humans, responsible for about 1/3 of human NHPH infections (Van den Bulck *et al.,* 2005). In this same study, *H. felis, H. bizzozeronii, H. salomonis* and *H. heilmannii* were detected in 8.9%, 2.4%, 11.4% and 7.3% of NHPH-infected humans, respectively. Remarkably, a very high prevalence rate has been reported in human patients diagnosed with Idiopathic Parkinsonism (Cantet *et al.*, 1999, Roosendaal *et al.*, 2000, Choi *et al.*, 2001, Trebesius *et al.*, 2001, Van den Bulck *et al.*, 2005, Baele *et al.*, 2009, Flahou *et al.*, 2010, Blaecher *et al.*, 2013).

Humans infected with an NHPH species have been described to suffer from gastrointestinal (GI) complaints, which may include dyspepsia, epigastric pain, acid reflux, etc. (McNulty *et al.*, 1989, Hilzenrat *et al.*, 1995, Singhal *et al.*, 2005). Histopathologically, a chronic gastritis is often observed which is generally less severe compared to *H. pylori*-induced gastritis, and some patients infected with NHPH bacteria may benefit from anti-*H. pylori* therapy (Oliva *et al.*, 1993, Holck *et al.*, 1997, Singhal *et al.*, 2005, Joosten *et al.*, 2013c).

Although NHPH infections are much less frequent in humans, it is of interest to note that the risk of developing gastric MALT lymphoma has been described to be higher for NHPH infection compared to *H. pylori* infection. A report from Stolte and colleagues described, in a period from 1988 to 1998, eight MALT lymphomas among 543 patients with *H. heilmannii* gastritis (1.47%) compared to 1745 MALT lymphomas among 263 680 patients with *H. pylori* gastritis (0.66%) (Stolte *et al.*, 1997, Morgner *et al.*, 2000b, Stolte *et al.*, 2002, Joo *et al.*, 2007a).

Contrary to *H. pylori*'s predominate colonization in the human stomach and transmission from human to human, the natural reservoir of *H. suis* is the domesticated pig. Therefore, it can be speculated that direct and indirect contact with pigs and/or pig products are the main sources of infection. Indeed, Stolte et al. discovered that 70% of patients infected by "*H. heilmannii*" had frequent contact with one or more domestic animals or pets compared to only 37% in the healthy control population (Stolte *et al.*, 1994, Meining *et al.*, 1998). Foss et al. have found that *H. suis* spreads rapidly from infected pigs to adjacent groups of animals and they have identified *H. suis* DNA in the saliva from the infected pigs (Foss *et al.*, 2013). In addition, De Cooman and colleagues have demonstrated viable *H. suis* bacteria in 2 out of 50 retail pork samples, and viable *H. suis* bacteria were shown to persist for at least 48 hours

in experimentally contaminated pork (De Cooman *et al.*, 2013a). Recently, these same authors reported a relatively high prevalence of *H. suis* on pork carcasses, thus providing new evidence for potential routes of transmission of *H. suis* from pigs to humans (De Cooman *et al.*, 2014). However, the exact transmission route(s) between pigs and humans still need to be elucidated.

4. *Helicobacter*-induced gastric inflammation

It is estimated that *H. pylori* has co-evolved with its human host since before the major migrations of *Homo sapiens* about 50 000 – 100 000 years ago. Currently, *H. pylori* is a highly successful human pathogen colonizing more than 50% of the human population (Covacci *et al.*, 1999b). Despite the development of an immune response, *H. pylori* can persist in the challenging stomach of humans and establish chronic infection for decades or even lifelong, thanks to some ingenious escape mechanisms suppressing and modulating the host defense (Ernst *et al.*, 2000, Allison *et al.*, 2010, Salama *et al.*, 2013). In this section, the innate and adaptive host immune response induced by *H. pylori* will be briefly described as well as the recent progress in the field of *H. suis*.

4.1 The innate immune response against gastric *Helicobacter* infection

Both epithelial cells lining the GI tract and innate immune cells including macrophages, dendritic cells (DC) and neutrophils form the first barrier against foreign microorganisms encroaching the GI tract of the host. Epithelial innate immune cells express a series of receptors known as pattern recognition receptors (PRRs) (Kumar *et al.*, 2013). PRRs can sense or recognize the conserved pathogen-derived molecular structures, the so-called pathogen-associated molecular patterns (PAMPs) of the invading pathogens (Ishii *et al.*, 2008, Kumar *et al.*, 2013). PAMPs produced by distinct microorganisms are generally unique, chemically diverse products with conserved motifs (Cullen *et al.*, 2011), and can be recognized by at least 4 distinct varieties of PRRs identified to date: toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide binding oligomerization domain containing protein (NOD)-like receptors (NLRs) and RIG-like receptors (RLRs) (Muller *et al.*, 2011). Each PRR triggers a distinct innate signalling pathway, and the combination between different signalling pathways determines the final adaptive immune response against the invading pathogen (Gringhuis *et al.*, 2009). Previous studies have shown that *H. suis* infection induces an

increased infiltration of the gastric mucosa of mice and Mongolian gerbils with macrophages, neutrophils and DC (Flahou *et al.*, 2010, Nobutani *et al.*, 2010b). The innate immune response to invading microbes plays an important role in eliminating pathogens as well as further tailoring of pathogen-specific adaptive immunity, typically mediated by effective T and B cells (Pasare *et al.*, 2004).

4.2 The adaptive immune response against gastric *Helicobacter* infection

In view of the fact that *H. pylori* persistently colonizes the human stomach, it can be anticipated that this pathogen can not only trigger and modulate the innate immune response, but also possesses mechanisms to deal with the robust adaptive immune response, to establish chronic infections in humans. Generally, the adaptive immune response against *Helicobacter* infection consists of the immunoregulatory effects of tolerant DC and the activation of T and B lymphocytes.

DC

DCs are highly specialized antigen-presenting cells and they serve as an important mediator linking the innate and adaptive immune response. DCs reside in the secondary lymphoid organs and peripheral tissues, including the gastric mucosa, and it has been shown that DCs are more prevalent and common in *H. pylori* infected patients compared to healthy subjects, and that *H. pylori* infection can induce the maturation of human DCs (Sarsfield *et al.*, 1996, Necchi *et al.*, 2009, Bimczok *et al.*, 2010, Sundquist *et al.*, 2010, Shiu *et al.*, 2013). This is also the case in experimentally infected mice with both acute and chronic gastritis after *H. pylori* infection (Kao *et al.*, 2006, Kao *et al.*, 2010, Flach *et al.*, 2012) or *H. felis* infection (Drakes *et al.*, 2006). It has been demonstrated that *H. pylori* immune evasion can be partly mediated by DCs by inducing the conversion of naïve T cells into regulatory T cells (Tregs), thus favouring a persistent *H. pylori* infection (Kao *et al.*, 2010, Zhang *et al.*, 2010, Hitzler *et al.*, 2011). Also, *in vivo* depletion of Tregs in infected mice leads to increased gastric inflammation and decreased bacterial colonization (Rad *et al.*, 2006). With regards to *H. suis*, it has been shown that lymphoid follicles induced by *H. suis* infection can indeed be regulated by local DC in the gastric mucosa of mice (Mimura *et al.*, 2011). Moreover, GGT, also produced by *H. suis*, contributes critically to the tolerizing effect of *H. pylori* on murine DCs towards a tolerogenic phenotype *in vivo* and *in vitro* and it also induces Tregs with highly

suppressive properties (Oertli *et al.*, 2013), which still needs to be explored for *H. suis* infection.

Tregs

In general, Tregs are a minor specialized population, comprising 5-10% of $CD4^+$ T cells both in mice and humans, which also co-express CD25, the α-chain of the interleukin-2 receptor (IL-2R) (Shevach, 2002). It has been shown that $CD4^+CD25^+$ Tregs exert their immuno-suppressive function through a variety of mechanisms. They play critical roles both in prevention of autoimmunity and in the control of tumor immunity and transplantation tolerance (Sakaguchi, 2002, Josefowicz *et al.*, 2012b). Forkhead/winged helix transcription factor (Foxp3) is considered to be a unique marker of Tregs, and it is specifically required for the development and suppressive function of Tregs (Fontenot *et al.*, 2003). Tregs have been shown to execute their suppressive function through cell-to-cell contact as well as secretion of the anti-inflammatory cytokine IL-10 and transforming growth factor-β (TGF-β) (Levings *et al.*, 2002, Gondek *et al.*, 2005). Notably, an increasing amount of reports have shown that elevated numbers of Tregs are found both in patients with *H. pylori* infection (Lundgren *et al.*, 2005, Kandulski *et al.*, 2008, Iwaya *et al.*, 2013) as well as in mice experimentally infected with *H. pylori* (Rad *et al.*, 2006, Oertli *et al.*, 2013), suggesting a significant role for Tregs in suppressing *H. pylori* induced inflammation and maintaining the chronic nature of the infection (Rad *et al.*, 2006, Raghavan *et al.*, 2012). A recent report has demonstrated that GGT from *H. pylori* promotes an efficient induction of Tregs *in vivo* (Oertli *et al.*, 2013), and just recently the same group provided preclinical data proving that GGT can prevent allergen-induced asthma (Engler *et al.*, 2014). Interestingly, the suppressive cytokine IL-10, often produced by Tregs (Bazzoni *et al.*, 2010), is significantly up-regulated in the stomach of mice experimentally infected with *H. suis* (Flahou *et al.*, 2012). So most likely, Tregs are also involved in immune modulation in *H. suis* infected hosts.

T helper cells

Besides the involvement of DCs and Tregs in the adaptive immune response, gastric helicobacters harbour several key virulence factors that can directly or indirectly interfere with adaptive immune cells. Convincing evidence indicates that the vacuolating toxin (VacA) and GGT of *H. pylori* can impair the proliferation of human T cells, and the GGT of *H. bilis* has been shown to exhibit a similar inhibitory effect in a direct manner (Gebert *et al.*, 2003, Sundrud *et al.*, 2004, Schmees *et al.*, 2007, Rossi *et al.*, 2012). Moreover, recent data have

demonstrated that *H. pylori* can also modulate the T cell response in an indirect manner through the induction of Treg properties in naive T cells by *H. pylori*-experienced DCs depending on both VacA and GGT (Oertli *et al.*, 2013). Despite the fact that the exact role of both secreted factors during the shaping of specific DC tolerance is still unclear (Salama *et al.*, 2013), the ability of induction of Tregs facilitated by VacA and GGT will undoubtedly affect the *H. pylori* specific memory T cell response.

In general, based on cytokine expression patterns, most researchers support the notion that infection with *H. pylori* or its bacterial components triggers a T helper (Th)1 predominant immune response, as detected in gastric biopsies from patients (Bamford *et al.*, 1998, Sommer *et al.*, 1998), in experimental rodent models (Crabtree *et al.*, 2004b, Flahou *et al.*, 2012), in cell models *in vitro* (Hafsi *et al.*, 2004, Amedei *et al.*, 2006), in an experimental pig model (Kronsteiner *et al.*, 2013) as well as in rhesus macaques (Mattapallil *et al.*, 2000). Nevertheless, it has been occasionally reported that gastric mucosa from patients with *H. pylori* infection exhibits a mixed Th1-Th2 cytokine expression profile (Goll *et al.*, 2007).

A growing number of reports have shown that *H. suis* infection using bacteria from a pure culture or an inoculum prepared from pig, mouse or monkey stomach can trigger a T cell response in the stomach (Flahou *et al.*, 2010, Nobutani *et al.*, 2010b, Yamamoto *et al.*, 2011). With regards to the type of T helper immune response, several reports have demonstrated that mice inoculated with homogenized pig or mouse stomach mucosa develop a Th1 predominant immune response, based on the measurement of IFN-γ secretion (Cinque *et al.*, 2006, Mimura *et al.*, 2011). Another report has shown that enhanced levels of IFN-γ and IL-10 were observed in mice upon infection with homogenates from mouse stomach, suggesting that a mixed Th1/Th2 immune response is involved in this process (Park *et al.*, 2008). For the first time, Flahou and colleagues demonstrated that infection in Th2-prone BALB/c mice as well as Th1-prone C57BL/6 mice with pure *in vitro* isolated strains of *H. suis* results in a significant up-regulation of IL-4 and IL-10, however without a significant increase in IFN-γ expression, revealing that a Th2 predominant immune response is induced upon *H. suis* infection in this animal model (Flahou *et al.*, 2012). It remains to be determined what causes the different outcome of these studies, but possibly, other micro-organisms present in the pig or mouse stomach homogenates used in the former studies may be involved.

The Th17 lineage is defined as IL-23 or IL-21 induced, IL-17 producing $CD4^+$ T effector cells, and this response is distinct from classically assigned Th1 and Th2 subsets (Harrington *et al.*, 2005, Fantini *et al.*, 2007). It has been shown that, besides a Th1 (or Th2)-directed immune response, infection with *Helicobacter*, including *H. suis*, will also induce a

Th17 immune response in experimentally infected mice (Shi *et al.*, 2010, Flahou *et al.*, 2012) or infected human patients (Zhang *et al.*, 2008, Pinchuk *et al.*, 2013). At the ulcer site of human patients with *H. pylori* infection, there is a strong association between IL-17 expression level and the number of infiltrating mononuclear cells and neutrophils (Mizuno *et al.*, 2005). In addition, Shi and colleagues have demonstrated that the Th17/IL-17 pathway can modulate the Th1 immune response during *H. pylori* infection, supporting bacterial growth and contributing to the development of gastric pathology (Shi *et al.*, 2010). In contrast, results from other research groups have shown that IL-17 secretion induced by *H. pylori* infection promotes gastric inflammation, and plays an important role in elimination of the bacteria during the acute infection stage (Luzza *et al.*, 2000). Several virulence factors of *H. pylori* have been shown to be involved in the Th17-directed polarization of the immune response. $CD4^+$ T cells stimulated by DCs pulsed with an *H. pylori* strain lacking the cytotoxin-associated gene A (CagA) show a decreased production of IL-17 (Kabir, 2011). In contrast, more recent data suggest that CagA is in part responsible for evading Th17-mediated clearance by modulating expression of B7-H2 (inducible co-stimulator ligand), thus contributing to the establishment of chronic *H. pylori* infection (Lina *et al.*, 2013). Moreover, the urease β subunit is considered to be an important bacterial factor which can elicit a Th17 response against *H. pylori* infection both *in vivo* and *in vitro* (Zhang *et al.*, 2011). The urease enzyme is also a crucial virulence factor for *H. suis* (Vermoote *et al.*, 2011, Vermoote *et al.*, 2012). Compared to wild-type *H. pylori*, both *H. pylori* Δ*ggt* and Δ*vacA* strains induce a relative higher Th17 immune response (Oertli *et al.*, 2013). Collectively, conflicting reports have been described about the protective role or pathogenic significance of a Th17 response during *H. pylori* infection, and the exact role of Th17/IL-17 probably depends on the bacterial strain, infectious stage, and the experimental models used (Kabir, 2011).

With regards to the gastric NHPH, relatively few studies are available about the possible involvement of a Th17 response. It has nevertheless been demonstrated that infection with *H. felis* (Obonyo *et al.*, 2011, Ericksen *et al.*, 2014) as well as *H. suis* (Flahou *et al.*, 2012) can induce a strong Th17-directed immune response. In addition, elevated levels of IL-17 have been observed in immunized mice after *H. suis* challenge (Vermoote *et al.*, 2012) and these elevated levels were shown to correlate with protection against *H. suis* challenge infection.

B cells

Besides the involvement of T lymphocytes, DC and macrophages, increased numbers of B lymphocytes have also been observed in gastric biopsies from *H. pylori* infected individuals (Goll *et al.*, 2005, Munoz *et al.*, 2007). High levels of immunoglobulin (Ig)A, IgG and IgM are detected in patients with *H. pylori* infection or volunteers challenged with *H. pylori* (Rathbone *et al.*, 1985, Mitchell *et al.*, 1988, Perezperez *et al.*, 1988, Nurgalieva *et al.*, 2005), although there are contrary reports regarding the IgM antibody level, showing no differences between infected subjects and un-infected subjects (Perezperez *et al.*, 1988). Possible reasons for these conflicting results may be the short life-span of the IgM response as well as variation in the age of the patients (Gold *et al.*, 1997, Soares *et al.*, 2005). Regarding *H. suis*, mice and Mongolian gerbils infected with pure cultures of *H. suis* show a clear mucosal/submucosal infiltration with B lymphocytes at 8 months post infection (Flahou *et al.*, 2010) and often, these infiltrates are organized into lymphoid follicles containing large, irregular, hyperproliferative B cell-containing germinal centers. In another study, mice infected with an *H. suis*-containing inoculum derived from pig stomach also exhibited the development of lymphoid follicles and acquired immune responses characterized by the activation of B cells and T cells (Yamamoto *et al.*, 2011).

5. *Helicobacter* and the gastric epithelium

5.1 Parietal cells and gastric acid secretion

Parietal cells

Secretion of HCl into the gastric lumen is mediated by the oxyntic (acid-secreting) cells, and these cells from the mammalian gastric mucosa are commonly called gastric parietal cells (Yao *et al.*, 2003). Due to the massive secretion of H^+ by parietal cells into the gastric lumen, the pH of pure gastric juice can reach values as low as 0.8, and this means that the proton gradient is 4 million times greater than that in the blood stream (Urushidani *et al.*, 1997). Acid facilitates the activation of pepsinogen, the digestion of food and absorption of iron, calcium, and vitamin B-12, and it also controls bacterial overgrowth and enteric infection (Schubert *et al.*, 1990).

Gastric parietal cells originate from progenitor or stem cells located in the isthmus region of the gastric unit and they constitute highly specialized epithelial cells with several distinctive morphological characteristics (Schubert, 2002). The capacity to persistently and

abundantly secrete gastric acid is associated with morphological and functional characteristics of parietal cells. The H^+, K^+-ATPase of parietal cells is the proton pump composed of a catalytic subunit (α-subunit) and an accessory subunit (β-subunit). H^+, K^+-ATPase transports protons against a huge gradient into the gastric lumen in exchange for K^+, which is an energy-consuming process requiring a mass of adenosine triphosphate (ATP). Consistent with the high demand of ATP, parietal cells are known to have the highest density of mitochondria in the human body, with an occupation ratio of nearly 40% of the total cell volume (Duman *et al.*, 2002, Kopic *et al.*, 2010). Branching secretory canaliculi course through the cytoplasm and are connected by a common outlet to the cell's luminal surface. Microvilli line the surface of the secretory canaliculi. The cytoplasm of unstimulated parietal cells contains numerous tubules and vesicles, which is called the tubulovesicular system (Ogata, 1997).

In general, there are two typical stages for parietal cells: the resting state and stimulated stage. In the resting state, H^+ K^+ -ATPase is sequestered within cytoplasmic vesicles called tubulovesicles, and the low permeability of tubulovesicular membranes to KCl limits the turnover of the pump even though there is ample ATP around the enzyme (Figure 7, left). Parietal cells undergo a profound morphological conversion upon the activation of acid secretion. Activation of acid secretion is achieved by two concomitant functional changes, namely, tubulovesicles fuse with the apical secretory membrane thus recruiting functional pumps to the expanded microvillar surface and the apical membrane acquires a permeability to KCl (Figure 7, right) (Urushidani *et al.*, 1997, Forte *et al.*, 2010). It is estimated that the human stomach contains 1×10^9 parietal cells (with typically 70-90 parietal cell per fundic gland), comprising 80% of the corpus (Joseph *et al.*, 2003), thus making it possible to deliver adequate amounts of HCl into the stomach whenever needed.

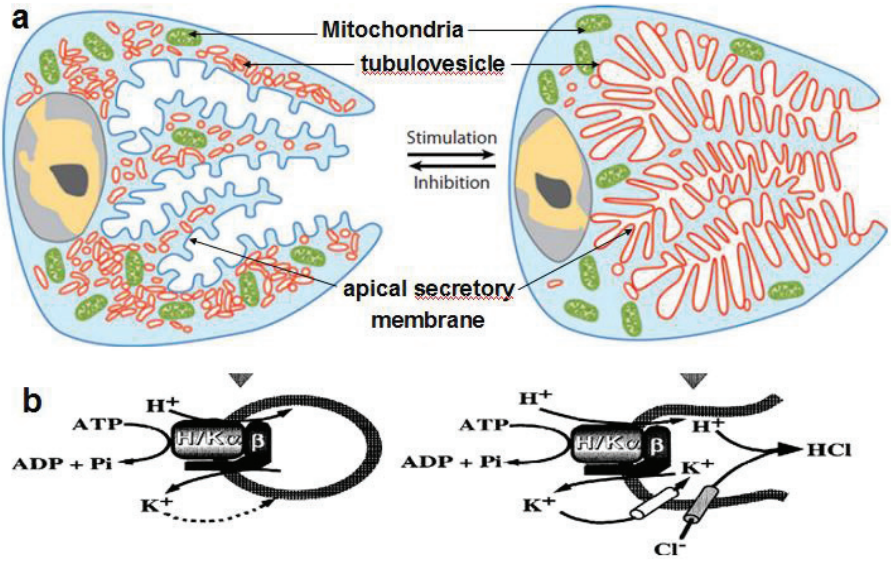

Figure 7. Schematic representation of parietal cells at rest and under stimulation stages.

(a) Graphic representation describing the morphological changes in parietal cells upon stimulation by the release of histamine, or acetylcholine. In the resting state (left), the apical canaliculi extend into the cell, presenting short microvilli. Tubulovesicles containing H+/K+ ATPase (red) abound in the cytoplasmic space. In the stimulation stage (right), tubulovesicles are recruited at and fused with the apical membrane, greatly expanding the canalicular microvilli (red membrane) and putting H+/K+ ATPase pumps in position to power acid secretion. (b) Schematic representation of ion transport in resting and stimulated parietal cells. In the resting stage (left), the low permeability of tubulovesicular membranes to KCl limits the turnover of the pump even though there is ample ATP around the enzyme. In stimulation stage, tubulovesicles fuse with the apical secretory membrane thus recruiting functional pumps to the expanded microvillar surface and the apical membrane acquires a permeability to KCl, resulting in the transport of H^+ into lumen. (Adapted from T. Urushidani and John G. Forte, 1997; John G. Forte and Lixin Zhu, 2010)

Gastric acid secretion

Control of acid secretion by gastric parietal cells is achieved by a highly-regulated and complex interaction between endocrine, paracrine, autocrine and neuronal mediators that transmit stimulatory or inhibitory signals as well as neural regulation (Schubert *et al.*, 2008).

Triggering of acid secretion typically involves an initially increased level of intracellular calcium and/or adenosine 3',5'-cyclic monophosphate (cAMP) followed by activation of a cAMP-dependent protein kinase cascade that triggers the translocation and insertion of the proton pump into the apical plasma membrane of parietal cells. Parietal cells, ECL cells, G cell and D cells are the most important cell types responsible for regulation and secretion of gastric acid. Histamine released by ECL cells can stimulate the parietal cell directly by binding to H2 receptors, coupled to activation of adenylate cyclase and generation of cAMP (Soll *et al.*, 1979). Histamine release can also stimulate acid secretion indirectly by

binding to H3 receptors, coupled to inhibition of somatostatin leading to the stimulation of histamine secretion and acid secretion (Tari *et al.*, 1997). Gastrin is produced by G cells located in the antrum of the stomach, and this hormone can also regulate the secretion and synthesis of histamine. Gastrin is considered to be the most important stimulator for acid secretion by ECL cells (Kidd *et al.*, 1998, Barocelli *et al.*, 2003). It was shown that gastrin stimulates the activation of cholecystokinin (CCK2 or CCKb) receptors on ECL cells leading to the release of histamine and a concurrent stimulation of gastric acid secretion (Tari *et al.*, 1997, Schubert *et al.*, 2008).

The excessive increase of HCl in the gastric lumen will in turn activate the process to attenuate the acidity via a pathway involving the release of somatostatin. Somatostatin produced by D-cells is the main inhibitor of acid secretion in the stomach. In the stomach, the inhibitory effect of somatostatin has been shown to be mediated by the somatostatin subtype 2 receptor (SST2) (Zaki *et al.*, 1996). A summary of the regulations of HCl secretion is shown in Figure 8 (Barocelli *et al.*, 2003), indicating that the process of regulation of gastric acid secretion is in fact even more complicated than described above.

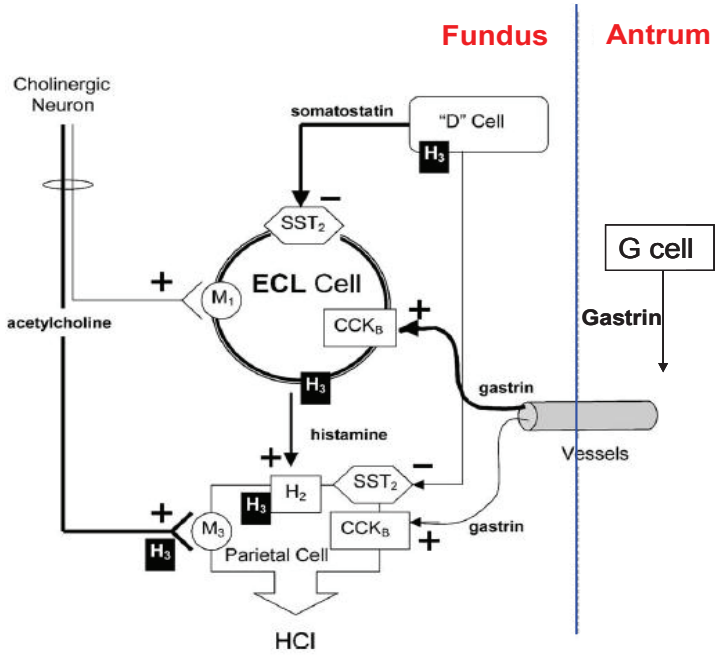

Figure 8. Diagram showing the interaction of acetylcholine, histamine, somatostatin and gastrin in the regulatory pathways involved in gastric acid secretion. Histamine (from Enterochromaffin-like cells), gastrin (from G cells), and acetylcholine (from postganglionic neurons) are the main stimulatants for acid secretion. Somatostatin (from D cells) can inhibit the acid secretion by blocking the release of histamine. Acetylcholine (M), histamine (H), somatostatin (SST), gastrin receptors (CCK) are indicated; − inhibitory signal; + stimulatory signal. (Adapted from Elisabetta Barocelli, Vigilio Ballabeni, 2003)

5.2 The importance of amino acids in maintaining stomach health

Amino acids (AA) are defined as organic substances containing both amino and acid groups, and they are not only building blocks for tissue proteins and polypeptides but also key regulators of metabolic pathways in cells (Wu, 2009, Wu, 2013). In general, AA are classified as nutritionally essential or non-essential for humans and animals based on the nitrogen balance in animals. Essential AA (EAA) are AA that must be provided from the diet to meet optimal requirements, and non-essential AA (NEAA) are AA which can be synthesized *de novo* in sufficient quantity to meet optimal requirements of the body. It should be noted that the actual list of EAA differs from species to species, e.g. arginine is an EAA for rats and cats, but not for pigs. In addition, the nutritional importance also differs within the same species at different stages. Young animals, pregnant animals, and aging animals indeed have different needs compared to 'normal' adult animals (McDonald *et al*., 2011). Conditionally essential AA are AA that can be synthesized in adequate amounts by the organism under normal conditions, but which must be provided from the diet to meet optimal needs under special conditions, such as reproduction, malnutrition, and disease. A general classification of AA in mammals, poultry, and fish is summarized in Table 1. AA are generally stable in an aqueous solution at physiological conditions except for glutamine (Gln) and cysteine (Wu, 2009). Recent studies have revealed that AA play an important role during the modification of exocrine and endocrine secretion, modulation of protein digestion, metabolism and nutrient utilization, support of the epithelial barrier integrity and defense of the GI mucosa, all contributing to a healthy life of animals and humans (Kim *et al.*, 2007b, Kitamura *et al.*, 2012, San Gabriel *et al.*, 2013).

Mammals[a]			Poultry			Fish		
EAA	NEAA	CEAA[b]	EAA	NEAA	CEAA[b]	EAA	NEAA	CEAA[b]
Arg[c]	Ala	Gln[c]	Arg[c]	Ala	Glnv	Arg[c]	Ala	Gln[c]
Cys[c]	Asn	Glu[c]	Cys[c]	Asn	Glu[c]	Cys[c]	Asn	Glu[c]
His	Asp[c]	Gly[c]	Gly[c]	Asp[c]	Tau[c]	His	Asp[c]	Gly[c]
Ile	Ser	Pro[c]	His	Ser		Ile	Ser	Tau[c]
Leu[c]		Tau[c]	Ile			Leu[c]		
Lys			Leu[c]			Lys		
Met[c]			Lys			Met[c]		
Phe			Met[c]			Phe		
Thr			Phe			Pro[c]		
Trp[c]			Pro[c]			Thr		
Tyr[c]			Thr			Trp[c]		
Val			Trp[c]			Tyr[c]		
			Tyr[c]			Val		
			Val					

Table 1. Classification of AA in animal and human nutrition.
Classification of AA as nutritionally ‘‘essential’’ or ‘‘nonessential’’ or conditionally essential depends on species, age, physiological factors, environmental factors, and pathological states
CEAA: conditionally essential AA, EAA: nutritionally essential AA, NEAA: nutritionally nonessential AA
[a] Preweaning ruminants have qualitatively similar requirements for dietary AA to those for nonruminants. In postweaning ruminants, the microbial source of protein and AA is inadequate for supporting their maximal growth or milk production when the animals are fed roughage diets
[b] For neonates (including human infants and piglets), adults under stress conditions (e.g., heat stress, burns, and infection), and breeding stocks (both males and females). Taurine (Tau) is a nutritionally essential AA for cats
[c] Functional AA
(Adapted from Guoyao Wu, 2013)

Fundamental role of AA in the maintenance of GI health

Several AA are an important fuel for both epithelial cells and leukocytes, e.g Gln, glutamate (Glu) and arginine (Arg). Gln is one of the most abundant AA in alimentary protein and also the most abundant extracellular AA *in vivo* (Newsholme *et al.*, 2003). It is the precursor for glutathione (GSH), Glu, alanine, proline, and ornithine (Blachier *et al.*, 2009), and it is of great importance for the intestinal metabolism and physiology (Wang *et al.*, 2009). Small intestinal epithelial cells can utilize Gln from both the arterial circulation and intestinal lumen, whereas Glu is absorbed mainly from the intestinal lumen (Wu, 2009). Gln is considered a conditionally essential AA, which is known to support a proper intestinal mucosal metabolic function as well as a normal function of epithelial cells (DeMarco *et al.*, 1999, Reeds *et al.*, 2001). Gln-supplemented parenteral nutrition has been shown to maintain the height of the intestinal villi, the thickness of the mucosa and intestinal wall in rats (Chen *et al.*, 1994b). The integrity of the barrier function of the lining epithelium of the GI mucosa as well as the secretion of mucus are the first and fundamental lines of defense against acid and pathogenic organisms in the lumen. Lymphocytes (e.g. T and B lymphocytes) residing in the lamina propria or deeper layers of the mucosa are another important component of the

host defense against pathogens (Salama *et al.*, 2013). Sufficient amounts of Gln have been shown to be essential for an intact proliferation capacity and normal function of T lymphocytes (Yaqoob *et al.*, 1997). Indeed, it has been shown that dietary Gln supplementation results in higher mucosal densities of macrophages and intraepithelial lymphocytes in the ileum (Domeneghini *et al.*, 2004) and that it significantly enhances the proliferative response of naive $CD4^+$ T cells from piglets as well as the T lymphocyte response in surgical patients (O'Riordain *et al.*, 1994, Johnson *et al.*, 2006), which clearly stresses the importance of this amino acid in the maintenance of a normal immune response in the body.

Glu, one of the most abundant intracellular AA with reported concentrations ranging from 2 to 20 mM, is the immediate product of Gln metabolism in most cells (Newsholme *et al.*, 2003). Similar to Gln, Glu is also involved in a number of key functions of cells, including nutrient metabolism and oxidative defense in epithelium, lymphocytes, macrophages, and neutrophils. In these Glu/Gln-utilizing cells, the Glu-Gln pathways can not be replaced by other metabolic inputs due to their critical roles in maintaining cell function (Newsholme *et al.*, 2003, Wu, 2009).

Arg is the most abundant carrier of nitrogen in humans and animals containing four nitrogen atoms per molecule (Wu *et al.*, 1999). It has been shown to be an EAA for the fetus and neonate and a CEAA for adults, especially under certain disease conditions (Wu *et al.*, 2000). Convincing evidence demonstrates that Arg plays a crucial role in reproduction, immune function and tissue integrity of the body (Wu *et al.*, 2009). Furthermore, Arg can stimulate intestinal fluid secretion through a nitric oxide **(**NO) mediated mechanism, and NO plays an important role in supporting the integrity of the intestinal mucosal barrier (Alican *et al.*, 1996). Beneficial effects of Arg have also been described in terms of its role in improving GI function and gastric ulcer healing, accelerating intestinal mucosal renewal, speeding up bacterial clearance, and reducing histological signs of bowel necrosis (Wang *et al.*, 2009). A sufficient supply of Arg is also neccesary for lymphocyte proliferation and functional development, and dietary Arg supplementation enhances immune responses in various models of immunological challenge (Li *et al.*, 2007).

Besides Gln, Glu, and Arg, other AA, like aspartate, glycine and lysine are also hypothesized to possess the ability to influence a series of GI-related disorders in both animals and humans (Wang *et al.*, 2009).

Regulatory role of AA in the GI system

AA are not only the biosynthetic precursors of many biologically relevant small molecules and a metabolic fuel but they also modulate physiological functions in the GI tract (Shimizu, 2010). Monitoring the luminal content in the GI tract is of vital importance for adjusting the activities of the stomach. AA appear to function by binding to a chemical communication system in the GI such as G protein-coupled receptors (GPCRs) that serve as nutrient-sensing systems, leading to relevant signalling pathways (San Gabriel *et al.*, 2013). So far, metabotropic glutamate receptors (mGluRs), GPRC6A, and calcium-sensing receptors (CaSRs) are the most relevant GPCRs in the GI tract (Busque *et al.*, 2005, Nakamura *et al.*, 2010, San Gabriel *et al.*, 2013).

San Gabriel et al. revealed that mGluRs are located in the apical membrane of chief cells and possible also in the parietal cells, and both cell types are responsible for secretion (of pepsinogen and gastric acid, respectively) involved in regulation of gastric phase protein digestion (San Gabriel *et al.*, 2007). A study by Uneyama et al. has shown that among the 20 natural AA, only Glu evokes firing in the afferents of gastric branches of the vagus. The same group also demonstrated that an intrinsic cascade involving NO and serotonin (5-HT) mediates the electrophysiological response of afferents from the gastric branch of the vagus nerve, which is specifically activated in response to glutamate in the rat gastric mucosa (Uneyama *et al.*, 2006). Molecular phenotyping has revealed that CaSRs, exposed to the luminal content, are expressed in G cells and a subpopulation of D cells in swine and men (Haid *et al.*, 2012). Reports also showed that GPRC6A is expressed in different regions (mainly in the antrum) of the murine stomach. CaSRs and GPRC6A are considered to act as efficient physiological sensors to protein breakdown products influencing gastric secretion (Haid *et al.*, 2011, San Gabriel *et al.*, 2013). Other AA have also been shown to be involved in gastric acid secretion, as shown by studies revealing that intragastric administration of aromatic AA such as phenylalanine and tryptophan can facilitate acid secretion (Strunz *et al.*, 1978, Taylor *et al.*, 1982). Considering the extensive and important role of AA sensoring on the physiological activities in the GI tract, it can be assumed that AA GPCRs are potential pharmaceutical targets for GI dysfunctions.

5.3 Interaction of *H. suis* and other gastric helicobacters with host epithelial cells

H. suis has been shown to actively cause apoptosis and necrosis of a human gastric epithelial cell line *in vitro*. This was accompanied by elevated levels of extracellular H_2O_2,

leading to lipid peroxidation. The GGT from *H. suis* was shown to be one of the main virulence factors responsible for this induction of epithelial cell death and the *H. pylori* GGT was shown to work in a very similar way (Flahou *et al.*, 2011).

Recent studies carried out by our research group as well as others have shown that *H. suis* interacts with host cells in a different way than the well-known *H. pylori*. Although some studies have reported that *H. pylori* can induce apoptosis of rat parietal cells, which was shown to be dependent on the activation of NF-κB and production of NO (Neu *et al.*, 2002), the majority of *H. pylori* bacteria remain in the mucus layer of infected human patients, whereas only a small number adheres to the gastric epithelial (mucus-secreting) cells (Linden *et al.*, 2008, Magalhaes *et al.*, 2010). *H. suis*, on the other hand, is most often observed in the vicinity of or inside the canaliculi of acid-producing parietal cells in pigs, humans and experimentally infected mice and Mongolian gerbils, and these cells often show signs of degeneration, necrosis or apoptosis (Joo *et al.*, 2007, Flahou *et al.*, 2010). Besides *H. suis*, other NHPH such as *H. felis*, *H. bizzozeronii* and *H. heilmannii* s.s. have often been observed near or inside the canaliculi of parietal cells in experimentally infected rodents or naturally infected carnivores. An almost complete loss of parietal cells has been described in the stomach of *H. felis* infected Mongolian gerbils (De Bock *et al.*, 2006, Joo *et al.*, 2007, Flahou *et al.*, 2010, Lanzoni *et al.*, 2011). Currently, only little information is available on the direct interactions of *H. suis* with parietal cells *in vivo* and *in vitro*.

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Chapter 2
Scientific Aims

Helicobacter (H.) pylori is considered to be the primary aetiological agent causing gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma in humans. Besides *H. pylori*, other non-*H. pylori Helicobacter* (NHPH) species also cause gastric disease in humans. This group of NHPH comprises several *Helicobacter* species naturally colonizing the stomach of various animal species. *H. suis* is a NHPH that colonizes the stomach of the majority of pigs, leading to the development of chronic gastritis, decreased daily weight gain and possibly also ulceration of the keratinized epithelium of the pars oesophagea. This bacterium is also the most prevalent gastric NHPH in humans.

H. suis infection triggers the development of a chronic inflammatory response in the stomach of its hosts, however without an effective clearance. This indicates that *H. suis* possesses immune suppressing properties. For *H. pylori,* several virulence factors have been described to modulate the host immune response, including the *H. pylori* γ-glutamyl transpeptidase (GGT). A similar enzyme has been described to be present in *H. suis*, but it was unknown at the onset of these studies whether this enzyme also plays a role in the modulation of the host immune response by *H. suis*. **The first aim** of this thesis was to investigate a possible effect of *H. suis* GGT on the function of various lymphocyte subsets. In addition, we aimed at unravelling the mechanisms and modes of action involved.

Besides effects on the function of lymphocytes *in vitro*, *H. suis* GGT has also been shown to induce death of gastric epithelial cells *in vitro*. No experiments have, however, attempted to determine the true relevance of GGT in the pathogenesis of *H. suis* infection *in vivo*. Therefore, **the second aim** of this thesis was to determine the role of this virulence factor in the pathogenesis of long-term *H. suis* infection, using wild-type and isogenic *ggt* mutant strains of *H. suis*. At the same time, we aimed at comparing its relative importance with that of the GGT of *H. pylori* by using wild-type and isogenic *ggt* mutant strains of *H. pylori*. Experiments were performed in mice and Mongolian gerbils, which are both considered excellent rodent models to investigate the pathogenesis of gastric *Helicobacter* infection.

In experimentally infected mice and Mongolian gerbils, *H. suis* bacteria are often seen in close association with gastric acid-producing parietal cells, sometimes showing signs of necrosis. In addition, degeneration of parietal cells has been described in the stomach of

humans infected with gastric NHPH species. At the onset of this PhD study, little or no information was available on the interaction between *H. suis* and this cell type in pigs. Hence, **the third aim** of this thesis was to investigate the interactions between pig parietal cells and *H. suis* and to determine the direct effects of *H. suis* infection on the health and function of this cell type.

Chapter 3
Experimental Studies

Study 1: Effects of *Helicobacter suis* γ-glutamyl transpeptidase on lymphocytes: modulation by glutamine and glutathione supplementation and outer membrane vesicles as a putative delivery route of the enzyme

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Abstract

Helicobacter (H.) suis colonizes the stomach of the majority of pigs as well as a minority of humans worldwide. Infection causes chronic inflammation in the stomach of the host, however without an effective clearance of the bacteria. Currently, no information is available about possible mechanisms *H. suis* utilizes to interfere with the host immune response. This study describes the effect on various lymphocytes of the γ-glutamyl transpeptidase (GGT) from *H. suis*. Compared to whole cell lysate from wild-type *H. suis*, lysate from a *H. suis ggt* mutant strain showed a decrease of the capacity to inhibit Jurkat T cell proliferation. Incubation of Jurkat T cells with recombinantly expressed *H. suis* GGT resulted in an impaired proliferation, and cell death was shown to be involved. A similar but more pronounced inhibitory effect was also seen on primary murine $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells. Supplementation with known GGT substrates was able to modulate the observed effects. Glutamine restored normal proliferation of the cells, whereas supplementation with reduced glutathione strengthened the *H. suis* GGT-mediated inhibition of proliferation. *H. suis* GGT treatment abolished secretion of IL-4 and IL-17 by $CD4^+$ T cells, without affecting secretion of IFN-γ. Finally, *H. suis* outer membrane vesicles (OMV) were identified as a possible delivery route of *H. suis* GGT to lymphocytes residing in the deeper mucosal layers. Thus far, this study is the first to report that the effects on lymphocytes of this enzyme, not only important for *H. suis* metabolism but also for that of other *Helicobacter* species, depend on the degradation of two specific substrates: glutamine and reduced glutatione. This will provide new insights into the pathogenic mechanisms of *H. suis* infection in particular and infection with gastric helicobacters in general.

Introduction

Helicobacter pylori can cause gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma in humans (Axon, 1999, Ernst *et al.*, 2000). It is, however, not the only bacterial pathogen capable of colonizing the human gastric mucosa. Indeed, gastric non-*H. pylori* helicobacters (NHPH) have also been detected in humans and these bacteria are capable of causing disease in both humans and animals (Lee *et al.*, 1989, Solnick *et al.*, 1993, Trebesius *et al.*, 2001, O'Rourke *et al.*, 2004b, Van den Bulck *et al.*, 2005, Haesebrouck *et al.*, 2009, Flahou *et al.*, 2010, Suzuki *et al.*, 2010, Joosten *et al.*, 2013d). *H. suis* has been shown to be the most prevalent gastric NHPH in humans (Flahou *et al.*, 2010). Similar to *H. pylori*, *H. suis* generally causes a life-long infection, suggesting that the bacterium possesses immune suppressing properties.

Lymphocyte responses are involved in a wide range of immunoregulatory activities, both *in vivo* and *in vitro* (Reinherz *et al.*, 1980). So far, no information is available on the influence of *H. suis* virulence determinants on the function of lymphocytes. For *H. pylori,* several factors have been described having an effect on the host lymphocyte response, including the vacuolating cytotoxin (VacA) and *H. pylori* GGT (Schmees *et al.*, 2007, Gong *et al.*, 2010a, Beigier-Bompadre *et al.*, 2011). The former is absent in *H. suis* (Vermoote *et al.*, 2011). The latter enzyme, synthesized as a precursor enzyme with a molecular weight (MW) of ~60 kDa, followed by processing into a large (~40 kDa) and small (~20 kDa) subunit (Flahou *et al.*, 2011), is also produced by a number of other *Helicobacter* species, including *H. suis*, and the enzyme has been shown to play an important role during the metabolism of extracellular L-glutamine (L-Gln) and reduced glutathione (GSH) (Flahou *et al.*, 2011, Rossi *et al.*, 2012). Degradation of GSH, an important antioxidant, by GGT results in the development of extracellular oxygen radicals, leading to oxidative damage of epithelial cells, or inhibition of cellular proliferation (Perego *et al.*, 1997, Shibayama *et al.*, 2007, Flahou *et al.*, 2011). In contrast, Gln, another substrate of GGT, is a major metabolic fuel for rapidly dividing cells, including enterocytes and immunologically challenged lymphocytes (Curthoys *et al.*, 1995, Rhoads *et al.*, 1997). In addition, regulation of L-Gln utilization seems to be an important component of T cell activation and the development of an immune response and Gln is also a key regulator of gene expression and cell signalling pathways (Johnson *et al.*, 2006, Carr *et al.*, 2010). Currently, no information exists regarding a possible regulatory effect of L-Gln or GSH (supplementation) on the proliferation of lymphocytes affected by the GGT of gastric helicobacters.

It has been demonstrated that the GGT secreted from gastric helicobacters as well as other secreted factors such as the VacA from *H. pylori* can access the lymphocytes in the lamina propria. These secreted factors may affect the lymphocyte function in a direct and indirect manner, for instance by inflicting damage to epithelial cells, resulting in small epithelial defects (Rieder *et al.*, 2005, Flahou *et al.*, 2010, Flahou *et al.*, 2011, Salama *et al.*, 2013). Interestingly, *H. pylori* outer membrane vesicles (OMV) have been shown to contain the *H. pylori* GGT (Olofsson *et al.*, 2010) and they have been shown to be internalized by epithelial cells (Parker *et al.*, 2010). In general, OMV are released by Gram-negative bacteria under natural conditions *in vitro* or in infected tissue *in vivo*, and they can act as a delivery vehicle of virulence factors to reach a distant target (Beveridge, 1999, Kuehn *et al.*, 2005, Ellis *et al.*, 2010b, Kulp *et al.*, 2010). Thus far, no information is available on the formation of *H. suis* OMV, the content thereof, their internalization by epithelial cells and the putative delivery of bacterial components, such as the *H. suis* GGT, to the deeper mucosal layers.

In the present study, Jurkat T cells as well as murine splenocyte subsets ($CD4^+$ T cell, $CD8^+$ T cell, $CD19^+$ B cell) were used as cell models to investigate the immunosuppressive effect of *H. suis* GGT through the action on its substrates. AGS cells, intestinal porcine epithelial (IPEC-J2) cells, and human Caco-2 cells were used to investigate the putative translocation of GGT, present in *H. suis* OMV, across an epithelial cell monolayer.

Materials and methods

Animals

For isolation of splenic lymphocytes, female specific-pathogen-free (SPF) 4-6-week-old BALB/c mice were purchased from Harlan NL (Horst, The Netherlands). Housing and euthanasia of experimental animals were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2012/156).

Construction of a *H. suis ggt* isogenic mutant strain

Deletion of *H. suis ggt* was introduced by allelic exchange using pBluescript II SK (+) phagemid vector (Agilent Technologies, California, USA) in which ~650 bp of the 5′ –end and ~750 bp of the 3′ –end of the target gene and the chloramphenicol resistance gene from pUOA14 (Wang *et al.*, 1990, Rossi *et al.*, 2012) were ligated through a PCR-mediated

strategy (Fischer *et al.*, 2001, Huang *et al.*, 2011). All primers used for PCR-mediated construction of the recombinant plasmid are shown in table 1.

Table 1. Primers used for construction of a *H. suis ggt* isogenic mutant strain (HS5Δ*ggt*)

Primer name	Sequence (5′ - 3′)	Primer use
pBlue linear Fwd 1	GGGGATCCACTAGTTCTAGAGCG	Linearization of plasmid
pBlue linear Rev1	CGGGCTGCAGGAATTCGATATCAAG	Linearization of plasmid
HsGGT_flank_fusion1F	CTTGATATCGAATTCCTGCAGCCCGGAGGCGTTGCACAATAGCTTTAGGG	Amplification *H. suis ggt* and partial up- and downstream flanking genes
HsGGT_flank_fusion1R	GCCGCTCTAGAACTAGTGGATCCCCATAAAACCAGTTAGGCTGGGCAAAG	Amplification *H. suis ggt* and partial up- and downstream flanking genes
pBluelinear_Hsggtflank1F	CCACGCAAGGAATTTTAAATGCAAC	Linearization of the recombinant plasmid
pBluelinear_Hsggtflank1R	GATCTCCTCAAATTTTAAAAAATACGC	Linearization of the recombinant plasmid
Hschloram_fusion_1F	GCGTATTTTTTAAAATTTGAGGAGATCTATCAACAAATCGGAATTTACGG	Amplification chloramphenicol resistance gene
Hschloram_fusion_1R	GCATTTAAAATTCCTTGCGTGGTTATTTATTCAGCAAGTCTTGTAA	Amplification chloramphenicol resistance gene
T7 prom3	TAATACGACTCACTATAGGG	Sequencing
M13R	CAGGAAACAGCTATGAC	Sequencing

The resultant plasmid was amplified in XL1-Blue MRF′ *E. coli* (Agilent Technologies) and used as a suicide plasmid in *H. suis* strain HS5, isolated from the stomach of a sow. Transformation of *H. suis* strain HS5 was perfomed by electroporation as described for *H. felis* (Josenhans *et al.*, 1999) with some modifications. Briefly, 1.5 µg suicide plasmid was used for electroporation. Then, the *H. suis 5 ggt* mutant strain (HS5Δ*ggt*) was first cultured for 2 days on biphasic Brucella culture plates without chloramphenicol, as described previously (Flahou *et al.*, 2012a). Subsequently, bacteria were transferred onto biphasic Brucella culture plates supplemented with chloramphenicol (20 µg/mL) for 4 days, after which they were finally selected on biphasic Brucella plates supplemented with chloramphenicol (30 µg/mL) for 7-14 days. The site of recombination was verified by a GGT activity assay (Flahou et al., 2011), PCR and nucleotide sequencing.

Recombinant expression and purification of *H. suis* γ-glutamyl transpeptidase

The expression and subsequent purification of recombinant *Helicobacter suis* γ-glutamyl transpeptidase (GGT) were performed as described previously (Flahou *et al.*, 2011). Briefly, the enzyme was expressed in *E. coli* strain BL21-AI™. Subsequently, the protein was

purified to homogeneity by immobilized metal affinity chromatography (IMAC) on a Ni-sepharos column (His GraviTrap; GE Healthcare Bio-Science AB, Uppsala, Sweden) and gel filtration using a Superdex^{TM} 75 gel filtration column (GE Healthcare Bio-Sciences AB). The purified protein was stored at -80°C until further use.

Preparation of *H. suis* outer membrane vesicles (OMV)

72-hour-old cultures of *H. suis* were harvested, and the bacteria were removed by centrifugation (12000 × g, 15 minutes, 4 °C). The supernatant fluid was subjected to ultracentrifugation (200000 × g, 2 hours, 4 °C) to recover the OMV. After two washing steps in Hank's Balanced Salt Solution (HBSS), the OMV were stored at -70 °C until further use. The obtained OMV were visualized by a negative staining technique. Hereby a copper grid with formvar membrane was placed on top of a drop of OMV suspension for 10 seconds and counterstained with uranylacetate for 1 minute. After rinsing and drying the grids were analysed by Transmission Electron Microscopy (TEM). The presence of GGT activity in *H. suis* OMV was validated with a GGT activity assay as described previously (Flahou *et al.*, 2011).

Cell cultures

Jurkat E6.1 cells (Human leukaemic T cell line; ECACC; Salisbury, UK) were cultured in RPMI 1640 with 5% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 millimolar (mM) L-Gln (Invitrogen, Carlsbad, CA, USA) and penicillin (50 units/mL) and streptomycin (50 µg/mL) (Invitrogen) at 37°C with 5% CO_2.

$CD4^+$ and $CD8^+$ T cells, as well as $CD19^+$ B lymphocytes were isolated from mouse spleens using EasySep™ Mouse $CD4^+$ and $CD8^+$ T cell, and $CD19^+$ B cell Enrichment Kits (StemCell Technologies, Grenoble, France). Culture was performed in RPMI 1640 containing 10% (v/v) FBS, 1 mM L-Gln, 50 micromolar (µM) 2-mercaptoethanol (Sigma-Aldrich St. Louis, MO, USA), penicillin (50 units/mL) and streptomycin (50 µg/mL) at 37°C with 5% CO_2.

The culture conditions of AGS cells (a human gastric adenocarcinoma cell line), IPEC-J2 cells, and Caco-2 cells have been described elsewhere (Flahou *et al.*, 2011, Verbrugghe *et al.*, 2011, Eeckhaut *et al.*, 2013). Briefly, AGS cells were cultured in Ham's F12 (Invitrogen; 1 mM glutamine) supplemented with 10% (v/v) FBS, penicillin (50 units/mL) and streptomycin (50 µg/mL). IPEC-J2 cells were cultured in Dulbecco's Modified Eagle's

medium (DMEM; Gibco, Life Technologies, Paisley, Scotland) supplemented with 47% (v/v) Ham's F12 medium (Gibco), 5% (v/v) FBS, 1% (v/v) insulin-transferrin-selenium-A supplement (ITS, Gibco), penicillin (50 units/mL), and streptomycin (50 µg/mL). Caco-2 cells were cultured in DMEM (Gibco) supplemented with 10% (v/v) FBS, 1 mM glutamine, 1% (v/v) non-essential amino acids (Gibco), penicillin (50 units/mL) and streptomycin (50 µg/mL).

Internalization of *H. suis* OMV by AGS, IPEC-J2 and Caco-2 cells

AGS, IPEC-J2 and Caco-2 cells were used to examine the putative internalization of *H. suis* OMV. AGS cells labeled with green CellTracker™ (Invitrogen) were incubated for 4 hours with *H. suis* OMV labeled with red fluorescent Vybrant® DiD (Invitrogen). AGS cells were fixed with 4% paraformaldehyde for 15 minutes, washed 5 times extensively with HBSS and analysed by confocal laser scanning microscopy for uptake of *H. suis* OMV. IPEC-J2 and Caco-2 cells were labeled with red fluorescent CellTracker Red CMTPX (Invitrogen) and incubated for 8 hours with *H. suis* OMV labeled with green fluorescent Vybrant® DiO (Invitrogen). Subsequently, cells were fixed with 4% paraformaldehyde for 15 minutes, washed 5 times extensively with HBSS and analysed by confocal laser scanning microscopy for uptake of *H. suis* OMV.

Translocation across a differentiated IPEC-J2 monolayer of active GGT present in *H. suis* OMV

In order to examine the putative translocation ability of active *H. suis* GGT contained in *H. suis* OMV across an epithelial cell monolayer, a translocation assay was performed as described elsewhere (Verbrugghe *et al.*, 2012). IPEC-J2 cells (1×10^4 cells/250 µl/insert) were seeded on the apical side of the Transwell® polycarbonate membrane inserts with a pore size of 3.0 µm and a membrane diameter of 6.5 mm (Corning Costar Corp., Cambridge, MA, USA), and the basolateral side was filled with 1 mL fresh culture medium. Cell medium was refreshed every 2 to 3 days and cells were cultured for 3 to 4 weeks in order to allow differentiation to a complete monolayer as described elsewhere (Verbrugghe *et al.*, 2012, Eeckhaut *et al.*, 2013). When differentiated, 100 µg (based on the total protein content) *H. suis* OMV were added to the apical compartment. After incubation for up to 48 hours (37°C; 5% CO_2), the presence of GGT activity in the basolateral compartment was determined with a GGT activity assay (Flahou *et al.*, 2011). The transepithelial electrical resistance (TEER) was

measured before and after the incubation with *H. suis* OMV to assess the barrier integrity of the differentiatedepithelial cell monolayer as described previously (Verbrugghe *et al.*, 2012).

Cell proliferation assays

Jurkat T cells (4×10^4/well), $CD4^+$ and $CD8^+$ T, and $CD19^+$ B lymphocytes (1.5×10^5/well) were cultured in 24-well or 96-well flat-bottom cell-culture plates (Greiner Bio One, Frickenhausen, Germany) as described above.

$CD4^+$ and $CD8^+$ T cells were stimulated by incubating the cells in wells of a microtiter plate that had been precoated with an anti-CD3 antibody (4 µg/mL and 8 µg/mL respectively, clone 145-2C11; eBioscience, Vienna, Austria) and in the presence of a soluble anti-CD28 antibody (2 µg/mL, clone 37-51; eBioscience). $CD19^+$ B cells were stimulated by F(ab')2 Goat anti-mouse IgM (12 µg/mL, Jackson Immunoresearch, West Grove PA, USA) and recombinant mouse IL-2 (100 U/mL, eBioscience).

All cells were incubated in the presence or absence of whole-cell lysate from wild-type *H. suis* strain HS5 and mutant *H. suis* strain HS5Δ*ggt*, as well as different concentrations of recombinant *H. suis* GGT for 24 - 72 hours, depending on the experiment and cell type. Cellular proliferation was determined by incorporation of [^{3}H]-thymidine (Amersham ICN, Bucks, UK). In brief, all cells were pulse-labeled with 1 µCi [^{3}H]-thymidine during the final 18 hours of experimental incubation, and then harvested onto glass fiber filters (Perkin-Elmer, Life Science, Brussels, Belgium). The incorporated radioactivity was detected using a β-scintillation counter (Perkin-Elmer).

Evaluation of cell death (apoptosis and necrosis) by flow cytometry

Jurkat T cells (4×10^4/well) were treated with 2 µg/mL recombinant *H. suis* GGT for 24 - 72 hours. Controls consisted of HBSS-treated Jurkat T cells. All samples were subjected to flow cytometric analysis (FCM) on a BD FACSCanto II flow cytometer with FACSDiva software (Becton Dickinson, Erembodegem, Belgium).

Propidium iodide (PI) staining was used to detect loss of plasma membrane integrity as a marker for necrosis. Briefly, cells were washed with HBSS, incubated with 1 µg/mL PI in HBSS for 15 minutes on ice, followed by FCM analysis. Staining for activated caspase-3 was performed to detect apoptosis. Briefly, cells were washed with HBSS, fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.1% Triton X-100 in HBSS for 2 minutes. Subsequently, cells were incubated with a primary rabbit antibody directed against

activated caspase-3 (R&D Systems Europe) for 1 hour at 37°C, followed by an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Invitrogen). Cells treated with 0.5 µM staurosporine (Sigma-Aldrich) for 20 hours served as positive controls for apoptosis.

Ammonia assay

Two µg/ml *H. suis* GGT was added to HBSS supplemented with 2 mM L-Gln and incubated at 37°C for 2 hours, after which the concentration of released ammonia was determined by the Ammonia Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions.

Supplementation of cell cultures with L-Gln and GSH

Jurkat T cells (4×10^4/well) were incubated in medium supplemented with L-Gln (0 - 10 mM; Sigma-Aldrich) or GSH (0 - 5 mM; Sigma-Aldrich) and treated with HBSS or 2 µg/mL recombinant *H. suis* GGT for 48 or 72 hours. $CD4^+$ and $CD8^+$ T cells (1.2×10^5/well) were incubated in medium supplemented with L-Gln (0 - 5 mM) or GSH (0 - 2 mM) and treated with HBSS or 1 µg/mL recombinant *H. suis* GGT for 68 hours. Cellular proliferation was determined by [^{3}H]-thymidine incorporation as mentioned above.

Measurement of cytokine release

$CD4^+$ T cells (1.5×10^5/well), activated by CD3/CD28 mAbs, were incubated in medium supplemented with 0.1 µg/mL or 0.5 µg/mL recombinant *H. suis* GGT for 68 hours. Secretion levels of IFN-γ, IL-4, and IL-17A were determined in cell supernatant by enzyme-linked immunosorbent assay (ELISA) (eBioscience).

Statistical analysis

All experiments were repeated at least 3 times with at least 3 replications for each treatment. Combined data from these experiments are used for statistical analysis, and all data were expressed as mean $\pm$ SD. A Student *t* test was used for statistical analysis between two groups, and one-way ANOVA was performed for comparison of control cells with multiple treatments. For both statistical analyses methods, *P* values less than 0.05 were considered statistically significant.

Results

H. suis OMV contain GGT activity and can be internalized by AGS, IPEC-J2, and Caco-2 cells

Ultrastructural examination revealed that most OMV isolated from *H. suis* culture supernatant ranged from 20 - 200 nm in size (Figure 1). A GGT activity level up to 4.5 - 9.5 mU/mg was detected in the OMV, confirming that GGT is one of the components of *H. suis* OMV.

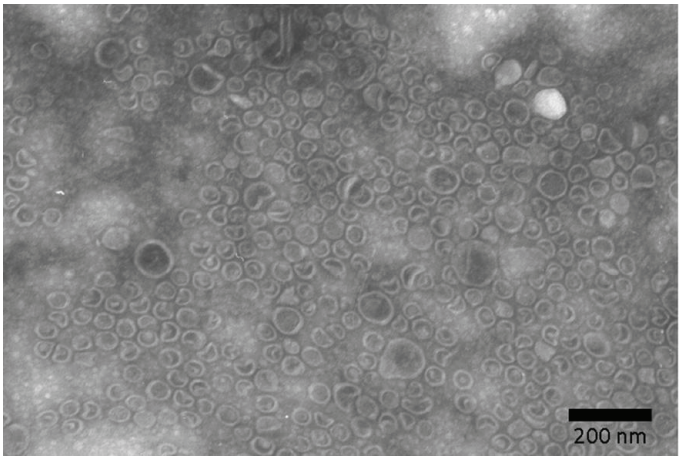

Figure 1. Ultrastructural examination of purified *H. suis* outer membrane vesicles (OMV). Shown are transmission electron microscopic images of *H. suis* OMV purified by repeated ultracentrifugation.

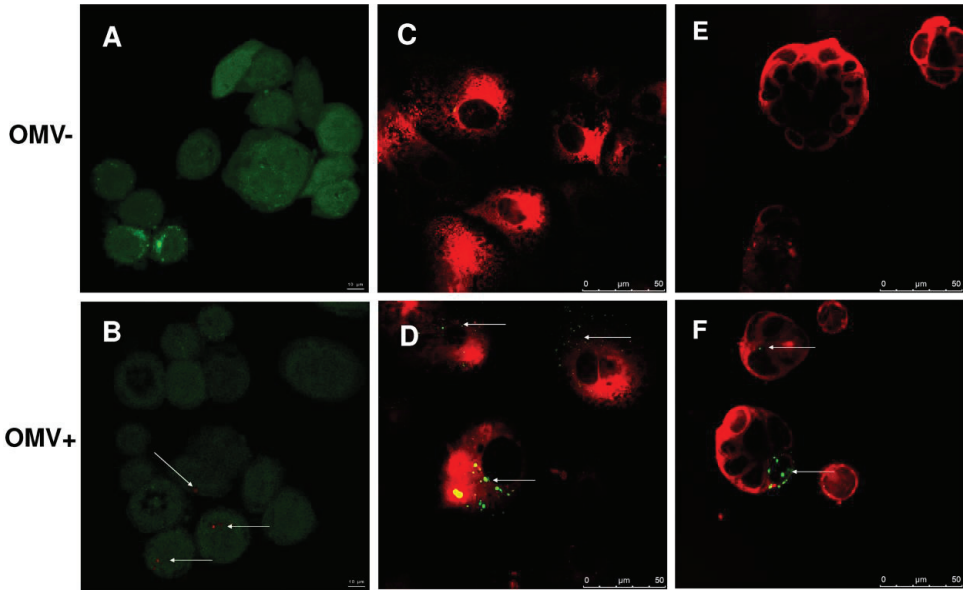

Figure 2. The uptake of *H. suis* OMV by AGS, IPEC-J2, and Caco-2 cells. AGS cells labeled with green CellTracker™ were incubated for 4 hours with HBSS (Figure 2A) or *H. suis* OMV labeled with red fluorescent Vybrant® DiD (Figure 2B). IPEC-J2, and Caco-2 cells labeled with red fluorescent CellTracker Red CMTPX were incubated for 8 hours with HBSS (Figure 2C, 2E, respectively) or *H. suis* OMV labeled with green fluorescent Vybrant® DiO (Figure 2D, 2F, respectively). The visualization of OMV was done by confocal laser scanning microscopy (indicated by arrows). HBSS: Hank's balanced salt solution; *H. suis* OMV: *Helicobacter. suis* outer membrane vesicles.

In order to further examine if *H. suis* OMV carrying GGT can be internalized by gastric or intestinal epithelial cells, AGS, IPEC-J2, and Caco-2 cells were incubated with *H. suis* OMV for 4 hours or 8 hours. Our results reveal that *H. suis* OMV can be internalized by all three types of epithelial cell lines (Figure 2 A - F).

Active *H. suis* GGT from *H. suis* OMV translocates across a differentiated IPEC-J2 cell monolayer

After 3 - 4 weeks culture, a differentiated IPEC-J2 cell monolayer was established, indicated by a stable TEER value of approximately 2400 Ohm/insert. Compared to the IPEC-J2 cells treated with HBSS, incubation of a differentiated IPEC-J2 cell monolayer with 100 µg *H. suis* OMV for 48 hours resulted in the detection of higher GGT activity in the basolateral compartment (Figure 3, p=0.058) without disrupting the integrity of IPEC-J2 cell monolayer, as shown by a stable TEER: an average value of 2421 Ohm/insert was detected at the onset of the experiment and an average value of 2361 Ohm/insert was detected at the end of the experiment (p=0.72, Student t test). This translocation may constitute one of the routes

by which GGT from *H. suis* can access lymphocytes residing in the lamina propria underneath the lining epithelium.

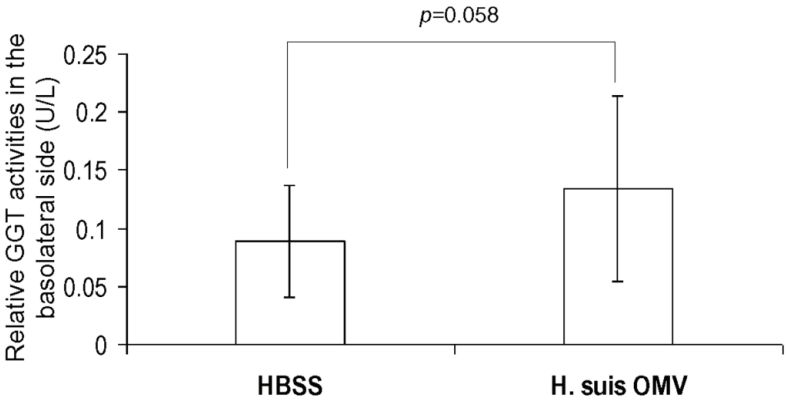

Figure 3. Translocation of active *H. suis* GGT through a differentiated IPEC-J2 cell monolayer. IPEC-J2 cells were seeded on the insert (with a pore size of 3.0 µm and a membrane diameter of 6.5 mm) for 3 - 4 weeks until the cells were differentiated to a complete cell monolayer. The cells were treated with HBSS or 100 µg *H. suis* OMV for 48 hours, and the presence of GGT in the baselateral compartment was determined by a GGT activity assay as described before. Results are presented as the relative GGT activity level compared to control cells treated with HBSS. Shown are the mean values (± SD) of 3 independent experiments (n=9). Student *t* test was used for analysis of statistically significant difference. HBSS: Hank's balanced salt solution; *H. suis* OMV: *Helicobacter. suis* outer membrane vesicles.

Effect of *H. suis* whole-cell lysate on Jurkat T cells

Cellular proliferation of Jurkat T cells was inhibited after incubation with whole cell lysate of wild-type *H. suis* strain HS5 for 48 or 72 hours in a dose-dependent manner (data not shown). Concentrations of 250 µg/mL of this lysate almost completely inhibited cellular proliferation of Jurkat T cells (Figure 4A). Compared to treatment with whole-cell lysate from wild-type *H. suis* strain HS5, treatment of Jurkat T cells with lysate (48 h; 62.5 to 250 µg/mL) from strain HS5Δ*ggt* resulted in a marked decrease (minus 15.3 - 49.3%) of the inhibitory effect on T cell proliferation (Figure 4A).

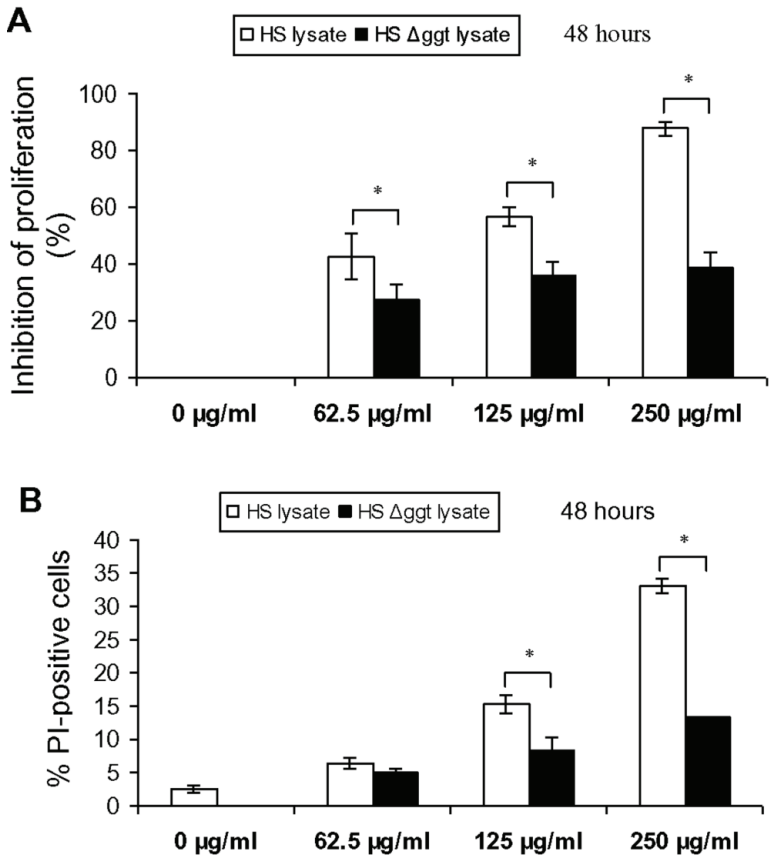

Figure 4. Effect of *H. suis* whole-cell lysate on cell proliferation and viability of Jurkat T cells. (*A*) Jurkat T cells were incubated in medium supplemented with whole-cell lysate (62.5 to 250 µg/mL) from wild-type *H. suis* strain HS5 and strain HS5Δ*ggt* for 48 hours, and cell proliferation levels are determined by cpm (counts per minute), as a measure of [^{3}H]-thymidine uptake. Shown are the rates of proliferation inhibition, relative to Jurkat T cells treated with HBSS instead of whole-cell lysate. Both whole-cell lysate from *H. suis* strain HS5 and strain HS5Δ*ggt* induced a statistically significant inhibition of T cell proliferation, although this was far less pronounced for the mutant strain (one-way ANOVA). (*B*) Jurkat T cells were incubated in medium supplemented with whole-cell lysate (62.5 to 250 µg/mL) from *H. suis* strain HS5 and strain HS5Δ*ggt* for 48 hours, and loss of plasma membrane integrity (as a marker for necrosis) was determined by PI staining. Both whole-cell lysate from *H. suis* strain HS5 and strain HS5Δ*ggt* induced a statistically significant increase of PI-positive cells (one-way ANOVA), although this was far less pronounced for the mutant strain. Shown in *A* and *B* are the mean values (± SD) of 3 independent experiments (n=9). An * represents a statistically significant difference ($p < 0.05$) between HS lysate- and HS Δ*ggt* lysate-treated cells. Control: Jurkat T cell treated by Hank's balanced salt solution. HS lysate: *H. suis* strain 5 lysate. HS Δ*ggt* lysate: *H. suis* strain HS5Δ*ggt* lysate.

Inhibitory effect of *H. suis* GGT on Jurkat T cells and mouse splenocyte subsets

Treatment of Jurkat T cells for 72 hours with up to 2 µg/mL recombinant *H. suis* GGT resulted in an inhibition of cellular proliferation (Figure 5A). Treatment for 48 hours showed similar results (data not shown). Further increasing the concentration of the enzyme, however, did not cause a significant increase of the inhibitory effect. Subsequently, we investigated the

effect of recombinant *H. suis* GGT on primary immune cells, including $CD4^+$ and $CD8^+$ T cells and $CD19^+$ B lymphocytes. A concentration of 1 µg/mL recombinant *H. suis* GGT inhibited the proliferation of $CD4^+$ and $CD8^+$ T splenocytes by about 80% (Figure 5B) and the proliferation of the $CD19^+$ B cells by more than 95% (Figure 5C). A concentration of 2 µg/mL recombinant *H. suis* GGT almost completely inhibited the proliferation of all three lymphocyte subsets.

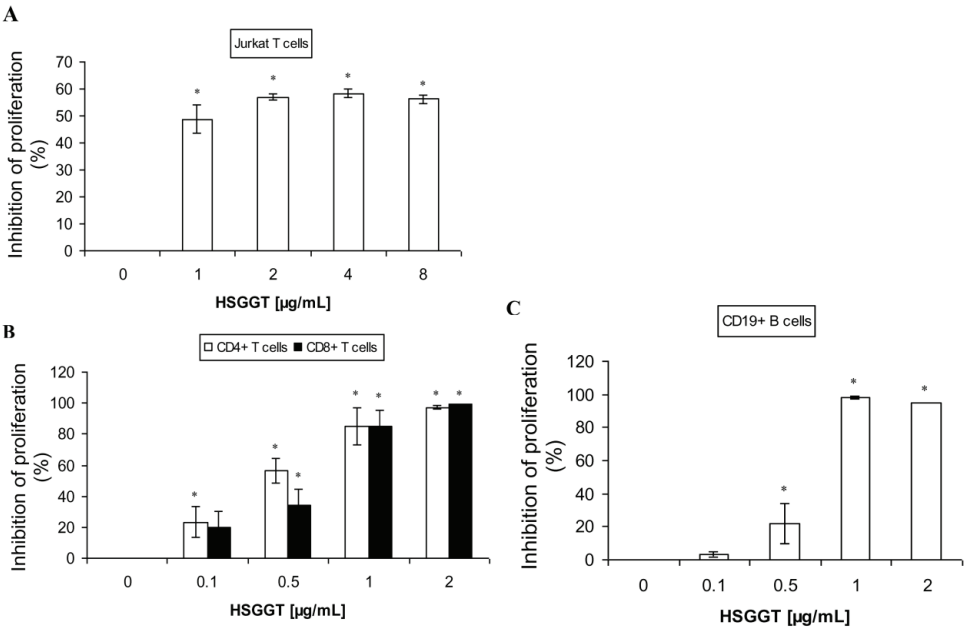

Figure 5. Inhibitory effect of *H. suis* γ-glutamyl transpeptidase (GGT) on Jurkat T cells and mouse splenocyte subsets. (*A*) Jurkat T cells were incubated in medium supplemented with recombinant *H. suis* GGT (1 to 8 µg/mL) for 72 hours, and cell proliferation levels are determined by cpm (counts per minute), as a measure of [^{3}H]-thymidine uptake. (*B*) $CD4^+$ or $CD8^+$ splenic T lymphocytes were purified, stimulated by CD3/CD28 mAbs, and incubated with recombinant *H. suis* GGT (0.1 µg/mL to 2 µg/mL) for 68 hours, resulting in a dose-dependent inhibition of proliferation. (*C*) $CD19^+$ B splenocytes were purified, stimulated by anti-IgM (12 µg/mL) and recombinant mouse IL-2 (100 U/mL), followed by treatment with recombinant *H. suis* GGT (0.1 µg/mL to 2 µg/mL) for 44 hours. Shown are the rates of proliferation inhibition, relative to stimulated splenocytes treated with HBSS instead of recombinant *H. suis* GGT. Shown are the mean values (± SD) of 3 independent experiments or one representative experiment (out of 3 performed in total). An * represents a statistically significant difference ($p < 0.05$) compared to HBSS-treated control cells. HSGGT: recombinant *H. suis* GGT.

The role of cell death (apoptosis and necrosis) during *H. suis* GGT-mediated inhibition of T cell proliferation

Compared to treatment with whole-cell lysate from wild-type *H. suis* strain HS5, treatment of Jurkat T cells with lysate (48 h; 62.5 to 250 µg/mL) from strain HS5Δ*ggt* resulted in a considerably lower (1.3 - 19.6%) cell death-inducing capacity (Figure 4B).

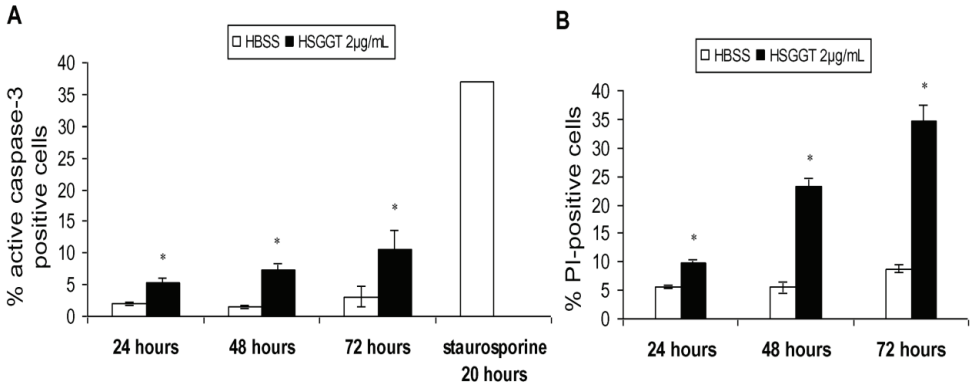

Figure 6. Cell death analysis of Jurkat T cells treated with recombinant *H. suis* γ-glutamyl transpeptidase (GGT) evaluated by flow cytometry. Jurkat T cells were incubated in medium supplemented with 2 µg/mL recombinant *H. suis* GGT for 24, 48, or 72 hours and (*A*) cell apoptosis and (*B*) loss of plasma membrane integrity (as a marker for necrosis) were determined by staining for activated caspase-3 and PI staining, respectively. Jurkat T cells treated with 0.5 µM staurosporine for 20 hours served as positive control for apoptosis. Shown are the mean values (± SD) of one representative experiment (n=3) or 3 independent experiments (n=9). An * represents a statistically significant difference ($p < 0.05$) between HSGGT- and HBSS-treated cells (Student *t* test). HBSS: Hank's balanced salt solution; HSGGT: recombinant *H. suis* GGT; PI: propidium iodide.

Compared to the HBSS-treated cells, incubating Jurkat T cells with 2 µg/mL recombinant *H. suis* GGT for 24, 48 or 72 hours resulted in an increase (+3 - 7%) of the number of active caspase-3 positive cells (Figure 6A). PI staining demonstrated a higher increase (+26%, compared to HBSS-treated cells) of the number of Jurkat T cells showing loss of plasma membrane integrity, as a marker for necrosis, after treatment with *H. suis* GGT for 72 hours (Figure 6B).

Identification of catalytic activity of *H. suis* GGT on L-Gln

L-Gln and reduced glutathione (GSH) are 2 putative substrates of *H. suis* GGT. In a previous report, we indeed showed that *H. suis* GGT catalyzes the degradation of GSH [9]. To investigate whether also L-Gln can serve as a substrate for *H. suis* GGT, 2 mM Gln was incubated in HBSS with or without 2 µg/mL *H. suis* GGT at 37°C. After 2 hours of incubation the concentration of ammonia was determined. Data showed that *H. suis* GGT indeed hydrolyses Gln *in vitro*, with the formation of ammonia as by-product (Figure 7). Compared to HBSS-treated Gln, 2 mM Gln treated with 2 µg/mL *H. suis* GGT released 5.3 µg/mL ammonia after incubation for 2 hours, showing that >15% of Gln was degraded by 2 µg/mL *H. suis* GGT under these conditions. More than 70% of Gln was degraded by using a

higher concentration of *H. suis* GGT (10 µg/mL), after incubation under the same conditions as described above (data not shown).

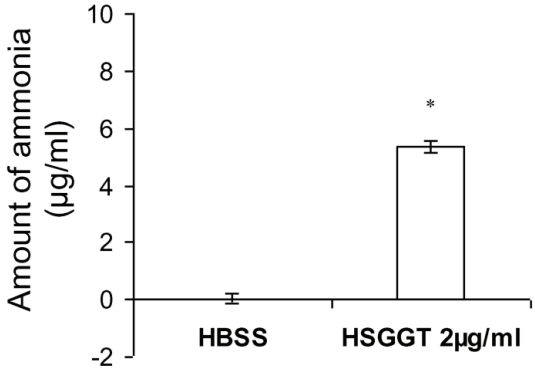

Figure 7. Determination of catalytic activity of *H. suis* GGT on L-Gln. Two mM Gln was incubated with HBSS or 2 µg/mL *H. suis* GGT at 37°C for 2 hours, after which the concentration of released ammonia was determined using the Ammonia Assay Kit. The mean data (± SD) of one representative experiment are shown (n=3). An * represents a statistically significant difference ($p < 0.05$) compared to HBSS-treated L-Gln (Student *t* test). HSGGT: recombinant *H. suis* GGT; HBSS: Hank's balanced salt solution.

Modulation of *H. suis* GGT-mediated inhibition of lymphocyte proliferation by L-Gln and GSH

To investigate the role of L-Gln and GSH, two important substrates of GGT, in the above described inhibition of lymphocyte proliferation, Jurkat T cells and stimulated $CD4^+$ or $CD8^+$ T cells isolated from mice, were treated with a series of concentrations of L-Gln or GSH in the presence or absence of 1 or 2 µg/mL recombinant *H. suis* GGT. Data from HBSS-treated control cells showed that the presence of L-Gln is essential for a normal proliferation of Jurkat T cells (Figure 8A). As described above, treatment of Jurkat T cells with recombinant *H. suis* GGT resulted in an inhibition of cellular proliferation. Interestingly, supplementation of 2 µg/mL *H. suis* GGT-treated Jurkat T cells with L-Gln was able to restore the normal proliferation rate of the cells, incubated for 72 hours (Figure 8A), in a dose (up to 10 mM L-Gln)-dependent manner. For primary $CD4^+$ or $CD8^+$ T lymphocytes isolated from mouse spleens, supplementation with L-Gln showed a similar effect (Figure 8B, 8C). Supplementation with 5 mM L-Gln was able to restore the cellular proliferation of 1 µg/mL recombinant *H. suis* GGT treated $CD4^+$ and $CD8^+$ T cells to normal levels after incubation for 68 hours (Figures 8B, 8C).

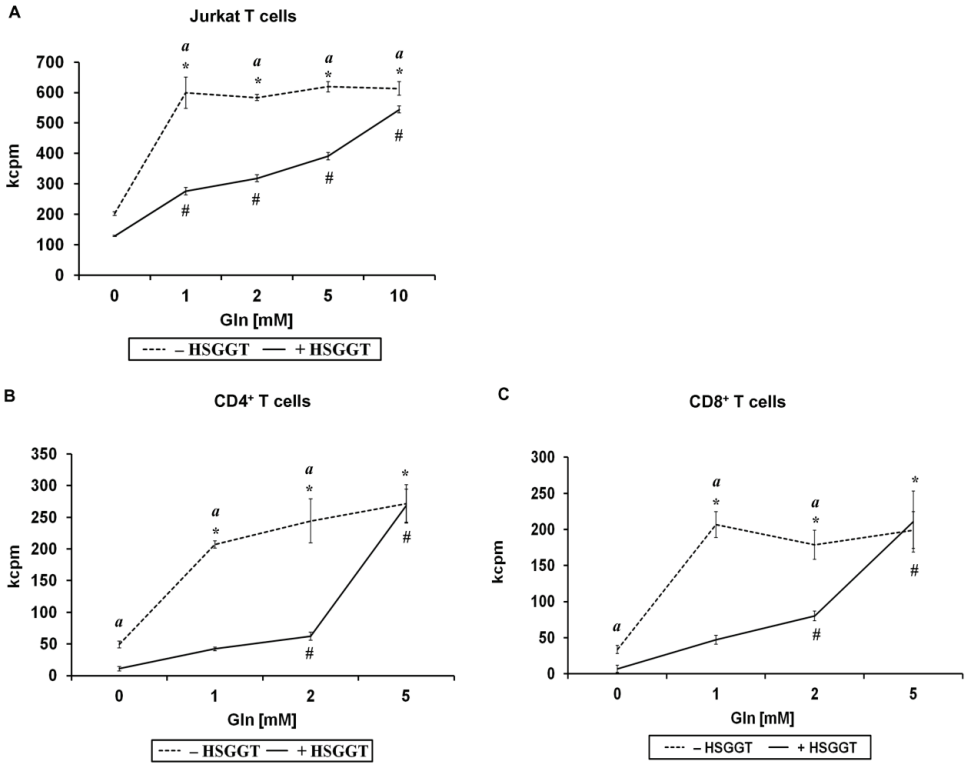

Figure 8. The effect of L-Gln supplementation on *H. suis* γ-glutamyl transpeptidase (GGT)-treated Jurkat T cells and murine splenocytes. Jurkat T cells were incubated in medium supplemented with L-Gln (0 mM to 10 mM) for 72 hours (*A*) in the presence or absence of 2 µg/mL recombinant *H. suis* GGT, followed by cell proliferation detection by determining [^{3}H]-thymidine uptake. CD4$^+$ T cells (*B*) or CD8$^+$ T cells (*C*) activated by anti-CD3 and anti-CD28 mAbs, were supplemented with L-Gln (0 mM to 10 mM) for 68 hours in the presence or absence of 1 µg/mL recombinant *H. suis* GGT, followed by cell proliferation detection by measuring [^{3}H]-thymidine uptake. The mean data (± SD) of one representative experiment (out of 3 performed in total) are shown for *A*-*C* (n=3). * and # represent a statistically significant increase ($p < 0.05$) of cell proliferation by supplementing cells with a given L-Gln concentration, compared to HBSS- or *H. suis* GGT-treated cells, respectively, without L-Gln supplementation (0 mM L-Gln) (one-way ANOVA). An (*a*) indicates a higher proliferation rate of HBSS-treated cells, compared to *H. suis* GGT-treated cells for a given L-Gln concentration (Student *t* test). *, #, and (*a*): $p < 0.05$. kcpm: the number of counts per minute (x1000) determined by β-scintillation counting, as a measure of cellular proliferation; HSGGT: recombinant *H. suis* GGT; CD3/CD28 mAbs: anti-mouse CD3/CD28 monoclonal antibodies; Gln: L-glutamine; -HSGGT: treated without recombinant *H. suis* GGT; +HSGGT: treated with recombinant *H. suis* GGT.

On the other hand, GSH supplementation induced a slightly higher stimulation of cellular proliferation of primary T splenocyte subsets (Figure 9B, $P< 0.05$), treated with HBSS (control cells). Interestingly and in contrast, supplementation of *H. suis* GGT-treated Jurkat T cells with GSH aggravated the inhibitory effect of *H. suis* GGT, both after 48 and 72 hours of incubation (Figure 9A, $P< 0.05$). For CD4$^+$ or CD8$^+$ T cells, however, we did not observe similar effects (Figure 9B, 9C).

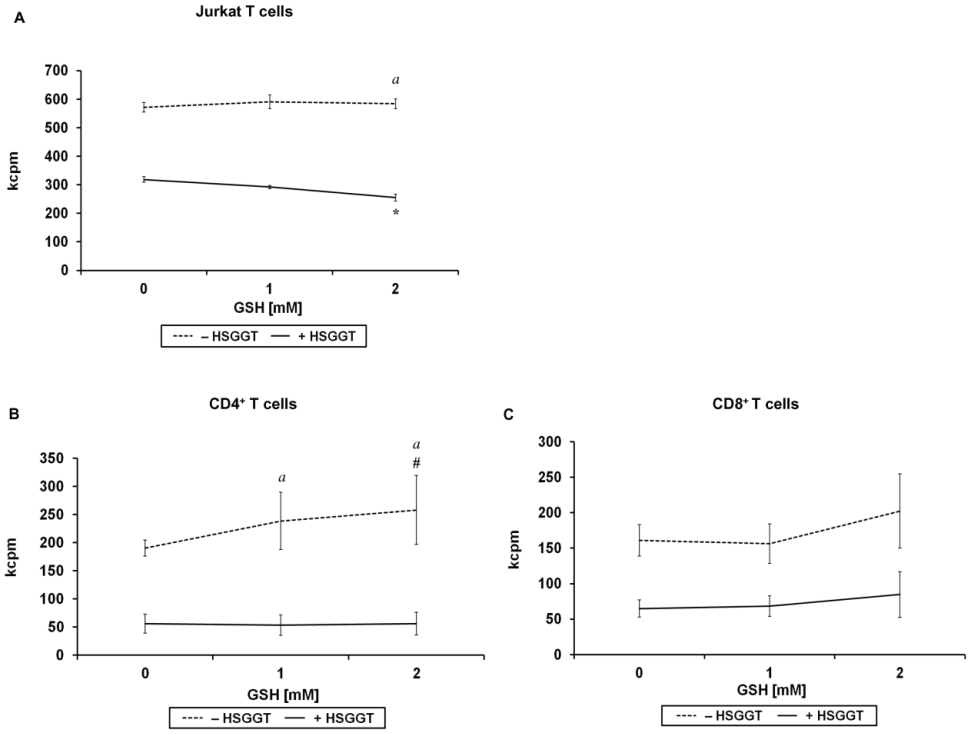

Figure 9. The effect of GSH supplementation on *H. suis* γ-glutamyl transpeptidase (GGT)-treated Jurkat T cells and murine splenocytes. Jurkat T cells were incubated in medium supplemented with GSH (0 mM to 2 mM) for 72 hours (*A*) in the presence or absence of 2 µg/mL recombinant *H. suis* GGT, followed by cell proliferation detection by measuring [^{3}H]-thymidine uptake. The mean data (± SD) of one representative experiment are shown (n=3). CD4$^+$ T cells (*B*) or CD8$^+$ T cells (*C*) activated by CD3/CD28 mAbs, were incubated in medium supplemented with GSH (0 mM to 2 mM) for 68 hours in the presence or absence of 1µg/mL recombinant *H. suis* GGT, followed by cell proliferation detection by measuring [^{3}H]-thymidine uptake, as shown by kcpm (counts per minute; x1000) values. Shown are the mean values (± SD) of 3 independent experiments (n=9). An * indicates a decrease of cell proliferation of *H. suis* GGT-treated cells supplemented by a given GSH concentration, compared to *H. suis* GGT treated cells without GSH supplementation (0 mM GSH) (one-way ANOVA). An # indicates an increase of cell proliferation of HBSS-treated cells supplemented by a given GSH concentration, compared to HBSS-treated cells without GSH supplementation (0 mM GSH) (one-way ANOVA). An (*a*) indicates the relative increase of the difference of cell proliferation between HBSS-treated cells and *H. suis* GGT-treated cells at an indicated concentration of GSH, compared to 0 mM GSH-treated cells (Student *t* test). *, #, and (*a*): $p < 0.05$. kcpm: the number of counts per minute (x1000) determined by β-scintillation counting, as a measure of cellular proliferation; HSGGT: recombinant *H. suis* GGT; CD3/CD28 mAbs: anti-mouse CD3/CD28 monoclonal antibodies; GSH: reduced glutathione; -HSGGT: treated without recombinant *H. suis* GGT; +HSGGT: treated with recombinant *H. suis* GGT.

Effects of *H. suis* GGT on T helper cytokine secretion by murine CD4$^+$ T cells

CD4$^+$ T cells are known to play a pivotal role in the immune response directed against *Helicobacter* infection (Aebischer *et al.*, 2000, Eaton *et al.*, 2001, Akhiani *et al.*, 2002). The results described above show that *H. suis* GGT inhibits the proliferation of this lymphocyte subset. We investigated whether this also implies a change in cytokine secretion by these cells. Murine CD4$^+$ T cells were incubated with 0.1 µg/mL or 0.5 µg/mL recombinant *H. suis* GGT

for 68 hours. Enzyme-linked immunosorbent assay (ELISA) for IFN-γ, IL-4 and IL-17A performed on supernatant fluids of these cells revealed a significant suppression of IL-4 and IL-17A secretion, a Th2 and Th17 signature cytokine, respectively, in the presence of 0.5 µg/mL recombinant *H. suis* GGT (Figure 10B, 10C, $P< 0.05$). For IFN-γ secretion by these same cell populations, however, no effects were observed upon treatment with *H. suis* GGT (Figure 10A).

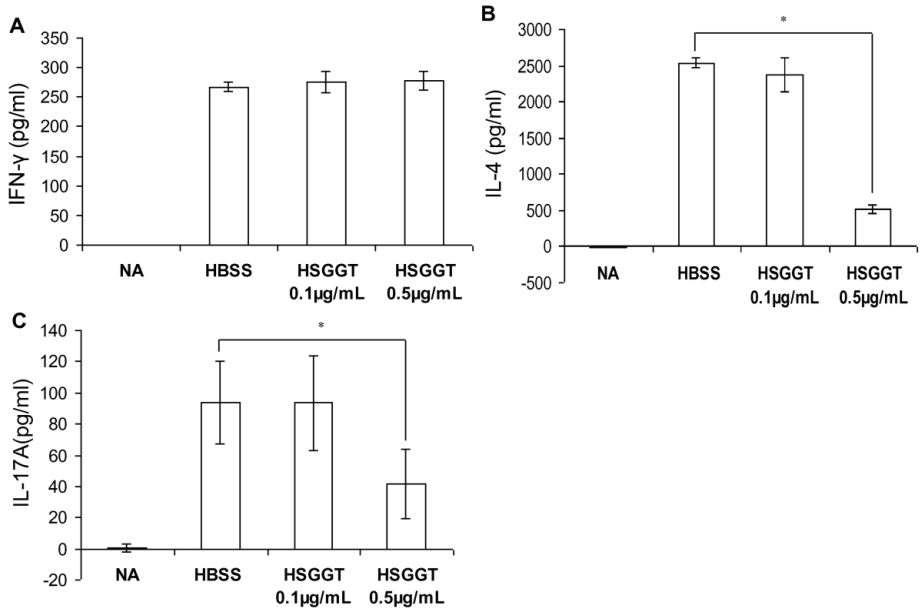

Figure 10. Influence of *H. suis* γ-glutamyl transpeptidase (GGT) on Th1, Th2, and Th17 type cytokine secretion by murine CD4$^+$ T cells. IFN-γ (*A*), IL-4 (*B*), and IL-17A (*C*) secretion by CD4$^+$ T cells activated by anti-CD3 and anti-CD28 mAbs was measured after 68 hours by enzyme-linked immunosorbent assay. Data represent mean ± SD of one representative experiment (n=4). * $p < 0.05$ (one-way ANOVA). NA: unstimulated CD4$^+$ T cells in the absence of *H. suis* GGT; HBSS: stimulated CD4$^+$ T cells in the absence of *H. suis* GGT; HSGGT: stimulated CD4$^+$ T cells in the presence of recombinant *H. suis* GGT.

Discussion

To date, limited information is available on the virulence mechanisms of *H. suis* (Haesebrouck *et al.*, 2009). The development, in 2008, of a method for *in vitro* isolation and culture of *H. suis*, facilitated research on the interactions between *H. suis* and its hosts (Baele *et al.*, 2008). In a previous study, *H. suis* was shown to cause a chronic infection, leading to severe gastric lesions in mouse and Mongolian gerbil models of human gastric disease (Flahou *et al.*, 2010). For *H. pylori*, inhibition of lymphocyte proliferation is considered to contribute to the immune evasion of *H. pylori*, enabling the bacterium to establish a chronic infection (Sundrud *et al.*, 2004, Fischer *et al.*, 2009). Several *H. pylori* factors have been

described to be involved in inhibition of T lymphocyte proliferation, including the *H. pylori* GGT (Sundrud *et al.*, 2004, Schmees *et al.*, 2007, Fischer *et al.*, 2009, Beigier-Bompadre *et al.*, 2011). Similarly, *H. bilis* GGT was reported to inhibit T cell proliferation at a similar level compared to *H. pylori*, and both *H. bilis* and *H. pylori* GGT possess a similar suppressive effect on gastric epithelial cell proliferation mediated by an apoptosis-independent mechanism (Rossi *et al.*, 2012). In a recent study, we identified part of the mechanism by which *H. pylori* and *H. suis* GGT cause gastric epithelial cell death (Flahou *et al.*, 2011). An important role was attributed to the extracellular cell-independent formation of prooxidant metabolites through *H. suis* GGT-mediated degradation of GSH (Flahou *et al.*, 2011). In the present study, we investigated a potential effect of *H. suis* GGT on the proliferation of lymphocytes and more importantly demonstrated a possible role for degradation of its known substrates in this process.

In the present study, recombinantly expressed *H. suis* GGT, as well as whole-cell lysate of wild type *H. suis* strain HS5 had an inhibitory effect on the proliferation of Jurkat T cells, whereas this effect was much lower when Jurkat T cells were incubated with whole-cell lysate of the isogenic *H. suis ggt* mutant strain HS5Δ*ggt*. Recombinantly expressed *H. suis* GGT also inhibited the proliferation of different subsets of primary mouse lymphocytes and these effects were more pronounced than those observed in Jurkat T cells, since in primary splenocytes, 0.1 µg/mL *H. suis* GGT already caused a detectable inhibitory effect. However, using different concentrations of whole-cell lysate from strain HS5Δ*ggt* did not completely abolish the inhibitory effect on Jurkat T cell proliferation, suggesting that other factors are also involved. Putative virulence factors of *H. suis* other than GGT contributing to the inhibition of lymphocyte proliferation need to be further investigated in future experiments.

In Jurkat T cells, *H. suis* GGT-mediated inhibition of proliferation was correlated with an increase of both apoptosis and necrosis. Apparently, this is in contrast to what has been described for *H. pylori* GGT, which does not seem to induce apoptosis in Jurkat T cells, although it has to be mentioned that no other types of cell death were investigated in the study by Schmees et al. (Schmees *et al.*, 2007). On the other hand, in a previous study, we demonstrated that *H. suis* GGT can induce death of gastric epithelial cells, both by necrosis/oncosis and apoptosis, depending on the amount of extracellular reactive oxygen species, generated by GSH degradation(Flahou *et al.*, 2011). Most likely, these increased concentrations of reactive oxygen species in the extracellular environment are also involved in causing death of Jurkat T cells.

In the supernatant of a 24-hour-old to 48-hour-old *H. suis* culture (containing 1 - 4 x 10^8 bacteria/mL with a viability of >99%), approximately 2 - 5 mU/mL GGT activity can be detected (Flahou *et al.*, 2011). Currently, no exact data are available on the colonization density of *H. suis* in human stomachs. Average numbers of *H. suis* colonizing the stomach of experimentally infected mice can reach approximately 10^8 - 10^9/g tissue (Flahou *et al.*, 2012a) and 10^8/g tissue in the stomach of experimentally as well as naturally infected pigs, with colonization densities as high as 10^{10} - 10^{11}/g tissue in some cases [unpublished results]. These values thus correspond in general to the numbers of bacteria per mL in *in vitro* cultures, as mentioned above. Extrapolation clearly shows that the amounts of *H. suis* lysate or GGT used in the current study most likely are similar to what can be expected to be present *in vivo*. Indeed, *H. suis* lysate (containing 25 mU GGT activity/mg total protein) (Flahou *et al.*, 2011) was added to the Jurkat T cells at a final concentration of 62.5 to 250 µg/mL to reach a final concentration of 1.5 to 6.25 mU/mL GGT activity. Recombinant *H. suis* GGT (containing 8 mU GGT activity/µg purified *H. suis* GGT) was added to Jurkat T cells and murine splenocyte subset cultures at a final concentration of 0.1 to 2 µg/mL to reach similar levels of GGT activity (0.8 to 16 mU/mL) in the supernatant fluid of the cells.

As shown in the present and previous studies, L-Gln and GSH are two important substrates of GGT enzymes, including that of *H. suis* (Flahou *et al.*, 2011, Hu *et al.*, 2012, Rossi *et al.*, 2012). The present report is the first one describing that the effects induced by *H. suis* GGT on the function of lymphocytes can be largely attributed to its catalytic activity on extracellular L-Gln and GSH. As GGT activity and function are considered to be conserved among the genus *Helicobacter* (Rossi *et al.*, 2012), similar effects can be expected for GGT from other helicobacters.

L-Gln is the most abundant free amino acid in the blood, and is in fact a major fuel for immune cells, especially lymphocytes (Calder, 1994, Grimble, 2001, Newsholme, 2001). Sufficient L-Gln is essential for both a complete proliferation capacity and normal immune functions of T lymphocytes (Yaqoob *et al.*, 1997, Aledo, 2004). In addition, several reports indicate that L-Gln supplementation has a general protective effect on eukaryotic cells, especially lymphocytes (O'Riordain *et al.*, 1994, Chang *et al.*, 2002, Nakamura *et al.*, 2002). Treatment of lymphocytes with *H. suis* GGT, as in the present study, causes a depletion of extracellular L-Gln, due to the deamidation of L-Gln to L-glutamate (L-Glu), with formation of ammonia as a by-product (Shibayama *et al.*, 2007, Leduc *et al.*, 2010). Results of the present *in vitro* study also show that supplementation of *H. suis* GGT-treated lymphocyte cultures with a series of concentrations of L-Gln strongly counterbalances the inhibitory effect

of *H. suis* GGT, stressing the importance of this amino acid for the proliferation of lymphocytes.

It has been extensively studied and accepted that the mammalian intestine can absorb and utilize L-Gln both from the bloodstream as well as the intestinal lumen (Windmueller *et al.*, 1980, Wilde *et al.*, 1991, Curthoys *et al.*, 1995, Reeds *et al.*, 2001, Wu, 2009). Little information is available on the L-Gln transport or utilization by epithelial cells or other cell types in the gastric mucosa (Hagen *et al.*, 2009, Kaparakis *et al.*, 2010). Transcripts from several amino acid transporter systems for L-Gln have been shown to be expressed in murine and human stomach tissue, including amino acid transporter systems N, A, and L (Wilde *et al.*, 1991, Hatanaka *et al.*, 2000, Bode, 2001, Nakanishi *et al.*, 2001, Kirchhoff *et al.*, 2006). In any case, when Gln is partially delivered to lymphocytes from the gastrointestinal lumen, the link between Gln depletion and the GGT from *H. suis* (as well as other gastric *Helicobacter* species), is obvious, since the GGT can easily access the free Gln in the lumen. On the other hand, it is believed by many researchers that the GGT from gastric helicobacters as well as other secreted factors such as the VacA from *H. pylori* can access the lymphocytes in the lamina propria, in this way affecting the lymphocyte function in a direct and indirect manner. This can be achieved by inflicting damage to epithelial cells, causing local defects in the epithelial barrier (Rieder *et al.*, 2005, Flahou *et al.*, 2010, Flahou *et al.*, 2011, Oertli *et al.*, 2013, Salama *et al.*, 2013). In the present study, we have also provided data supporting our hypothesis that the active GGT enzyme from *H. suis* can cross a differentiated epithelial cell layer, in this way reaching the Gln (and GSH) provided to lymphocytes residing in the lamina propria. We were able to show that the active GGT is one of the components of OMV of *H. suis*, and that the OMV can be internalized, resulting in a translocation of the active *H. suis* GGT from the apical to the basolateral side of epithelial cells, enabling the GGT to locally access the nutrients (eg. Gln) provided from the arterial blood flow.

In vivo, gastric helicobacters induce a deamidation of extracellular L-Gln to L-Glu, after which the latter can be taken up by the bacteria (Leduc *et al.*, 2010), depriving host epithelial and immune cells from both amino acids (Salama *et al.*, 2013). In the present study, no viable bacteria were used, capable of using extracellular L-Glu. Therefore, no depletion of L-Glu is instilled under the experimental conditions described in this study. In theory, L-Glu could thus serve as an alternative cellular fuel, replacing L-Gln, since both amino acids have been described to be able to serve as a cellular fuel for lymphocytes and gastrointestinal epithelial cells (Newsholme *et al.*, 1999, Newsholme *et al.*, 2003, Aledo, 2004, Blachier *et al.*, 2009). The fact that L-Glu can not simply replace L-Gln with respect to cellular proliferation, most

likely depends on the wider array of functions of L-Gln. For instance, L-Gln, but not L-Glu, can be used for purine and pyrimidine synthesis (Blachier *et al.*, 2009), and L-Gln is involved in regulation of protein turnover (Wu, 2009). Possibly, some relevant pathways mentioned above are also involved in T cell proliferation modulated by *H. suis* GGT and L-Gln.

GSH, another substrate for *H. suis* GGT, is considered to be the most important free thiol in animal cells, playing an important role in antioxidant defense, nutrient metabolism, and regulation of cellular events (Paolicchi *et al.*, 2002, Wu *et al.*, 2004). However, several groups have also described pro-oxidative reactions associated with the metabolism of extracellular GSH, initiated by GGT, which may lead to the production of reactive oxygen species and lipid peroxidation, followed by cell death or inhibition of cellular proliferation (Perego *et al.*, 1997, Maellaro *et al.*, 2000, Paolicchi *et al.*, 2002, Flahou *et al.*, 2011). Large amounts of intracellular and extracellular GSH, indeed available in the stomach (Flahou *et al.*, 2011), may act as a substrate to GGT during *H. suis* infection. In the present study, we showed that supplementation with GSH could enhance the proliferation of untreated control Jurkat T cells and murine T lymphocytes to a certain extent. In sharp contrast, when supplementing GSH to *H. suis* GGT-treated Jurkat lymphocytes, this even aggravated *H. suis* GGT-induced inhibition of cell proliferation, possibly due to the pro-oxidative effect of GSH metabolites. However, supplementing GSH to *H. suis* GGT-treated primary mouse lymphocytes, caused no aggravation of *H. suis* GGT-induced inhibition of cell proliferation. Possibly, primary mouse lymphocytes are less sensitive to pro-oxidative products formed under the current experimental conditions, compared to the human-derived Jurkat cell line. Most likely, the balance between concentrations of the antioxidant GSH and its pro-oxidative degradation products is important. Further investigation in primary mouse lymphocytes, using different concentrations of *H. suis* GGT and/or GSH, will allow us to determine whether an effect similar to that seen in Jurkat cells occurs.

IFN-γ, IL-4 and IL-17A are considered to be signature cytokines secreted by T helper (Th) 1, Th2 or Th17 cells, respectively (Zhou *et al.*, 2009). In the present study, IFN-γ secretion by activated $CD4^+$ T cells seems unaffected by *H. suis* GGT treatment, whereas *H. suis* GGT treatment did inhibit IL-4 and IL-17A secretion by activated $CD4^+$ T cells, showing that the effects of *H. suis* GGT on the proliferation of $CD4^+$ helper T lymphocytes also affect the functional secretion of cytokines involved in the maintenance of an immune response.

In summary, *H. suis* GGT was found to inhibit the proliferation of lymphocytes, making it the first discovery of a virulence factor of *H. suis* that affects the functions of immune cells. Cell death plays an important role in this process. Supplementation of *H. suis* GGT-treated

lymphocytes with L-Gln or GSH was able to modulate the observed inhibitory effect, however in opposite ways. L-Gln was able to restore the normal proliferation of the cells whereas supplementation with reduced glutathione (GSH) aggravated the inhibition of lymphocyte proliferation induced by *H. suis* GGT. In addition, we demonstrated that the inhibition of T cell proliferation by *H. suis* GGT is not identical for different lymphocyte subsets, and that *H. suis* GGT also affects the cytokine secretion of $CD4^+$ lymphocytes. Finally, we have generated data supporting our hypothesis that the uptake and processing of *H. suis* OMV by epithelial cells may result in the delivery of active *H. suis* GGT to lymphocytes residing in the deeper mucosal layers. The above described findings may explain part of the mechanisms by which *H. suis* establishes a chronic infection in its preferred niche.

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Study 2: Role of γ-glutamyltranspeptidase in the pathogenesis of *Helicobacter suis* and *Helicobacter pylori* infections

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Abstract

Helicobacter (*H.*) *suis* can colonize the stomach of pigs as well as humans, causing chronic gastritis and other gastric pathological changes including gastric ulceration and mucosa-associated lymphoid tissue (MALT) lymphoma. Recently, a virulence factor of *H. suis*, γ-glutamyl transpeptidase (GGT), has been demonstrated to play an important role in the induction of human gastric epithelial cell death and modulation of lymphocyte proliferation depending on glutamine and glutathione catabolism. In the present study, the relevance of GGT in the pathogenesis of *H. suis* infection was studied in mouse and Mongolian gerbil models. In addition, the relative importance of *H. suis* GGT was compared with that of the *H. pylori* GGT. A significant and different contribution of the GGT of *H. suis* and *H. pylori* was seen in terms of bacterial colonization, inflammation and the evoked immune response. In contrast to *H. pylori*Δ*ggt* strains, *H. suis*Δ*ggt* strains were capable of colonizing the stomach at levels comparable to WT strains, although they induced significantly less overall gastric inflammation in mice. This was characterized by lower numbers of T and B cells, and a lower level of epithelial cell proliferation. In general, compared to WT strain infection, *ggt* mutant strains of *H. suis* triggered lower levels of Th1 and Th17 signature cytokine expression. A pronounced upregulation of B-lymphocyte chemoattractant CXCL13 was observed, both in animals infected with WT and *ggt* mutant strains of *H. suis*. Interestingly, *H. suis* GGT was shown to affect the glutamine metabolism of gastric epithelium through downregulation of the glutamine transporter ASCT2.

Introduction

Helicobacter (*H.*) *pylori* is a Gram-negative bacterium that colonizes the stomach of more than half of the world's population. Infection with this bacterium can cause gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Parsonnet *et al.*, 1991, Huang *et al.*, 1998, Ernst *et al.*, 2000). Besides *H. pylori*, non-*H. pylori* helicobacters (NHPH) have also been detected in the stomach of humans and these bacteria cause similar gastric diseases. The risk of developing gastric MALT lymphoma is higher during NHPH infection compared to infection with *H. pylori* (Lee *et al.*, 1989, Trebesius *et al.*, 2001, O'Rourke *et al.*, 2004, Haesebrouck *et al.*, 2009, Flahou *et al.*, 2010, Joosten *et al.*, 2013b). *H. suis* is the most prevalent gastric NHPH in humans. Pigs are the natural host of this bacterium, with prevalences reaching 90% or more (Hellemans *et al.*, 2007) and most likely, pigs and possibly also pork are the main sources of human *H. suis* infection (Van den Bulck *et al.*, 2005, Flahou *et al.*, 2010, De Cooman *et al.*, 2013, De Cooman *et al.*, 2014).

H. suis infection seems to persist for life, at least in pigs and rodents used as models for human infections (Yamamoto *et al.*, 2014). In pigs, infection causes development of gastritis and a decrease in body weight gain. Moreover, the bacterium seems to play a role in the development of ulceration of the non-glandular pars oesophagea (De Bruyne *et al.*, 2012). In mice and Mongolian gerbil models of human gastric disease, experimental *H. suis* infection causes severe gastric pathology (O'Rourke *et al.*, 2003, Rogers *et al.*, 2004, Flahou *et al.*, 2010), including gastritis, parietal cell necrosis and the development of gastric MALT lymphoma-like lesions, resembling the lesions observed in *H. suis*-infected humans.

Previous studies have shown that this bacterium lacks a homologue for several virulence factors of *H. pylori*, such as the *cytotoxin associated genes* pathogenicity island (*cag*PAI) and the vacuolating cytotoxin (VacA) (Vermoote *et al.*, 2011). We were, however, capable of identifying the γ-glutamyl transpeptidase (GGT) as an important virulence factor of *H. suis*. This enzyme has been described to cause gastric epithelial cell damage (Flahou *et al.*, 2011) and modulation of lymphocyte proliferation (Zhang *et al.*, 2013) through the interaction of the enzyme with two of its substrates, L-glutamine and reduced glutathione, making it the first identified and investigated *H. suis* virulence determinant.

The role of GGT during *H. pylori* infection *in vivo* has been investigated in mice. Conflicting conclusions have been drawn regarding the importance of GGT for colonization. Some groups

have concluded that *H. pylori* GGT is required for persistent infection in mice (Chevalier *et al.*, 1999), while others have made contrary conclusions (McGovern *et al.*, 2001). In addition, there is accumulating evidence that *Helicobacter* GGT is a crucial virulence factor involved in immune evasion and immune tolerance (Schmees *et al.*, 2007, Oertli *et al.*, 2013, Salama *et al.*, 2013).

Currently, it is unknown if and how *H. suis* GGT influences the course of *H. suis* infection *in vivo*. The aim of the present study was to extend our previous *in vitro* findings with *H. suis* GGT, and to study the role of this virulence factor in the pathogenesis of *H. suis* infection *in vivo*. At the same time, we aimed at comparing its relative importance with that of the GGT of *H. pylori*. The current experiments were performed in BALB/c mice and outbred Mongolian gerbils, since these animal models have indeed been shown to be valuable tools to investigate the role of *Helicobacter* species in gastric pathology. Typically, in Mongolian gerbils, a more rapid and severe development of gastric lesions can be observed compared to mice (Wiedemann *et al.*, 2009, Flahou *et al.*, 2010, Joosten *et al.*, 2013a).

Material and methods

Animal and bacterial strains

Sixty 4-week-old, female specific-pathogen-free (SPF) BALB/c mice were purchased from Harlan NL (Horst, The Netherlands). Twenty-five 4-week-old, female SPF outbred Mongolian gerbils (Crl:MON) were obtained from Charles River Laboratories (Lille, France).

For *H. suis* infection in mice and Mongolian gerbils, strain HS5cLP was used. This strain has been isolated in 2008 from the stomach of a slaughterhouse pig (Baele *et al.*, 2008). For experimental *H. pylori* infection in Mongolian gerbils, strain PMSS1 (Flahou *et al.*, 2012) was used, since this strain has no history of *in vivo* adaptation in mice, in contrast to the mouse-adapted strain SS1. In BALB/c mice, *H. pylori* strain SS1 (Flahou *et al.*, 2012) was used, since strain PMSS1 has previously been demonstrated not to be able to colonize the stomach of BALB/c mice (Flahou *et al.*, 2012).

Construction of isogenic *ggt* mutant strains of *H. suis* and *H. pylori*

An isogenic *H. suis ggt* mutant strain (HS5cLPΔ*ggt*) was prepared as described previously (Zhang *et al.*, 2013). The isogenic *ggt* mutant strain of *H. pylori* was obtained using the same strategy as for creation of the *H. suis* isogenic *ggt* mutant, except that a kanamycin resistance

cassette was used instead of a chloramphenicol resistance cassette (Zhang *et al.*, 2013). Very briefly, deletion of *ggt* in *H. pylori* SS1 and PMSS1 was introduced by allelic exchange using pBluescript II SK (+) phagemid vector (Agilent Technologies, California, USA) in which ~440 bp of the 5′ –end and ~430 bp of the 3′ –end of the target gene and the kanamycin resistance cassette from plasmid pKD4 (Van Parys *et al.*, 2012) were ligated through a PCR-mediated strategy with 2 cycles of inverse PCR and fusion PCR (Zhang *et al.*, 2013). All primers used for PCR-mediated construction of the recombinant plasmids are shown in Table 1. The resultant plasmid was amplified in XL1-Blue MRF′ *E. coli* (Agilent Technologies) and used as a suicide plasmid in *H. pylori* SS1 and PMSS1 (a kind gift from Sara Lindén and Anne Muller, respectively). *H. pylori* SS1 *ggt* mutant (SS1Δ*ggt*) and *H. pylori* PMSS1 *ggt* mutant (PMSS1Δ*ggt*) were obtained by electrotransformation (Ferrero *et al.*, 1992) or natural transformation (Wang *et al.*, 1993) as described previously. Finally, bacteria were selected on columbia agar plates (Oxoid, Basingstoke, UK) with Vitox supplement (Oxoid), 5% (v/v) defibrinated sheep blood (E&O Laboratories Ltd, Bonnybridge, UK), and kanamycin (25 μg/ml). The plates were incubated for 5-9 days. The isogenic *ggt* mutants were verified by a GGT activity assay (Flahou *et al.*, 2011), PCR and nucleotide sequencing.

Table 1 Primers used for construction of the *H. pylori ggt* isogenic mutant strains.

Primer name	Sequence (5′- 3′)	Primer use
pBlue linear Fwd 1	GGGGATCCACTAGTTCTAGAGCG	Linearization of plasmid
pBlue linear Rev1	CGGGCTGCAGGAATTCGATATCAAG	Linearization of plasmid
HpGGT-flank_fusion1F	CTTGATATCGAATTCCTGCAGCCCGT AACCGGTAAAATCAACACGGACGC	Amplification *H. pylori ggt* and partial up- and downstream flanking genes
HpGGT-flank_fusion1R	CGCTCTAGAACTAGTGGATCCCCGC GCTCTTATAAAAAGAAGCCGC	Amplification *H. pylori ggt* and partial up- and downstream flanking genes
pBluelinear_Hpggtflank1F	CCAAGGAAAGAATTTTAATCCTATTT AG	Linearization of the recombinant plasmid
pBluelinear_Hpggtflank1R	CTGTTTTCCTTTCAATCAACAATAAT C	Linearization of the recombinant plasmid
Hpkana_fusion_1F	ATTATTGTTGATTGAAAGGAAAACA GATGATTGAACAAGATGGATTGC	Amplification kanamycin resistance gene
Hpkana_fusion_1R	CTAAATAGGATTAAAATTCTTTCCTT GGTCAGAAGAACTCGTCAAGAAG	Amplification kanamycin resistance gene
T7 prom3	TAATACGACTCACTATAGGG	Sequencing
M13R	CAGGAAACAGCTATGAC	Sequencing

Culture conditions of bacterial strains

Wild-type (WT) *H. suis* strain HS5cLP was grown for 48 hours as described previously (Flahou *et al.*, 2012). HS5cLPΔ*ggt* bacteria were grown under the same conditions as strain

HS5cLP, except that the cultivation plates were supplemented with chloramphenicol (30 µg/ml) as described previously (Zhang *et al.*, 2013).

WT *H. pylori* strains SS1 and PMSS1 were grown on Columbia agar plates containing 5% (v/v) defibrinated sheep blood for 48-72 hours at 37°C under microaerobic conditions as described previously (Flahou *et al.*, 2012). Subsequently, colonies were picked up and cultured in Brucella broth supplemented with Vitox (Oxoid) and 5% fetal calf serum (HyClone) on a rotational shaker under microaerobic conditions (16 hours, 125 rpm). SS1Δ*ggt* and PMSS1Δ*ggt* strains were cultured under the same conditions as the corresponding WT strains on plates supplemented with kanamycin (25 µg/ml).

Experimental design

Upon arrival, sixty BALB/C mice and twenty-five Mongolian gerbils were divided into 5 groups, and the animals were allowed to acclimate to the new environment for 1 week. Animals were inoculated intragastrically 3 times at 48 hours intervals. Animals from group 1 and 2 (both mice and Mongolian gerbils) were inoculated with Brucella broth containing 8×10^7 viable bacteria of strains HS5cLP and HS5cLPΔ*ggt*, respectively. Animals in group 3 and 4 were inoculated with Brucella broth containing 3×10^8 viable bacteria of strains SS1 and SS1Δ*ggt* (mice) or 1×10^9 viable bacteria of strains PMSS1 and PMSS1Δ*ggt* (gerbils). Animals in the fifth group were inoculated with Brucella broth and served as uninfected controls. For mice, at 4 weeks, 9 weeks and 6 months p.i., 4 animals from each group were euthanized by cervical dislocation under isoflurane anaesthesia. For Mongolian gerbils, all animals were sacrificed at 9 weeks p.i.. The stomachs of the animals were resected for further processing as described previously (Flahou *et al.*, 2012, Joosten *et al.*, 2013a).

Animal experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2013/29).

Histopathological examination and immunohistochemistry (IHC)

Three longitudinal strips of gastric tissue from mice and Mongolian gerbils were cut from the oesophagus to the duodenum along the greater curvature. Tissue was fixed in 4% phosphate buffered formaldehyde, processed by standard methods and embedded in paraffin for light microscopy. Five serial sections of 5 µm were cut. The first section was stained with haematoxylin/eosin (H&E) to score the degree of gastritis according to the Updated Sydney System with some modifications (Stolte *et al.*, 2001). After deparaffinization and rehydration for the remaining sections, heat-induced antigen retrieval was performed in citrate buffer

(pH=6.0). In order to block endogenous peroxidase activity and non-specific reactions, all the slides were incubated with 3% H_2O_2 in methanol (5 min) and 30% goat serum (30 min), respectively. For the differentiation between T and B lymphocytes, CD3 and CD20 antigens were stained on sections two and three, using a polyclonal rabbit anti-CD3 antibody (1/100; DakoCytomation, Glostrup, Denmark) and a polyclonal rabbit anti-CD20 antibody (1/25; Thermo Scientific, Fremont, USA), respectively. These sections were further processed with Envision+System-HPR (DAB) (DakoCytomation) for use with the rabbit primary antibodies. On the fourth and fifth sections, epithelial cell proliferation and the number of parietal cells were determined by IHC staining, using a mouse monoclonal anti-Ki67 antibody (1/25; Menarini Diagnostics, Zaventem, Belgium) and mouse monoclonal anti-hydrogen potassium ATPase β-subunit (H^+/K^+ ATPase) antibody (1/25000; Abcam Ltd, Cambridge, UK), respectively. Subsequent visualization was done with Envision+System-HPR (DAB) (DakoCytomation) for use with the mouse primary antibodies. Quantification of T cells, B cells and epithelial cells were performed as described previously (Flahou *et al.*, 2010). Briefly, the numbers of cells belonging to defined cell populations (T cells, B cells, and epithelial cells) were determined by counting the positive cells in five randomly chosen High Power Fields (magnification: × 400), both in the antrum and corpus region.

In order to assess the possible development of pseudopyloric metaplasia induced by *Helicobacter* infection, alcian blue-periodic acid-schiff stain staining (AB/PAS) was performed.

Quantification of colonizing bacteria in the stomach of mice and Mongolian gerbils

Strips of gastric tissue containing all regions for mice and separate pieces (antrum and corpus) for Mongolian gerbils were stored in 0.5 mL RNA*later* solution (Ambion, Austin, TE, USA) at -70 °C until RNA and DNA extraction. Quantitative Real-Time PCR (qRT-PCR) was used to determine the number of colonizing bacteria in the gastric tissue as described previously (Flahou *et al.*, 2012, Blaecher *et al.*, 2013).

RNA extraction and reverse transcription

qRT-PCR was used to determine gene expression in the gastric tissue from mice and Mongolian gerbils. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of RNA was measured using a NanoDrop spectrophotometer (Isogen Life Science, PW De Meern, Utrecht, The Netherlands). The purity of the RNA was evaluated with the Experion automated

electrophoresis system using StdSens RNA chips (Bio-Rad, Hercules CA, USA). The RNA concentration from all samples was adjusted to 1 µg/µL and cDNA was synthesized immediately after RNA purification using the iScript™ cDNA Synthesis Kit (Bio-Rad).

Design and validation of primers and determination of gene expression

The housekeeping genes *H2afz, PPIA* and *HPRT* were included as reference genes for mice (Flahou *et al.*, 2012). For Mongolian gerbils, a set of reference genes was tested based on the fact that they are extensively used in other animal species. Primers were designed based on the conserved regions of *ACTB, β-actin, RPS18*, *GAPDH*, *HPRT1*, *SDHA* and *UBC* complete or partial coding sequences available for humans, pigs, mice and rats.

Table 2 List of genes and primers used for qRT-PCR in Mongolian gerbils.

Genes	Primer	Sequence (5′- 3′)	References
Foxp3	sense	GCCCCTMGTCATGGTGGCA	This study
	antisense	CCGGGCCTTGAGGGAGAAGA	
CXCL13	sense	GAATGGCTGCCCCAAAACTGAA	This study
	antisense	TCACTGGAGCTTGGGGAGTTGAA	
GAPDH	sense	AACGGGAAGCTCACTGGCATG	This study
	antisense	CTGCTTCACCACCTTCTTGATGTCA	
HPRT1	sense	GCCCCAAAATGGTTAAGGTTGCA	This study
	antisense	TCAAGGGCATATCCAACAACAAAC	
RPS18	sense	CGAGTACTCAACACCAACATCGATGG	This study
	antisense	ATGTCTGCTTTCCTCAACACCACATG	
IL-1β	sense	GGCAGGTGGTATCGCTCATC	(Sugimoto *et al.*, 2009)
	antisense	CACCTTGGATTTGACTTCTA	
IFN-γ	sense	CCATGAACGCTACACACTGCATC	(Crabtree *et al.*, 2004)
	antisense	GAAGTAGAAAGAGACAATCTGG	
IL-5	sense	AGAGAAGTGTGGCGAGGAGAGACG	(Joosten *et al.*, 2013a)
	antisense	ACAGGGCAATCCCTTCATCGG	
IL-6	sense	GAGGTGAAGGATCCAGGTCA	(Sugimoto *et al.*, 2011)
	antisense	GAGGAATGTCCTCAGCTTGG	
IL-10	sense	GGTTGCCAAGCCTTATCAGA	(Joosten *et al.*, 2013a)
	antisense	GCTGCATTCTGAGGGTCTTC	
IL-17	sense	AGCTCCAGAGGCCCTCGGAC	(Sugimoto *et al.*, 2009)
	antisense	AGGACCAGGATCTCTTGCTG	
ATP4b	sense	GGGGGTAACCTTGAGACCTGATG	(Joosten *et al.*, 2013a)
	antisense	AAGAAGTACCTTTCCGACGTGCAG	
β-actin	sense	TCCTCCCTGGAGAAGAGCTA	(Sugimoto *et al.*, 2011)
	antisense	CCAGACAGCACTGTGTTGGC	

The mRNA expression levels of various cytokines (IFN-γ, IL-4, IL-5, IL-17, IL-1β, IL-6, IL-10), previously shown to be differentially expressed during *H. suis* infection, as well as other genes (Foxp3, CXCL13, ASCT2, ATP4a, and ATP4b) were quantified using SYBR

Green based RT-PCR with iQ^{TM} SYBR Green Supermix. Reactions were performed using a CFX96 RT PCR System in a C1000 Thermal Cycler (Bio-Rad) as described previously (Flahou *et al.*, 2012). All reactions were performed in 12 µL volumes containing 0.05 µL of each primer (1.25 pmol/ µL), 6 µL iQ^{TM} SYBR Green Supermix, 3.9 µL HPLC water and 2 µL cDNA. The experimental program consisted of 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30s, and extension at 72°C for 30s. The threshold cycle values (Ct) were normalized to the geometric means of the reference genes and the normalized mRNA levels of all target genes were calculated using the method of $2^{-\Delta\Delta Ct}$ (Livak *et al.*, 2001).

Due to the unavailability of gene information for Forkhead/winged helix transcription factor (Foxp3) and the chemokine CXC ligand 13 (CXCL13) from Mongolian gerbils, primers were designed based on the conserved regions of Foxp3 and CXCL13 complete or partial coding sequences available for humans, pigs, mice and rats with the same strategy as described above.

The mRNA expression levels of Foxp3 and CXCL13 were determined using the same method as described above. Sequence information of all the primers for mice and for Mongolian gerbils is shown in Tables 2-3.

Table 3 List of genes and primers used for qRT-PCR in mice.

Gene	Primer	Sequence (5′- 3′)	Reference
IL-1β	sense	GGGCCTCAA AGGAAAGAATC	(Flahou *et al.*, 2012)
	antisense	TACCAGTTGGGGAACTCTGC	
IFN-γ	sense	GCGTCATTGAATCACACCTG	(Flahou *et al.*, 2012)
	antisense	TGAGCTCATTGAATGCTTGG	
IL-4	sense	ACTCTTTCGGGCTTTTCGAT	(Flahou *et al.*, 2012)
	antisense	AAAAATTCATAAGTTAAAGCATGGTG	
IL-10	sense	ATCGATTTCTCCCCTGTGAA	(Flahou *et al.*, 2012)
	antisense	CACACTGCAGGTGTTTTAGCTT	
IL-17	sense	TTTAACTCCCTTGGCGCAAAA	(Flahou *et al.*, 2012)
	antisense	CTTTCCCTCCGCATTGACAC	
Foxp3	sense	GCCCCTMGTCATGGTGGCA	This study
	antisense	CCGGGCCTTGAGGGAGAAGA	
CXCL13	sense	CTCTCCAGGCCACGGTATT	(Lee *et al.*, 2009)
	antisense	TAACCATTTGGCACGAGGAT	
ATP4a	sense	TGCTGCTATCTGCCTCATTG	(Jain *et al.*, 2006)
	antisense	GTGCTCTTGAACTCCTGGTAG	
ATP4b	sense	AACAGAATTGTCAAGTTCCTC	(Jain *et al.*, 2006)
	antisense	AGACTGAAGGTGCCATTG	
HPRT	sense	CAGGCCAGACTTTGTTGGAT	(Flahou *et al.*, 2012)
	antisense	TTGCGCTCATCTTAGGCTTT	
PPIA	sense	AGCATACAGGTCCTGGCATC	(Flahou *et al.*, 2012)
	antisense	TTCACCTTCCCAAAGACCAC	
H2afz	sense	CGTATCACCCCTCGTCACTT	(Flahou *et al.*, 2012)
	antisense	TCAGCGATTTGTGGATGTGT	

Statistical analysis

Differences in colonization capacity were analyzed using a non-parametric Mann-Whitney *U* test. Differences in lymphocytic infiltration, cytokine expression and IHC analysis were assessed with one-way ANOVA followed by a Bonferroni post hoc test. Statistical analyses were performed using SPSS Statistics 20 software (IBM). Pair-wise comparisons were done for each individual time-point and on pooled data using time as stratification factor. *P* values less than 0.05 were considered statistically significant. All data are expressed as mean $\pm$ SD. All the figures were created using GraphPad Prism5 software (GraphPad Software Inc., San Diego, CA).

Results

Colonization density

All control animals were negative for *Helicobacter*. Results of infected animals showed that WT *H. suis* can persistently colonize the mouse stomach with colonization levels as high as 5.42×10^4 ($\pm$ 1.46×10^4) bacteria/mg gastric tissue even at 6 months p.i. (Figure 1C). *H. pylori* strain SS1 was shown to colonize the mouse stomach at a much lower bacterial density, being 1.68×10^3 ($\pm$ 1.73×10^3) bacteria/mg tissue at 6 months p.i. (Figure 1C, $p<0.05$).

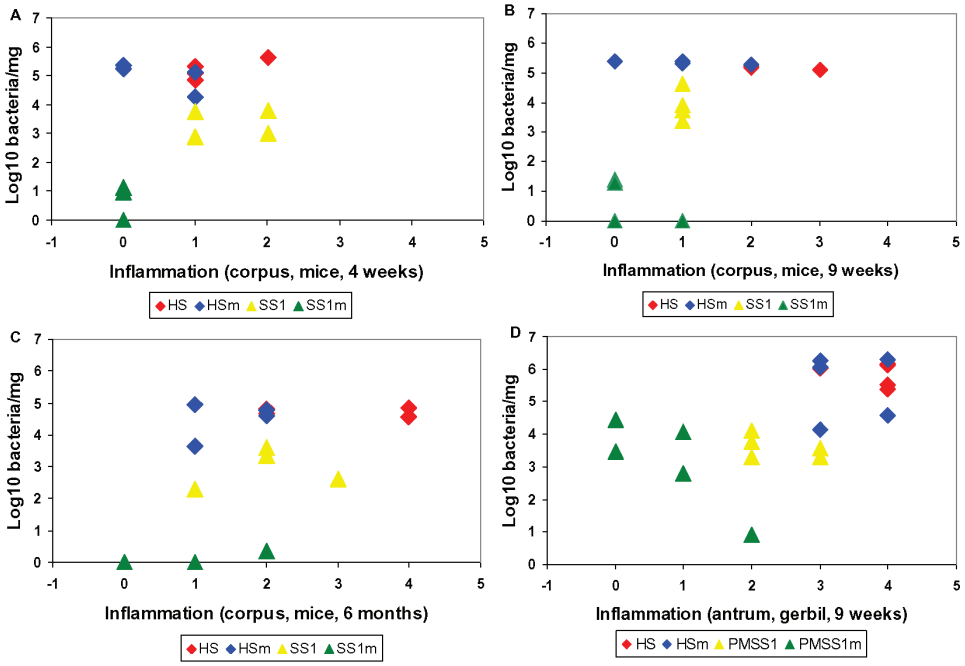

Figure 1 Correlation between bacterial colonization capacity and inflammation score in the stomach of mice and Mongolian gerbils.
The colonization capacity is shown as log10 values of *H. suis* or *H. pylori* per mg tissue, determined with qRT-PCR in the corpus of mice (Figure 1A-1C) and antrum of Mongolian gerbils (Figure 1D). 0, no infiltration with mononuclear and/or polymorphonuclear cells; 1, very mild diffuse infiltration with mononuclear and/or polymorphonuclear cells or the presence of one small (20-50 cells) aggregate of inflammatory cells; 2, mild diffuse infiltration with mononuclear and/or polymorphonuclear cells or the presence of one small (50-200 cells) aggregate of inflammatory cells; 3, moderate diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of 2-4 inflammatory aggregates; 4, marked diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of at least five inflammatory aggregates. HS vs. HSm: Colonization: $p > 0.05$; Inflammation: $p < 0.05$. SS1 vs. SS1m: Colonization: $p < 0.05$; Inflammation: $p < 0.05$. PMSS1 vs. PMSS1m: Colonization: $p > 0.05$; Inflammation: $p < 0.05$. HS: animals infected with WT *H. suis* srain HS5cLP; HSm: animals infected with *H. suis* strain HS5cLPΔ*ggt*; SS1: animals infected with WT *H. pylori* SS1; SS1m: animals infected with *H. pylori* SS1Δ*ggt*; PMSS1: animals infected with WT *H. pylori* PMSS1; PMSS1m: animals infected with *H. pylori* PMSS1Δ*ggt*.

Interestingly, *H. suis* strain HS5cLPΔ*ggt* was able to colonize the corpus of the stomach of the mice to a similar extent as the WT strain, and this was observed for all timepoints (Figure 1A-1C). In contrast, *H. pylori* strain SS1Δ*ggt* was shown to have an impaired colonization capacity in mice at all three timepoints (Figure 1A-C, $p<0.05$). Similar colonization data were demonstrated in the antrum of *Helicobacter* infected-mice at all three timepoints (data not shown).

Both the HS5cLP and HS5cLPΔ*ggt* strain successfully colonized the antrum and corpus of the stomach of Mongolian gerbils, although colonization rates were much lower in the corpus compared to the antrum. No statistically significant differences were observed between both strains (Figure 1D, $p>0.05$). *H. pylori* strain PMSS1Δ*ggt* was able to colonize the antrum and corpus of the stomach at similar levels compared to PMSS1 (Figure 1D, $p>0.05$), although 2 out of 5 Mongolian gerbils were negative for the presence of PMSS1Δ*ggt* in the corpus of the stomach (data not shown).

Infection-induced inflammation

All control mice and gerbils showed normal gastric histomorphology at all timepoints. The correlation between inflammation scores and bacterial colonization is displayed in Figure 1.

Compared to mice with WT strain infection, infection with *H. suis* strain HS5cLPΔ*ggt* generally induced significantly less overall inflammation both in the antrum ($p<0.01$) and corpus ($p<0.01$), whereas only in the corpus region ($p<0.01$), infection with *H. pylori* strain SS1Δ*ggt* induced less inflammation, compared to that seen in WT strain infected mice. At 6 months p.i., the corpus region in 2 out of 4 mice with HS5cLP infection contained large lymphoid aggregates or lymphoid follicles accompanied by destruction of the normal mucosal architecture (Figure 2A), which was not observed in animals from other groups.

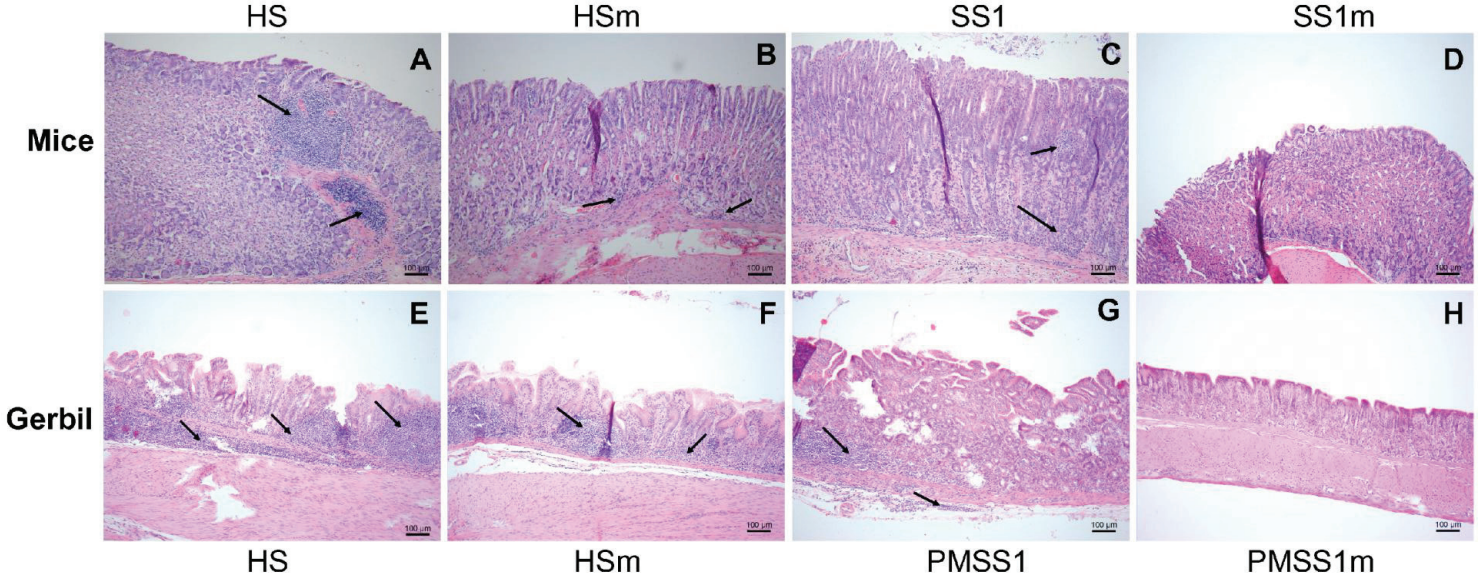

Figure 2 H&E staining of stomach sections from *Helicobacter*-infected mice and Mongolian gerbils.
Representative micrographs of H&E stained sections shown here were taken from mice orally inoculated with *H. suis* (A), *H. suis*Δ*ggt* (B), *H. pylori* SS1 (C) and *H. pylori* SS1Δ*ggt* (D) at 6 months post inoculation and Mongolian gerbils orally challenged with *H. suis* (E), *H. suis*Δ*ggt* (F), *H. pylori* PMSS1 (G) and *H. pylori* PMSS1Δ*ggt* (H) at 9 weeks post inoculation. Arrows indicate the presence of inflammatory cells, inflammatory aggregates, lymphocytic infiltration, or lymphocytic follicles. HS: animals infected with WT *H. suis* strain HS5cLP; HSm: animals infected with *H. suis* strain HS5cLPΔ*ggt*; SS1: animals infected with WT *H. pylori* SS1; SS1m: animals infected with *H. pylori* SS1Δ*ggt*; PMSS1: animals infected with WT *H. pylori* PMSS1; PMSS1m: animals infected with *H. pylori* PMSS1Δ*ggt*; WT: wild-type. Original magnification: 100×.

For Mongolian gerbils, infection with HS5cLP or PMSS1 induced severe antrum-dominant gastritis with formation of lymphocytic aggregates in the lamina propria and/or sub-mucosa of the stomach (Figure 1D, Figure 2E, 2G). No significant differences were observed between the WT and mutant strain of *H. suis* with respect to the inflammatory response induced in gerbils, although all animals infected with strain HS5cLP showed inflammation in the corpus region, whereas this was only the case for some animals infected with HS5cLPΔ*ggt* (Figure 1D, Figure 2E-2F). In one gerbil infected with *H. suis* strain HS5cLP, a pronounced inflammatory response was observed, in which more than 65% of the area in the lamina propria and submucosa of the antrum was densely infiltrated with inflammatory cells, fused lymphoid aggregates and lymphoid follicles (Additional file 1).
Inflammation induced by *H. pylori* strain PMSS1Δ*ggt* in the antrum of gerbils was less severe compared to that seen in WT infected animals ($p<0.05$) (Figure 1D, Figure 2G-2H).

Inflammatory cell infiltration

In general, an increase in T cell numbers was observed in the corpus (Figure 3A, $p<0.05$) of mice infected with *H. suis* strain HS5cLP and *H. pylori* strain SS1 at all three timepoints. Compared to the mice infected with WT *H. suis*, HS5cLPΔ*ggt* induced a lower T cell response in the corpus at 6 months p.i. ($p<0.01$). *H. pylori* strain SS1Δ*ggt* induced a reduced T cell response in the corpus region ($p<0.01$) compared to WT infected animals, at both 9 weeks and 6 months p.i. (Figure 3A). Similar results were observed in the antrum of mice (data not shown).
An increase in B cell numbers was observed in the corpus mucosa of mice infected with strain HS5cLP ($p<0.01$) and SS1 ($p<0.01$) at 6 months p.i. (Figure 3B). Compared to the WT *H. suis* infected mice, HS5cLPΔ*ggt* induced a lower B cell response in the corpus region of mice at 6 months p.i. ($p<0.05$) and a similar reduction was observed in SS1Δ*ggt* infected mice ($p<0.01$) (Figure 3B).

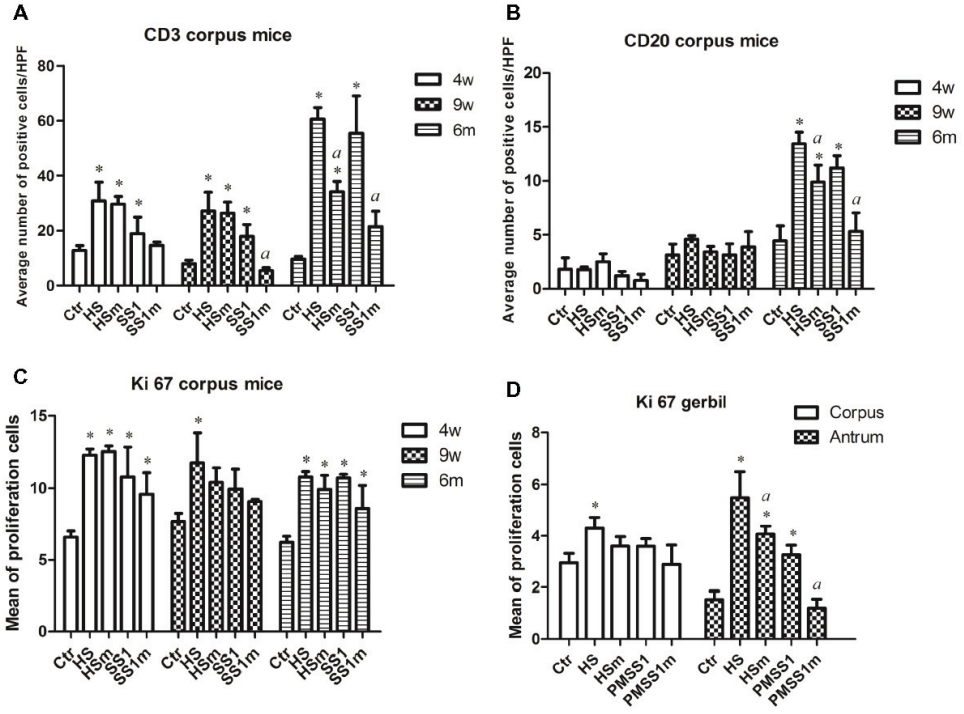

Figure 3 Quantitative analysis of defined cell populations with immunohistochemistry.
(A-B) Shown are the average (± SD) numbers of cells/ High Power Field, including T cells (CD3-positive) and B cells (CD20-positive) in the corpus of the stomach of mice. (C-D) Shown are the average (± SD) numbers of epithelial cells in five randomly chosen microscopic fields at the level of the gastric pits in the stomach from mice and Mongolian gerbils. An * represents a statistically significant difference ($p < 0.05$) between infected and control groups. An *a* represents a statistically significant difference ($p < 0.05$) between WT *Helicobacter* infected groups and isogenic *ggt* mutant infected groups. Ctr: animals from control group; HS: animals infected with WT *H. suis* strain HS5cLP; HSm: animals infected with *H. suis* strain HS5cLPΔ*ggt*; SS1: animals infected with WT *H. pylori* SS1; SS1m: animals infected with *H. pylori* SS1Δ*ggt*; PMSS1: animals infected with WT *H. pylori* PMSS1; PMSS1m: animals infected with *H. pylori* PMSS1Δ*ggt*; WT: wild-type; 3w: 3 weeks post infection; 9w: 9 weeks post infection; 6m: 6 months post infection.

For Mongolian gerbils, an exact quantification of T and B lymphocytes was not performed since the inflammation was characterized by a marked diffuse infiltration with large numbers of lymphocytes and large inflammatory aggregates. Histopathological analysis showed a pronounced increase of T cell numbers as well as lymphocytic aggregates and follicles in the lamina propria and tunica submucosa in all groups (Figure 4A-4D), although this was most pronounced in the antrum of both WT and mutant *H. suis* infected animals (Figure 4A-4B). T cell infiltration levels induced by PMSS1Δ*ggt* infection were lower compared to that seen in WT *H. pylori* infected animals (Figure 4C-4D).

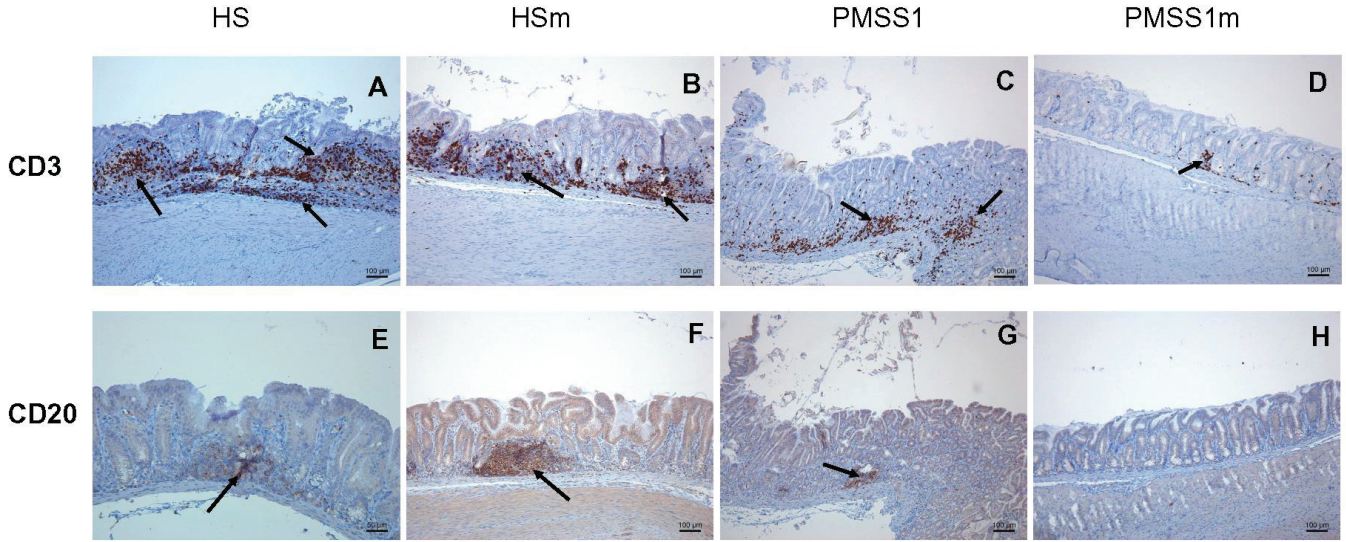

Figure 4 Gastric inflammation of *Helicobacter*-infected Mongolian gerbils.
CD3 staining of the antrum of the stomach from Mongolian gerbils inoculated with *H. suis* (A), *H. suis*Δ*ggt* (B), *H. pylori* PMSS1 (C) and *H. pylori* PMSS1Δ*ggt* (D) at 9 weeks post inoculation, showing T-lymphocytes (brown). CD20 staining of the antrum of a gerbil infected with WT *H. suis* (E), *H. suis*Δ*ggt* (F), WT *H. pylori* PMSS1 (G) and *H. pylori* PMSS1Δ*ggt* (H) at 9 weeks post inoculation, showing B lymphocytes (brown) in germinal centers of lymphoid follicles (arrows) or lymphoid aggregates (arrows). HS: animals infected with WT *H. suis* strain HS5cLP; HSm: animals infected with *H. suis* strain HS5cLPΔ*ggt*; PMSS1: animals infected with WT *H. pylori* PMSS1; PMSS1m: animals infected with *H. pylori* PMSS1Δ*ggt*; WT: wild-type. Original magnification: 100×.

WT and mutant strains of *H. suis* induced similar levels of B cell infiltration, mainly in the centre of lymphocytic aggregates/follicles in the antrum (Figure 4E-4F). WT *H. pylori* induced mild B cell infiltration in the antrum of gerbils, whereas animals with PMSS1Δ*ggt* infection did not show an obvious B cell infiltration (Figure 4G-4H). A marked proliferation of B cells in germinal centers was observed in gerbils infected with *H. suis* strains HS5cLP (Additional file 4A) and HS5cLPΔ*ggt* (Additional file 4B) but not in *H. pylori* infected animals.

Epithelial cell-related changes

For mice, IHC staining did not reveal a clear decrease of the number of parietal cells in the stomach, except for mice infected with *H. suis* strain HS5cLP for 6 months ($p<0.05$). For Mongolian gerbils, a clear loss of parietal cells was only observed in the transition zone between corpus and antrum in *H. suis* strain HS5cLP (Additional file 2B) and HS5cLPΔ*ggt* (Additional file 2C) infected animals, but not in *H. pylori* PMSS1 or PMSS1Δ*ggt* infected animals (Additional file 2D and 2E).

Data on gastric epithelial cell proliferation in the corpus region are summarized in Figure 3C-3D. Compared to control mice, an increased epithelial cell proliferation was seen in the corpus (Figure 3C, $p<0.05$) of HS5cLP infected mice at all timepoints, and a similar increase was observed for SS1 infected mice (Figure 3C, $p<0.05$). In general, mice infected with *H. suis* and *H. pylori* strains mutated for the GGT revealed somewhat lower epithelial cell proliferation rates compared to WT strain infected mice (Figure 4C), which was, however, not statistically significant. Compared to WT strain infected Mongolian gerbils, both HS5cLPΔ*ggt* ($p<0.05$) and PMSS1Δ*ggt* ($p<0.01$) infected animals revealed a significantly lower level of epithelial cell proliferation in the antrum (Figure 3D).

AB/PAS staining showed that *H. suis* infection triggered the development of pseudopyloric metaplasia to a varying degree in the corpus region of mice at 6 months p.i. (Additional file 3B-3C). Compared to WT *H. suis* infection, infection with HS5cLPΔ*ggt* in general led to less obvious regions affected by pseudopyloric metaplasia. Infection with WT *H. pylori* also induced pseudopyloric metaplasia to a varying degree in the corpus region of mice at 6 months p.i. (Additional file 3D), whereas strain SS1Δ*ggt* did not (Additional file 3E).

Cytokine secretion in response to bacterial infection

Data on gene expression levels are presented in Figure 5-6.

Primers for housekeeping genes of Mongolian gerbils were chosen based on the specificity and amplification efficiency of the primers, and stable expression levels of the genes. *β-actin*, *RPS18*, *GAPDH* and *HPRT1* were included as the final reference genes for qRT-PCR performed in gerbils.

IFN-γ and IL-1β

In general, only *H. pylori* strain SS1 infection induced a significant up-regulation of the Th1 signature cytokine IFN-γ in mice (Figure 5A, $p<0.05$). WT and mutant strains of *H. suis* ($p<0.01$) and *H. pylori* ($p<0.01$) induced a pronounced upregulation of IFN-γ expression in the antrum of infected Mongolian gerbils (Figure 5C), and no significant differences were observed between WT infected- and mutant infected animals.

No significant differences of IL-1β expression were observed between groups (data not shown). In Mongolian gerbils, similarly increased expression levels of IL-1β were seen in animals infected with WT and mutant strains of *H. suis* (data not shown).

IL-4, IL-5, IL-6, IL-10

In general, compared to control mice, the expression of anti-inflammatory IL-10 was upregulated in *H. suis* strain HS5cLP and HS5cLPΔ*ggt* infected mice (Figure 5A, $p<0.01$). A very similar expression pattern was observed for Foxp3 (Figure 5B, $p<0.01$), an important cell marker of $CD4^+/CD25^+$ regulatory T cells (Tregs), which are one of the most important cell types secreting IL-10 (Josefowicz *et al.*, 2012).

In Mongolian gerbils, a clear increase of IL-10 expression, compared to control animals, was demonstrated both in the antrum of gerbils infected with strain HS5cLP ($p<0.01$) and strain HS5cLPΔ*ggt* (Figure 5C, $p<0.01$). Compared to control animals, no significant changes of IL-10 and Foxp3 expression levels were observed in animals infected with *H. pylori* (Figure 5A, 5C).

Compared to control animals, an upregulation of IL-6 expression was only demonstrated in gerbils with HS5cLP and HS5cLPΔ*ggt* infection, but no difference was observed between both groups (data not shown). No significant differences in expression between control animals and infected animals could be demonstrated for IL-4 and IL-5 (data not shown).

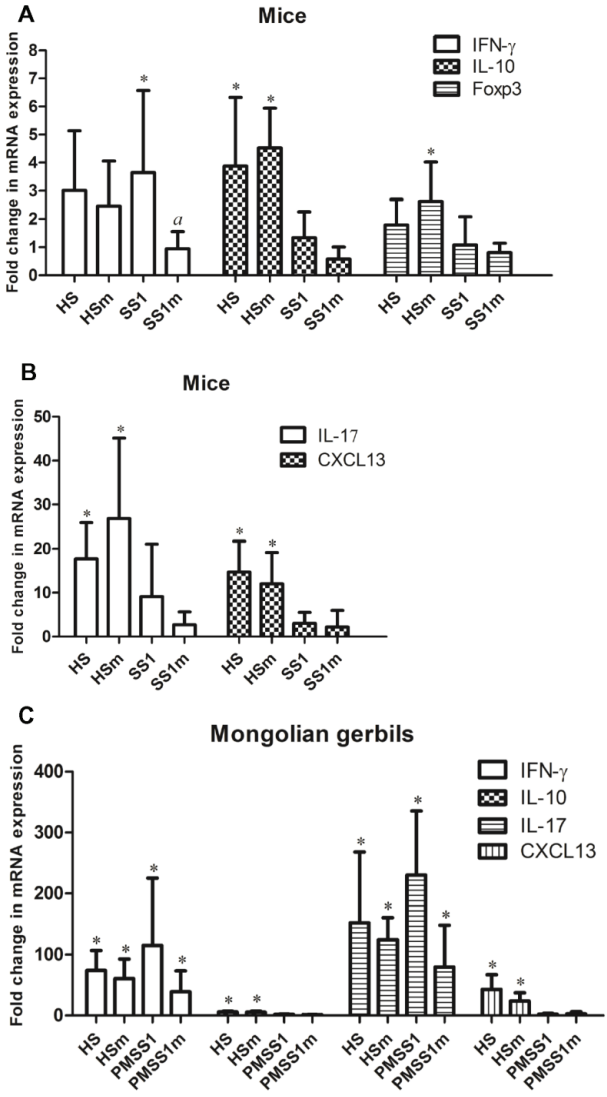

Figure 5 Cytokine expression patterns in the stomach of mice and Mongolian gerbils infected with *H. suis* and *H. pylori*.

Shown are the mean fold changes of mRNA expression in infected mice (A-B) and gerbils (C) for IFN-γ, IL-10, Foxp3, IL-17, CXCL13. The mean fold changes in the relevant uninfected control groups is equal to 1. An * indicates a statistically significant difference ($p < 0.05$) between infected and control groups. An *a* indicates a statistically significant difference ($p < 0.05$) between WT *Helicobacter* infected groups and isogenic *ggt* mutant infected groups. HS: animals infected with WT *H. suis* strain HS5cLP; HSm: animals infected with *H. suis* strain HS5cLPΔ*ggt*; SS1: animals infected with WT *H. pylori* SS1; SS1m: animals infected with *H. pylori* SS1Δ*ggt*; PMSS1: animals infected with *H. pylori* PMSS1; PMSS1m: animals infected with *H. pylori* PMSS1Δ*ggt*; WT: wild-type; 3w: 3 weeks post infection; 9w: 9 weeks post infection; 6m: 6 months post infection.

IL-17

IL-17 is a Th17 response signature cytokine. A notable increase of IL-17 expression was generally observed in mice infected with WT *H. suis* (Figure 5B, $p<0.05$). Similar expression levels were observed for HS5cLPΔ*ggt* infected mice (Figure 5B, $p<0.05$).

In Mongolian gerbils, both WT and mutant *H. suis* and *H. pylori* infection generally induced increased levels of IL-17 expression (Figure 5C, $p<0.01$). These levels were lower in HS5cLPΔ*ggt* and PMSS1Δ*ggt* infected gerbils compared to WT infected animals, which was, however, not statistically significant (Figure 5C, $p>0.05$).

CXCL13

CXCL13 plays an important role during the B-cell homing to follicles in lymph nodes and spleen and formation of gastric lymphoid follicles (Ansel *et al.*, 2000), and it is involved in the pathogenesis of *Helicobacter* infection (Ansel *et al.*, 2000, Yamamoto *et al.*, 2014). In general, infection with both HS5cLP and HS5cLPΔ*ggt* induced a marked upregulation of CXCL13 in mice (Figure 5B, $p<0.01$). Moreover, an even higher increase of CXCL13 expression levels was observed in the antrum of gerbils infected with *H. suis* strains HS5cLP and HS5cLPΔ*ggt* compared to control gerbils (Figure 5C, $p<0.01$). No statistically significant differences of CXCL13 expression levels were observed between HS5cLP and HS5cLPΔ*ggt* infected animals (Figure 5B-5C).

Changes in expression of epithelial cell-related factors in the stomach

The H+/K+ ATPase is responsible for gastric acid secretion by parietal cells (Chow *et al.*, 1995). Compared to uninfected control mice, a clear decrease of *Atp4a* (Figure 6A, $p<0.05$) and *Atp4b* ($p<0.05$, data not shown) mRNA expression levels was detected in the stomach of HS5cLP and SS1 infected mice at 9 weeks p.i.. In addition, a statistically higher expression of *Atp4a* (Figure 6A, $p<0.05$) and *Atp4b* ($p<0.05$, data not shown) was observed in HS5cLPΔ*ggt* infected mice compared to WT infected animals.

ASCT2 is an important glutamine transporter for the growth of epithelial cells and other cell types (McGivan *et al.*, 2007). Compared to control animals, infection with *H. suis* strain HS5cLP resulted in a downregulation of ASCT2 expression in mice at 9 weeks p.i. (Figure 6B, $p<0.05$), and infection with *H. suis* strain HS5cLPΔ*ggt* revealed significantly higher ASCT2 expression levels compared to WT *H. suis* infection (Figure 6B, $p<0.05$). Similar

results were observed in *H. pylori* infected mice, without being statistically significant (Figure 6, $p>0.05$).

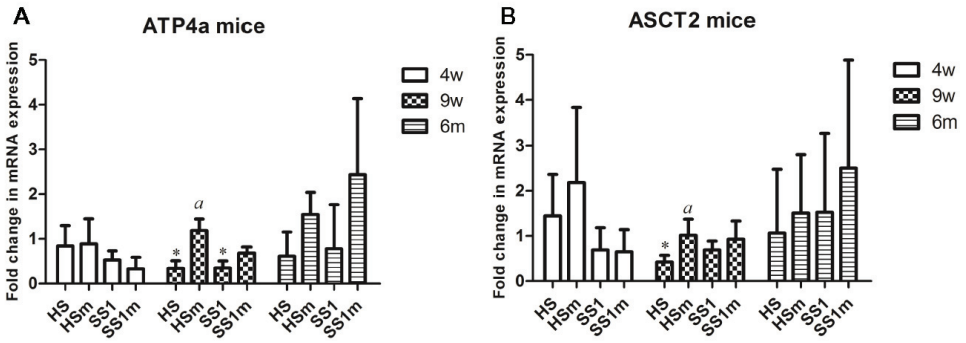

Figure 6 Expression of epithelial cell-associated factors.
Shown are the mean fold changes of mRNA expression in infected mice for ATP4a (A) and ASCT2 (B). The mean fold changes in the relevant uninfected control groups is equal to 1. An * indicates a statistically significant difference ($p < 0.05$) between infected and control groups. An *a* indicates a statistically significant difference ($p < 0.05$) between WT *Helicobacter* infected groups and isogenic *ggt* mutant infected groups. HS: animals infected with WT *H. suis* strain HS5cLP; HSm: animals infected with *H. suis* strain HS5cLPΔ*ggt*; SS1: animals infected with WT *H. pylori* SS1; SS1m: animals infected with *H. pylori* SS1Δ*ggt*; PMSS1: animals infected with *H. pylori* PMSS1; PMSS1m: animals infected with *H. pylori* PMSS1Δ*ggt*; WT: wild-type; 3w: 3 weeks post infection; 9w: 9 weeks post infection; 6m: 6 months post infection.

Discussion

Although, in the present study, *H. suis* strain HS5cLPΔ*ggt* was shown to be able to colonize the stomach of mice at similar levels compared to WT *H. suis*, it induced significantly less overall inflammation in both corpus and antrum. This suggests that the *H. suis* GGT is involved in the induction and regulation of the inflammatory response, without being an essential factor for colonization. However, in Mongolian gerbils, *H. suis* strain HS5cLPΔ*ggt* was shown to induce only a slightly milder inflammatory response compared to the WT *H. suis* strain. This implies that, besides GGT, *H. suis* harbours other virulence factors or bacterial components, involved in the generation and modulation of the host immune response. In a previous study performed *in vitro*, lysate from HS5cLPΔ*ggt* indeed was shown to still have an effect on the proliferation and function of T lymphocytes, further suggesting the presence of hitherto unidentified factors in *H. suis* that can modulate the host immune and inflammatory response (Zhang *et al.*, 2013). These factors remain to be investigated in the future.

Interestingly and in contrast to what we observed for *H. suis* lacking GGT, *H. pylori* strains SS1Δ*ggt* and PMSS1Δ*ggt* failed to persistently colonize the stomach of mice and gerbils,

highlighting the different relative contributions of *H. pylori* GGT and *H. suis* GGT to the colonization ability in these rodent models. In any case, data from the current study as well as previous studies on the *H. pylori* GGT show that the *H. pylori* GGT confers a benefit to *H. pylori* in terms of its colonization capacity, at least in mice and gerbils, whereas the *H. suis* GGT mainly affects the inflammatory response evoked during *H. suis* infection without having a notable impact on the levels of bacterial colonization. Since *H. suis* lacks several other virulence determinants of *H. pylori,* such as VacA, the role of *H. suis* GGT in inducing or shaping the host immune response appears to be relatively important.

Our study reveals that *H. suis* infection induces a Th17 response in mice, without a significant upregulation of Th1 cytokines such as IFN-γ. This confirms the results of a previous study in which both Th1- and Th2- prone mouse strains were used (Flahou *et al.*, 2012). However, the use of Mongolian gerbils in the present study demonstrated that *H. suis* infection can induce a marked upregulation of IFN-γ expression in this animal model, which is accompanied by a more pronounced gastritis compared to that seen in mice. For *H. pylori*, it has been demonstrated that infection induces the expression of IFN-γ in both mice and gerbils, which plays a pivotal role in promoting mucosal inflammation. This in turn contributes to more pronounced gastric mucosal damage (Smythies *et al.*, 2000). Thus, the higher levels of IFN-γ expression in gerbils infected with *H. suis* most likely contribute to the more pronounced inflammation observed in this animal model compared to that in mice.

IL-10 is considered an important anti-inflammatory cytokine, which is mainly produced by regulatory T cells and dendritic cells (Eaton *et al.*, 2001), and this cytokine has been described to be upregulated in WT *H. suis* infected mice (Flahou *et al.*, 2012). In the present study, we observed a similar expression pattern for IL-10 and Foxp3 in mice. This may indicate that the secretion of IL-10 mainly occurs through Tregs in the stomach, which needs to be confirmed in future studies. It may be postulated that the higher levels of IL-10 expression in HS5cLPΔggt infected mice are partially responsible for the attenuated inflammatory response, when compared to WT-infected animals. Previously published data from in vitro experiments have shown that H. pylori GGT suppresses IL-10 secretion by activated human $CD4^+$ T cells (Beigier-Bompadre *et al.*, 2011), which is supported by our findings. The enzyme has, however, also been described to reprogram DC towards a tolerogenic phenotype, which was shown to depend upon increased secretion of IL-10 (Engler *et al.*, 2014).

A pronounced upregulation of CXCL13 expression levels was observed in *H. suis*-infected animals, which was shown to be independent of the presence of *H. suis* GGT. Interestingly, a similar upregulation was completely absent in *H. pylori*-infected animals. Possibly, however,

a longer experimental period (e.g. 12-18 months) may induce upregulation of CXCL13 expression in the stomach of these animals as well. CXCL13, also named B-cell-attracting chemokine-1 or B-lymphocyte chemoattractant, is a CXC subtype member of the chemokine superfamily (Gunn *et al.*, 1998), and it may play a pivotal role in various immune and inflammatory conditions as well as *H. pylori*-associated gastritis in humans (Galamb *et al.*, 2008, Nakashima *et al.*, 2011). It has been shown that the expression of CXCL13 is significantly upregulated in gastric MALT lymphoma in both humans (Mazzucchelli *et al.*, 1999) and mice (Nobutani *et al.*, 2010). The pronounced upregulation of CXCL13 as well as the presence of a clear proliferation of B-cells in germinal centers in the present study seems to be in line with the higher risk to develop gastric MALT lymphoma in humans infected with NHPH compared to *H. pylori* infected patients (Stolte *et al.*, 1997, Morgner *et al.*, 2000, Joo *et al.*, 2007, Haesebrouck *et al.*, 2009). A recent report showed that the formation of gastric lymphoid follicles after challenge with gastric mucosal homogenate from a monkey harbouring *H. suis* was efficiently suppressed by the administration of anti-CXCL13 antibodies (Yamamoto *et al.*, 2014). Taken together, this shows that CXCL13 might be one of the key cytokines involved in the development of gastric MALT lymphoma associated with *H. suis* infection.

In previous experiments we have shown that *H. suis* GGT inhibits the proliferation of lymphocytes *in vitro* through the interaction with glutamine (Zhang *et al.*, 2013). This seems contradictory to the results of the present *in vivo* study showing that animals infected with *H. suis* strain HS5cLPΔ*ggt* exhibited a lower lymphocytic infiltration rate in the gastric mucosa. Besides lymphocytes, however, *H. suis* and its GGT also target gastric mucosal epithelial cells (Flahou *et al.*, 2011). The uncontrolled loss of epithelial cells by cell death, e.g. necrosis, also triggers the influx of inflammatory cells, in turn promoting the further development of inflammation. In line with some of our previous studies (Flahou *et al.*, 2010), *H. suis* infection indeed affected the function of gastric acid secreting parietal cells, as shown by the decreased expression levels of *Atp4a* and *Atp4b*, and the mutant work demonstrated that *H. suis* GGT indeed plays a role. In addition, the present study indicates that the epithelial (hyper)proliferation observed in WT *H. suis* infected mice is more pronounced than in HS5cLPΔ*ggt* infected mice. This suggests that *H. suis* lacking GGT causes less damage to the epithelium compared to WT bacteria. Probably, this also has an implication on the subsequent development of inflammation in the presence of a more or less damaged epithelium. However, it remains to be determined whether the impact of the *H. suis* GGT on the health of gastric

epithelial cells is stronger compared to its direct effects on lymphocytes residing in the deeper tissue layers, including the inhibitory effect on their proliferation.

As mentioned above, infection with *H. suis* strain HS5cLP in mice induced a clear downregulation of *Atp4a* and *Atp4b* expression levels in the stomach at 9 weeks and 6 months p.i., and such an effect was not observed in the HS5cLPΔ*ggt* infected animals, showing that *H. suis* GGT contributes to alterations in gastric acid secretion by parietal cells. Previous reports have shown that *H. suis* is often observed near or inside the canaliculi of parietal cells in the stomach of mice, and colonization of *H. suis* is also closely linked with necrosis of parietal cells in mice and Mongolian gerbils (Flahou *et al.*, 2010). Besides the direct effect of *H. suis* GGT on the acid secretion by parietal cells, altered expression levels of IL-1β may also affect the acid production through multiple pathways (Wallace *et al.*, 1991, Beales *et al.*, 1998), including a decreased histamine release from enterochromaffin-like cells (Prinz *et al.*, 1997). The impaired gastric acid secretion and subsequent development of mucous metaplasia observed in the present study, may lead to the development of gastric atrophy, hypochlorhydria and gastric cancer (Correa, 1992, Kapadia, 2003).

For the first time, we were able to show an effect of *H. suis* GGT on the glutamine metabolism of gastric epithelial cells. This amino acid, targeted by the enzymatic activity of *H. suis* GGT (Zhang *et al.*, 2013), is a major fuel for rapidly dividing cells, including enterocytes, macrophages and lymphocytes (Rhoads *et al.*, 1997, Wu, 2009). It is supportive in improving digestion, absorption, and retention of nutrients through affecting tissue anabolism, stress, and immunity, and it also plays an important role in animal nutrition and health. WT *H. suis* infection was shown to cause a significant downregulation of ASCT2 mRNA in mice, while HS5cLPΔ*ggt* did not show this effect. This suggests that glutamine depletion catalysed by GGT activity at the level of the gastric mucosa resulted in the downregulation of glutamine transporter ASCT2. ASCT2 is a Na^+-dependent, broad-scope neutral amino acid transporter (Kekuda *et al.*, 1996, Utsunomiya-Tate *et al.*, 1996), which is essential for glutamine uptake by fast growing epithelial cells and tumor cells (Bode *et al.*, 2002, McGivan *et al.*, 2007, Wang *et al.*, 2014), and ASCT2 expression levels depend on glutamine availability (Bungard *et al.*, 2004).

In summary, our data show that *H. suis* GGT is not an essential factor for colonization in mice and gerbils, whereas it is involved in the induction of an inflammatory response. This differs to what has been described for the *H. pylori* GGT. In addition, we demonstrated that *H. suis* infection causes a considerable increase of IFN-γ expression levels in Mongolian gerbils, which differs from the situation in mice, where *H. suis* infection is not accompanied by

increased expression of this Th1 signature cytokine. This Th1 response was shown to be attenuated in the absence of *H. suis* GGT. CXCL13 expression levels were shown to be upregulated during *H. suis* infection, in contrast to what we observed for *H. pylori* infection, and this was shown not to depend on the presence of *H. suis* GGT. WT *H. suis* infection was shown to suppress expression levels of *Atp4a* and *Atp4b*, involved in gastric acid secretion, and to suppress expression levels of the glutamine transporter ASCT2. These effects on the gastric epithelium were clearly related to the presence of *H. suis* GGT.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GZZ and BF coordinated the study, designed the study, carried out the experiments, analysed the data and drafted the manuscript. FH coordinated the study, designed the study and drafted the manuscript. EDB, MJ, IB and AS contributed to the experiments, and RD participated in the design of this study and drafting of the manuscript. All authors read and approved the final manuscript.

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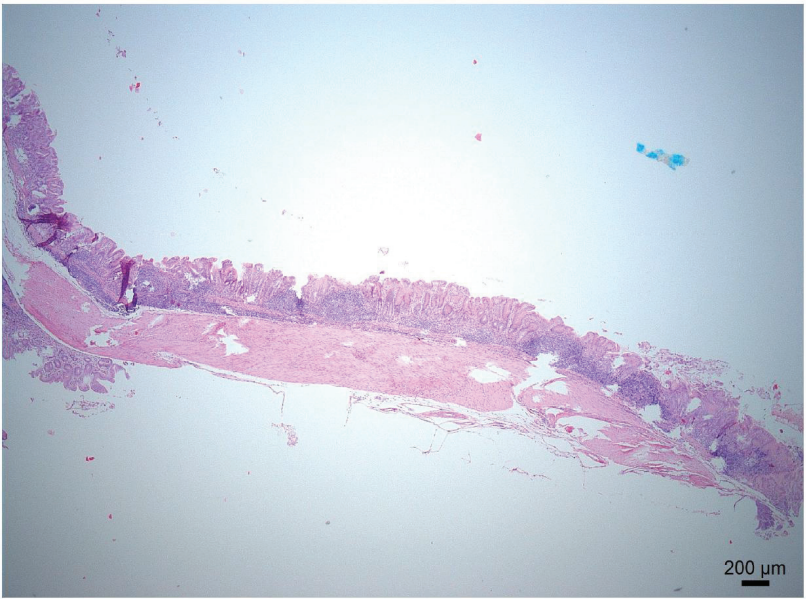
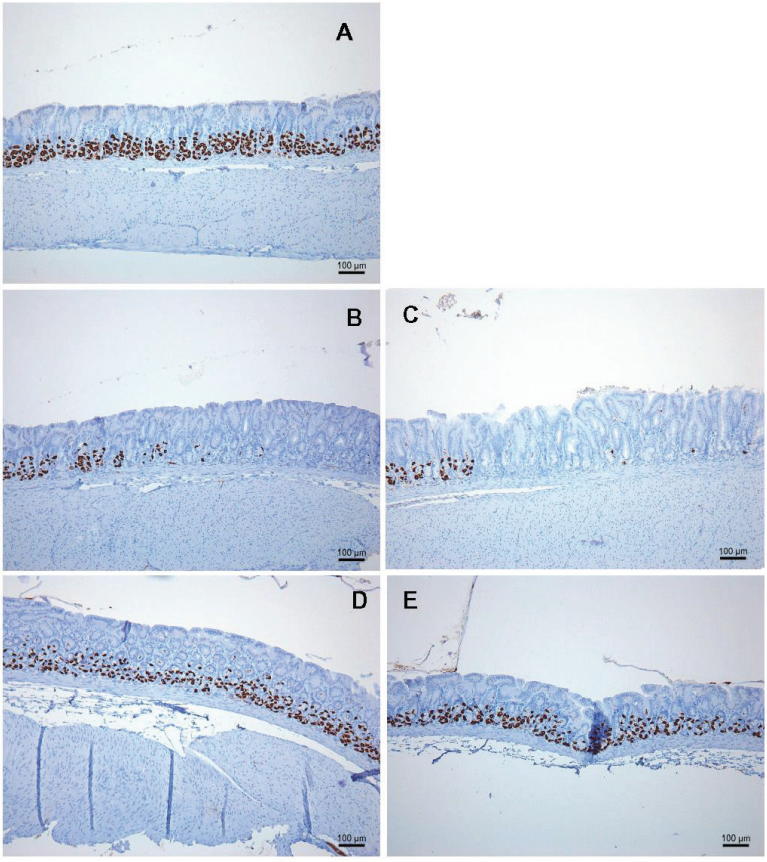
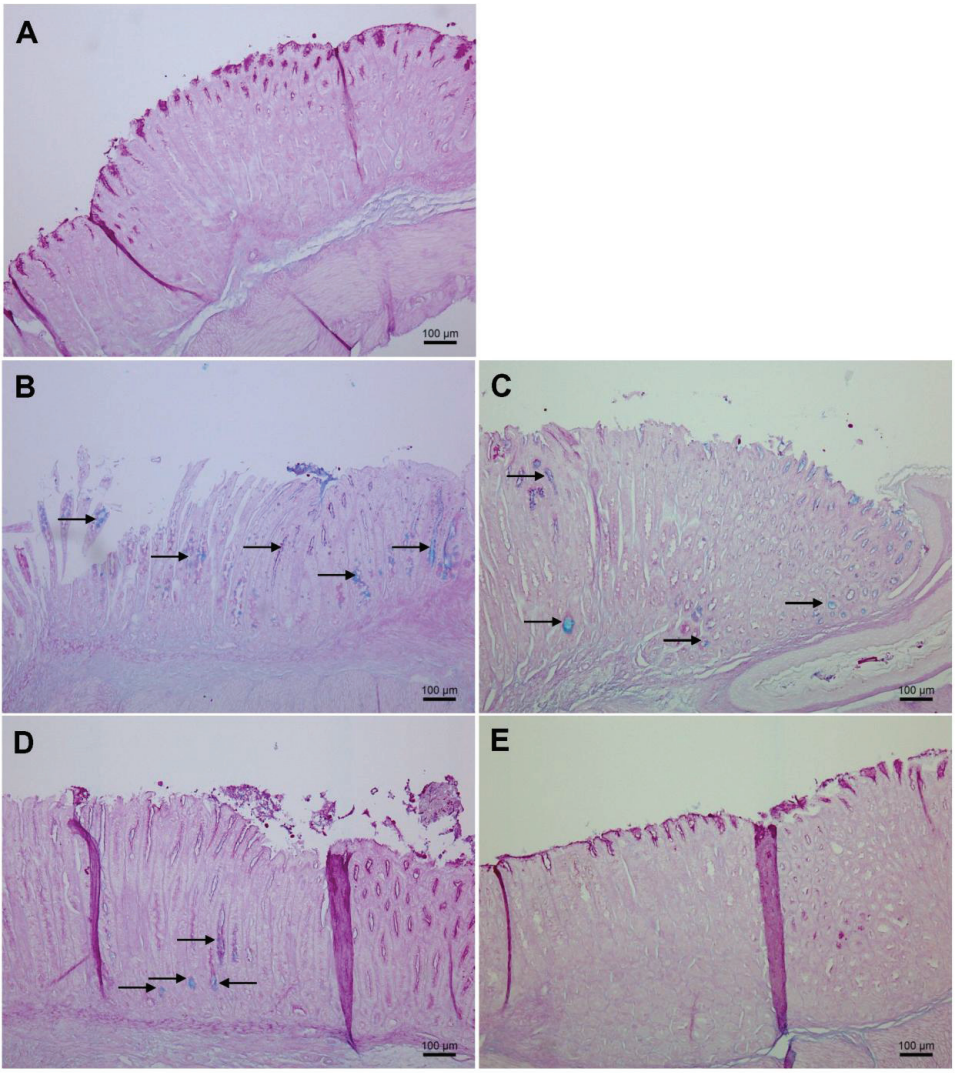
Additional files



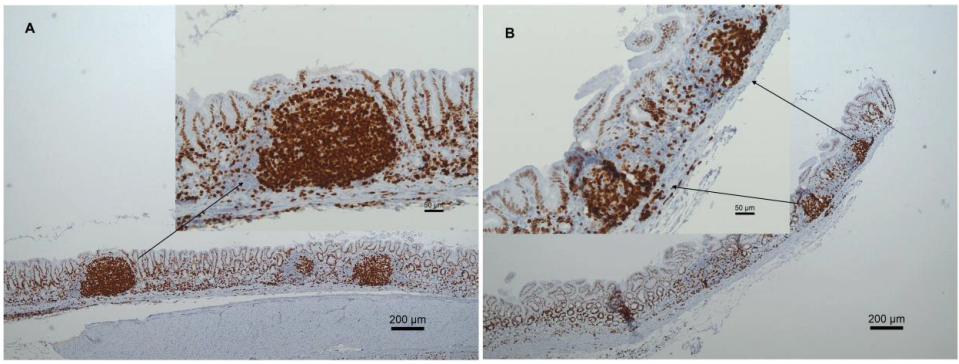
Additional file 1: H&E staining of the stomach section from a *Helicobacter suis* infected Mongolian gerbil. The vast majority of the antrum of the stomach from this WT *H. suis*-infected animal was densely infiltrated with inflammatory cells, fused lymphoid aggregates and lymphoid follicles. Original magnification: 25×.



Additional file 2: Immunohistochemical staining of the hydrogen potassium ATPase of parietal cells in the stomach mucosa of Mongolian gerbils.
Moderate numbers of parietal cells (brown) are present at the transition zone between the corpus and antrum of the stomach of control Mongolian gerbils (A). A clear loss of parietal cells is observed in the transition zone between the corpus and antrum of the stomach from Mongolian gerbils infected with WT *H. suis* strain HS5cLP (B) or *H. suis* strain HS5cLPΔ*ggt* (C) at 9 weeks post inoculation. No clear change of parietal cell numbers is seen in the transition zone between the corpus and antrum of the stomach from Mongolian gerbils infected with WT *H. pylori* PMSS1 (D) or *H. pylori* PMSS1Δ*ggt* (E) at 9 weeks post inoculation. WT: wild-type. Original magnification: 100×.



Additional file 3: Determination of mucous metaplasia in the stomach from *Helicobacter*-infected mice. An AB/PAS staining was applied to determine the presence of pseudopyloric metaplasia (arrows) in the stomachs of control mice (A), WT *H. suis* infected mice (B), *H. suis*Δ*ggt* infected mice (C), WT *H. pylori* infected mice (D), and *H. pylori*Δ*ggt* infected mice (E) at 6 months post infection. WT: wild-type; AB/PAS: alcial blue-periodic acid-Schiff stain. Original magnification: 100×.



Additional file 4: Proliferation of B cells in germinal centers.
Representative micrographs of a Ki67 staining of the stomach from a WT *H. suis* infected (A) and *H. suis*Δ*ggt* infected gerbil (B) are shown. Proliferating germinal centers were observed in animals from both groups, but mainly in WT *H. suis* infected animals. WT: wild-type. Original magnification: 50× and 200×.

Study 3: Pig parietal cells: isolation, culture and cellular dysfunction induced by *Helicobacter suis* infection

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Manuscript in preparation

Abstract

The stomach of the majority of pigs is colonized by *Helicobacer* (*H.*) *suis*, which is also the most prevalent gastric non-*H. pylori Helicobacter* (NHPH) species in humans. It is associated with chronic gastritis, gastric ulceration and other gastric pathological changes in both hosts. Parietal cells are highly specialized, terminally differentiated epithelial cells responsible for gastric acid secretion and regulation. Dysfunction of these cells is closely associated with gastric pathology and disease. Here we describe a method for isolation and culture of responsive parietal cells from slaughterhouse pigs. In addition, we investigated the interactions between *H. suis* and parietal cells both in *H. suis*-infected pigs, as well as in our *in vitro* parietal cell model. A close interaction of *H. suis* and parietal cells was observed in the fundic region of stomachs from *H. suis* positive pigs. The bacterium was shown to be able to directly interfere with cultured pig parietal cells, causing a significant impairment of cell viability. Transcriptional levels of Atp4a, essential for gastric acid secretion, showed a trend towards an up-regulation in *H. suis* positive pigs compared to *H. suis*-negative pigs. In addition, sonic hedgehog, an important factor involved in gastric epithelial differentiation, gastric mucosal repair, and stomach homeostasis, was also significantly up-regulated in *H. suis* positive pigs. In conclusion, this study describes a successful approach for the isolation and culture of pig parietal cells. The results indicate that *H. suis* affects the viability and function of this cell type.

Introduction

Helicobacter (*H.*) *suis* is a Gram-negative bacterium with a typical spiral-shaped morphology, which frequently colonizes the stomach of pigs as well as a minority of humans (Grasso *et al.*, 1996, Hellemans *et al.*, 2007, Joosten *et al.*, 2013). Indeed, gastric non-*H. pylori* helicobacters (NHPH) are found in 0.2-6% of gastric biopsies, depending on the study (Haesebrouck *et al.*, 2009), and *H. suis* is considered to be the most prevalent NHPH in humans (De Groote *et al.*, 2005, Haesebrouck *et al.*, 2009, Joosten *et al.*, 2013). In humans, infection with *H. suis* has been described to cause gastritis, gastric ulceration, as well as gastric mucosa-associated lymphoid tissue (MALT) lymphoma and sporadically gastric adenocarcinoma (Morgner *et al.*, 1995, Debongnie *et al.*, 1998, Morgner *et al.*, 2000). In naturally infected or experimentally infected pigs, *H. suis* infection has been shown to cause gastritis, reduced daily weight gain and other gastric pathological changes (Park *et al.*, 2000, De Bruyne *et al.*, 2012).

The gastric mucosa is composed of various cell types. Parietal (oxyntic) cells are abundant in the fundic gland region. They are responsible for the secretion of gastric acid and play a vital role in the maintenance of the normal structure and function of the gastric mucosa (Yao *et al.*, 2003). In some species, including humans, pigs, rabbits and cats, parietal cells can also secrete intrinsic factor which plays an important role in the absorption of vitamins and other nutrients by the small intestine (Chew, 1994). Hydrogen potassium ATPase (H^+/K^+ ATPase) is the proton pump composed of a catalytic subunit (α-subunit) and an accessory subunit (β-subunit) in parietal cells, and it mediates secretion of acid into the gastric lumen (Yao *et al.*, 2003). Various studies have shown that atrophic gastritis induced by *H. pylori* infection is characterized by the dysfunction or loss of parietal cells (Nozaki *et al.*, 2008, Saha *et al.*, 2010). While *H. pylori* is mainly observed in the mucus layer or close to mucus-producing cells, *H. suis* is often observed near or even inside the canaliculi of parietal cells in experimentally infected Mongolian gerbils and mice. Similar observations have been made in humans (Joo *et al.*, 2007). Both in rodent models and humans, these parietal cells can show signs of degeneration (Joo *et al.*, 2007, Flahou *et al.*, 2010).

Besides H^+/K^+ ATPase, sonic hedgehog (Shh) is another identified factor playing an important role in the regulation of gastric acid secretion, as well as maturation and differentiation of gastric epithelial cells and fundic glands in mice and humans under normal conditions (Ramalho-Santos *et al.*, 2000, van den Brink *et al.*, 2001). It has also been described to play a role in the pathogenesis of *H. pylori* infection and even in the development

of gastric cancer (Nielsen *et al.*, 2004, Katoh *et al.*, 2005). Currently, no information is available on potential effects of *H. suis* infection on the expression of Shh.

To date, there is no report illustrating the interactions between *H. suis* and parietal cells in pigs. Therefore, the aim of this study was to examine the direct effects of *H. suis* on porcine parietal cells, both using a newly developed *in vitro* parietal cell culture method and tissues from *H. suis*-infected pigs.

Materials and methods

Collection of pig stomachs

All pig stomachs were collected from 6-month-old slaughter pigs, brought to the laboratory immediately, and kept at 4 ℃ for further use.

Isolation and culture of primary pig parietal cells

Pig stomachs were opened, and washed successively several times with water (37°C) and phosphate buffered saline (PBS; 37°C). The mucus was removed using a glass slide, and the fundic region of the stomach was collected and kept in ice-cold PBS. The mucosa was separated gently from the underlying tunica submucosa and tunica muscularis, using the sharp side of a scalpel, and minced into small fragments. After washing the minced mucosa several times with PBS (37°C) and minimal essential medium-glutamax (37°C) (MEM; Invitrogen, Carlsbad, CA, USA), it was placed in MEM supplemented with dispase (1 mg/ml, Invitrogen) and BSA (5 mg/ml). This mixture was transferred to a tissue culture flask, and the tissue was digested at 37℃ for 25 min on a rotational shaker. The digestion was stopped by three-fold dilution with MEM, and the sample was subjected to centrifugation at 200 g for 10 min. The supernatant was discarded and the tissue was placed in MEM supplemented with collagenase type 1 (2.5 mg/ml, Invitrogen) and BSA (5 mg/ml) and incubated for another 50 min under the same conditions as described above. The resulting mixture was filtered through a 150 µm metal sieve, and centrifuged at 200 g for 10 min. The supernatant was removed carefully. The remaining cells were washed with MEM, and then filtered using a 70 µm and 40 µm cell strainer for 2 times each. The cell suspension was washed 2 times in MEM, and further purified using an OptiPrep™ gradient (Sigma-Aldrich St. Louis, MO, USA) according to the procedure described by Chew and Brown (Chew *et al.*, 1986). The purified cells were washed in MEM and incubated in cell culture flasks containing medium A (DMEM/F12 (Sigma-

Aldrich) supplemented with 20 mM Hepes, 0.2% BSA, 10 mM glucose, 8 nM EGF (Sigma-Aldrich), 1× Insulin, Transferrin, Selenium Solution (ITS) (Invitrogen), 1% penicillin-streptomycin, 50 µg/mL amphotericin B and 25 µg/mL gentamicin (Invitrogen)) for 40 min to kill most contaminating bacteria and fungi. Subsequently, the cells were washed in DMEM/F12 supplemented with 0.2% BSA, 10 mM glucose, and incubated in medium A without amphotericin B in 24-well flat-bottom cell-culture plates (Greiner Bio-One, Frickenhausen, Germany) containing Matrigel-coated glass coverslips (circular diameter 12 mm; Thermo Scientific, Leicestershire, UK). To coat these coverslips, Matrigel basement membrane (Corning B.V. Life Sciences, Amsterdam, LJ, Netherlands) was thawed on ice for at least 12 h. Subsequently, the glass coverslips were coated with Matrigel basement membrane, diluted six times in ice-cold sterile water, and left to dry in a laminar air flow over night.

Activation of parietal cells and visualization of gastric acid secretion

Twelve hours after seeding of parietal cells on coverslips, the medium was replaced by fresh medium. In order to stimulate cells to secrete HCl, they were incubated in medium supplemented with histamine (400 µM; Sigma-Aldrich) and 3-isobutyl-1-methylxanthine (IBMX) (30 µM; Sigma). Control cells were held in a resting state by administering cimetidine (100 µM; Sigma-Aldrich). After 30 min of incubation at 37℃, cells were incubated in medium A (without amphotericin B) supplemented with 2 µM LysoSensor™ Yellow/Blue DND-160 (Invitrogen) and 2 µM Cell Tracker Red CMTPX (Invitrogen) at 37℃ for 30 min. Subsequently, cells were washed 3 times, immediately mounted in a small volume of PBS (50% glycerol, v/v) on glass slides at room temperature, and analyzed using a confocal microscope within 30 min.

Preparation of *H. suis* and bacterial lysate

H. suis strain HS5cLP was grown on Brucella agar (BD, Franklin Lakes, NJ, USA) plates with a pH of 5 and supplemented with 20% fetal calf serum (HyClone), 5 mg/L amphotericin B (Fungizone; Bristol-Myers Squibb, Epernon, France), Campylobacter selective supplement (Oxoid, Basingstoke, UK) and Vitox supplement (Oxoid) under microaerobic and biphasic conditions (37°C; 85% N_2, 10% CO_2, 5% O_2) as described elsewhere (Flahou *et al.*, 2012). This strain was isolated in 2008 from the stomach of a

slaughterhouse pig (Baele *et al.*, 2008). Bacterial lysate was prepared as described previously (Flahou *et al.*, 2011).

Treatment of parietal cells and determination of cell viability

Parietal cells were cultured as described above in fresh medium without antibiotics. Parietal cells were inoculated with viable *H. suis* at a multiplicity of infection (MOI) of 100 or 200 or with whole bacterial lysate at a final concentration of 100 µg/ml or 200 µg/ml in 24-well plates. For the first 4 hours, incubation was done under microaerobic conditions, after which the cells were transferred to normal conditions (5% CO_2) for another 20 h. Parietal cell viability was determined using the neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) uptake assay as described previously with some minor modifications (Verbrugghe *et al.*, 2012). Briefly, 400 µL of pre-warmed neutral red solution (33 µg/mL in DMEM without phenol red) was added to each well and the plate was incubated at 37°C for 3 h. The cells were then washed twice with Hank's buffered salt solution (HBSS) with Ca^{2+} and Mg^{2+} (Gibco, Life Technologies, Paisley, Scotland). Two hundred µL of extracting solution (ethanol/ water/acetic acid, 50/49/1 (v/v/v)) was added to each well to release the dye, and the plate was shaken for another 30 min. The absorbance was then read at 540 nm with a microplate ELISA reader (Multiscan MS, Thermo Labsystems, Helsinki, Finland). The percentage of viable cells was estimated using the following formula:

% cell viability = 100 × (a-b) / (c-b)

(with a = OD540 derived from the wells incubated with live bacteria or lysate, b = OD540 derived from blank wells, c = OD540 derived from untreated control wells).

Indirect immunofluorescent staining

Cultured parietal cells treated as described above were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After fixation, the cells were washed three times with PBS, and permeabilized with 0.3% Triton X-100 in PBS (2% BSA) for 20 min followed by incubation in PBS (2% BSA) for another 30 min. The cells were washed 3 times with PBS. Subsequently, cells were incubated with a primary mouse monoclonal anti- H^+/K^+ ATPase β-subunit antibody (1/200; Abcam Ltd, Cambridge, UK) and a polyclonal rabbit anti-*Helicobacter pylori* antibody (1/320; Dako, Glostrup, Denmark) for 1 h at 37°C, followed by an Alexa Fluor 633-conjugated goat anti-mouse secondary antibody (1/200; Invitrogen) and Alexa Fluor 488-conjugated goat anti rabbit IgG (1/100; Invitrogen) for 1 h at 37°C. All antibodies were diluted in PBS and the cells were washed 5 times after incubation with the

primary and secondary antibodies. DAPI (0.5 µg/mL; Sigma) was used to counterstain the nuclei for 15 min and the cells were rinsed 5 times in PBS. Stained cells were mounted in ProLong® Gold antifade reagent medium (Invitrogen) and imaged by an Olympus BX61 fluorescence micro-scope (Olympus Belgium N.V.).

Immunohistochemical and immunofluorescent staining of pig gastric tissue slides

Stomachs from slaughterhouse pigs were opened along the greater curvature. For detection of *H. suis* colonization, a small piece of tissue from the fundic region of the stomach was collected, followed by DNA extraction and *H. suis*-specific Quantitative Real-Time PCR (qRT-PCR) as described previously (Blaecher *et al.*, 2013).

Gastric samples from the fundic gland zone were fixed in 10% phosphate-buffered formalin, processed by routine methods and embedded in paraffin**.** Consecutive sections of 5 µm were cut, and immunohistochemical staining for the identification and visualization of parietal cells was performed with these sections as described previously (Flahou *et al.*, 2010). Immunofluorescent staining was also perfomed to visualize co-localization of parietal cells and *H. suis*. Briefly, 5 µm formaldehyde-fixed tissue sections were deparaffinized in xylene and rehydrated in graded ethanol. Sections were boiled in antigen retrieval solution (850W, 1.5 min; 300W, 10 min) and washed respectively for 15 min in water and 5 min in PBS. Sections were permeabilized with 0.3% TritonX-100 in PBS (2% goat serum) for 15 min, and incubated in PBS (10% goat serum) for 45 min. Tissue sections were incubated with a primary mouse monoclonal anti- H^+/K^+ ATPase β-subunit antibody (1/3125; Abcam) and a polyclonal rabbit anti-*Helicobacter pylori* (1/320; Dako) antibody overnight at 4 °C. After washing with PBS, sections were incubated for 1 h with secondary Alexa Fluor 633 goat anti-mouse IgG (1/100; Invitrogen) and Alexa Fluor 488 goat anti rabbit IgG (1/100; Invitrogen). DAPI (0.5 µg/mL) was used to counterstain the nuclei. Tissue sections were washed extensively with PBS, mounted in ProLong® Gold antifade reagent medium and examined by fluorescence or confocal microscopy.

RNA extraction, reverse transcription and qRT-PCR

qRT-PCR was used to compare gene expression levels of gastric tissue from the *H. suis* negative pigs (n=15) and *H. suis* positive pigs (n=15). RNA was extracted and cDNA was prepared as described previously (Flahou *et al.*, 2012). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of RNA was measured using a NanoDrop spectrophotometer (Isogen Life

Science, PW De Meern, Utrecht, The Netherlands). The purity of the RNA was evaluated with the Experion automated electrophoresis system using StdSens RNA chips (Bio-Rad, Hercules CA, USA). The RNA concentration from all samples was adjusted to 1 µg/µL and cDNA was synthesized immediately using the iScript™ cDNA Synthesis Kit (Bio-Rad).

The housekeeping genes *ACTB, Cyc*-5 and *HPRT* were included as reference genes (Bosschem et al., unpublished data). Primers for *Atp4a* were referenced elsewhere (Bosi *et al.*, 2006), and primers for *Shh* were designed based on the conserved complete or partial coding sequences of *Shh* available for humans, pigs, mice and rats. The mRNA expression levels of reference genes and target genes were quantified using SYBR Green based RT-PCR with iQ^{TM} SYBR Green Supermix. Reactions were performed using a CFX96 RT PCR System in a C1000 Thermal Cycler (Bio-Rad). qRT-PCR was performed as described elsewhere (Flahou *et al.*, 2012). Sequence information of the primers was shown in Table 1.

Table 1 primers used in qRT-PCR

Gene	Primer	Sequence (5′- 3′)	Reference
Atp4a	sense	GCATATGAGAAGGCCGAGAG	Bosi *et al.*, 2006
	antisense	TGGCCGTGAAGTAGTCAGTG	
sonic hedgehog	sense	TGACCCCTTTAGCCTACAAGCA	This study
	antisense	TGGGGGTGAGTTCCTTAAATCG	

Results

Activation of parietal cells and stimulation of gastric acid secretion

Cultured parietal cells responded to stimulation with histamine/IBMX, as shown by the presence of more and bigger vacuoles under the light microscope (data not shown). In order to confirm the secretion of gastric acid by parietal cells after stimulation, a fluorescent acidic pH indicator, LysoSensor, was loaded both to resting and stimulated parietal cells.

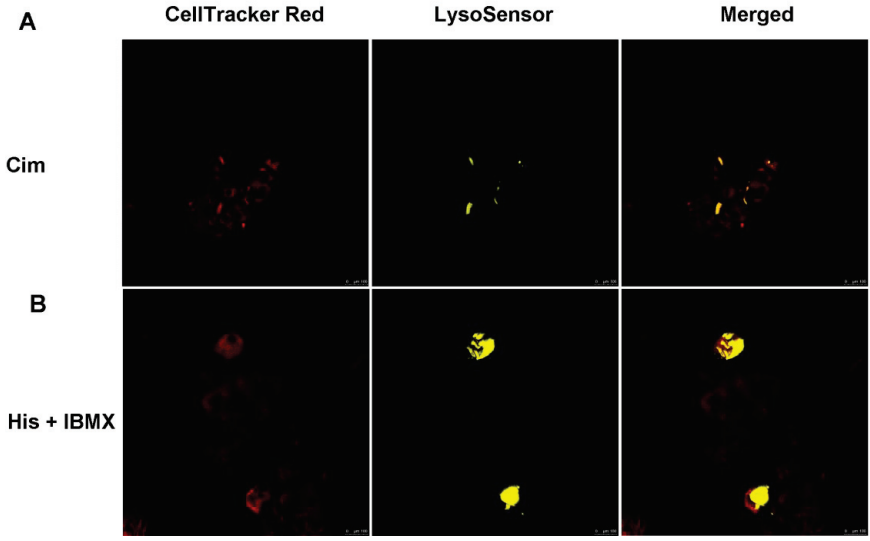

Figure 1 Acid secretion by parietal cells.
LysoSensor™ Yellow/Blue DND-160 was used to monitor the acid secretion by live parietal cells incubated either by Cim or His/IBMX. Cell Tracker Red CMTPX was used to track all live cells. Some apical vacuoles of parietal cells in resting stage revealed several small areas with weak yellow fluorescence, indicating basal acid secretion (A). Parietal cell treated with His/IBMX showed an expansion of apical vacuoles, as shown by much bigger zones with strong yellow fluorescence, indicating the continuous secretion of acid (B). Cim: Cimitidine; His: histamine; IBMX: 3-isobutyl-1-methylxanthine

An accumulation of LysoSensor was observed in the stimulated parietal cells, characterized by a predominantly strong yellow fluorescence (Figure 1B). Parietal cells in resting stage also showed several small areas with weak yellow fluorescence, indicating that vacuoles in parietal cells had a basal acid production (Figure 1A). Upon the stimulation by histamine/IBMX, an increase in the fluorescence intensity of LysoSensor in the vacuoles was observed and the size of the vacuoles was increased as well (Figure 1B), indicating the enhancement of gastric acid secretion.

H. suis bacteria interact with cultured parietal cells

Immunofluorescence staining showed adhesion of *H. suis* to parietal cells after incubation of cells with *H. suis* at an MOI of 10 or 100:1 for 6 h (Figure 2). Longer incubation times (12 hour) and a higher MOI (200) exhibited similar results (data not shown).

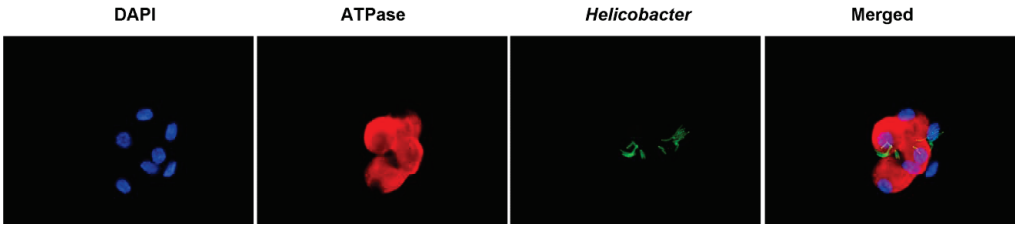

Figure 2 The presence of *H. suis* near or inside the cultured parietal cells.
Cultured parietal cells were inoculated with live *H. suis* at an MOI of 10 for 6 h, and a close relationship between *H. suis* (green) and parietal cells (red) could be observed. Nucleic acid was stained by DAPI (blue). Representative fluorescence micrographs were shown. Scale bars, 50 μm. MOI: multiplicity of infection.

Cell viability assay

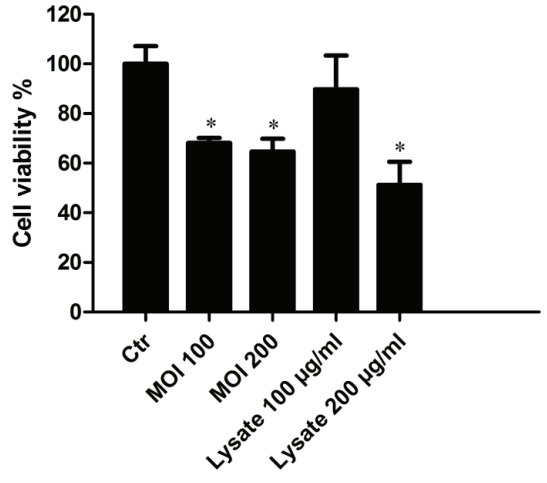

Figure 3 Effect of *H. suis* on parietal cell viability.
Parietal cells were treated with live *H. suis* (MOI: 100:1, 200:1) or whole bacterial lysate (100 μg/ml, 200 μg/ml), and control cells were treated with HBSS. After 24 h, cell viability was determined by a neutral red assay. Results of one representative experiment (out of 3 performed in total) are shown (n=5). An * represents a statistically significant difference between bacteria or lysate treated cells and HBSS treated cells (Student *t* test, $p<0.05$). MOI: multiplicity of infection; HBSS: Hank's buffered salt solution.

A neutral red assay was used to determine the effect of live *H. suis* bacteria and whole cell lysate of *H. suis* on parietal cell viability. Parietal cells were treated with live bacteria or bacterial lysate for 24 h. Compared to untreated control cells, a significant decrease of cell viability was observed in live bacteria-treated cells (MOI:100, 200) and lysate-treated cells (200 μg/ml) (Figure 3, $p<0.05$), confirming that both live bacteria and lysate affect parietal cell viability *in vitro*.

Interaction between *H. suis* and pig parietal cells *in vivo*

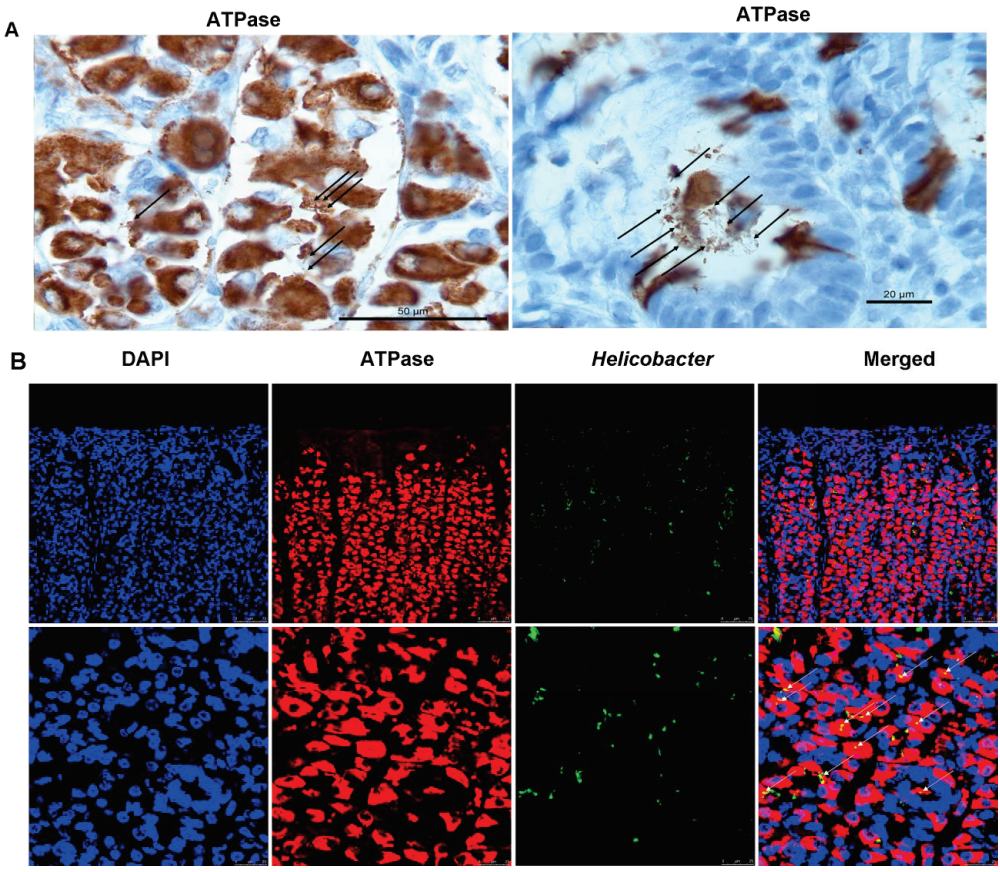

Figure 4 Co-localization of *H. suis* and pig parietal cells in the stomach of slaughterhouse pigs.
Shown are representative immunohistochemical (A) and fluorescent micrographs (B) of *H. suis* bacteria near or within the parietal cells in the fundic region of the stomach from slaughterhouse pigs. IHC staining (A) showed that *H. suis* bacteria (black arrows) were observed next to parietal cells, sometimes showing signs of degeneration (right panel) (brown). Immunofluorescence staining (B) revealed that a substantial number of *H. suis* bacteria (green, white arrows) are in close association or even inside the parietal cell (canaliculi) (red). Nuclei are stained with DAPI (blue). IHC: immunohistochemistry.

IHC staining did not reveal a clear change of parietal cell numbers in the stomach of *H suis*-infected pigs compared to *H. suis*-negative pigs (data not shown). However, a close relationship between parietal cells and *H. suis* was observed in the fundic region of the pig stomach (Figure 4A), and some *H. suis* bacteria stuck to the debris of parietal cells (Figure 4A, right panel). In order to further investigate the co-localization of parietal cells and *H. suis*, a double immunofluorescence staining for H^+/K^+ ATPase and *H. suis* was performed. The majority of the bacteria were observed in the vicinity of or inside the canaliculi or cytoplasm of parietal cells (Figure 4B).

qRT-PCR

Transcriptional changes of crucial genes involved in parietal cell function and gastric epithelial cell homeostasis were determined using qRT-PCR. Results showed that a tendency towards an up-regulation of *Atp4a* was observed in *H. suis* positive pigs compared to negative animals (Figure 5, p=0.14). Compared to *H. suis* negative pigs, a significant up-regulation of *Shh* was observed in *H. suis*-infected slaughter pigs (Figure 5, p=0.012).

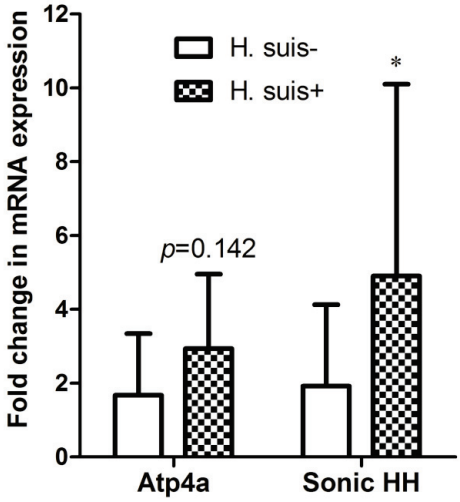

Figure 5 mRNA expression analysis Shown are the mean fold changes (±SD) of mRNA expression in *H. suis* positive pigs (n=15) for *Atp4a* and *Sonic HH*, compared to that in *H. suis* negative pigs (n=15). An * indicates a statistically significant difference (Student t test, $p < 0.05$) between *H. suis* positive pigs (H. suis-) and *H. suis* negative pigs (H. suis+). *Sonic HH*: *sonic hedgehog*.

Discussion

Pig stomachs are frequently inhabited by *H. suis*, a zoonotic bacterium, raising concerns regarding animal welfare, economic interests, public health and food safety (Haesebrouck *et al.*, 2009, De Bruyne *et al.*, 2012, De Cooman *et al.*, 2013). *H. suis* infection can cause a decreased body weight gain and gastritis in pigs (De Bruyne *et al.*, 2012), and chronic gastritis, peptic ulceration and the development of MALT lymphoma-like lesions in humans and rodent models of human gastric disease (Morgner *et al.*, 2000, O'Rourke *et al.*, 2004, Flahou *et al.*, 2010). In the latter, a close association between *H. suis* and parietal cells has been observed and these cells can show signs of degeneration or malfunction (Flahou *et al.*,

2010). In previous studies, it has been described that malfunction of acid secretion by parietal cells is closely associated with the development of gastritis (Chu *et al.*, 2012), indicating that the function of parietal cells might be influenced by gastritis. On the other hand, a direct effect of *H. suis* on the health and function of parietal cells might also be involved. At the onset of this study, very little information was available on the interactions between *H. suis* and parietal cells in its natural host, the pig.

In the present study, we explored and described an effective method for isolation and culture of pig parietal cells. This cell type is highly specialized and differentiated, requiring a specific approach. Our method was based on previously described methods for the isolation of rabbit parietal cells, and to a lesser extent on those described for dogs, rats and mice (Mihi *et al.*, 2013). At first, we used the protocols described for isolation of rabbit parietal cells, however without a great deal of success. Compared to rabbit stomach mucosa, it is more difficult to separate the pig stomach mucosa from the deeper layers, enzymatic digestion is less efficient, and the mucosa is covered by a thick layer of mucus, all of which give rise to some obstacles during the initial isolation of parietal cell. Some reagents that have previously been shown to be useful for the removal of mucus, including N-acetylcysteine and DTT (Risack *et al.*, 1978, Alemka *et al.*, 2010), did not contribute a lot to successful parietal cell isolation in the current study. In addition, some studies have shown that the use of EDTA can disrupt tight junctions between gastric epithelial cells, further facilitating the release of parietal cells from the gastric glands. In our study, however, the administration of EDTA did not exhibit beneficial effects. In view of the existing difficulties, we have optimized some steps that appeared to be essential for isolation of pig parietal cells. These include an adequate removal of mucus by scraping, separating the mucosa in small pieces from the underlying tissue using a sharp blade and taking care to minimize the presence of submucosa and other connective tissues. Finally, using a combination of dispase and collagenase also proved to contribute to the release of parietal cells from the mucosa. Several matrices were tested for their ability to stimulate adhesion of parietal cells to coverslips, including fibronectin, collagen type I, collagen type IV, gelatine and Matrigel. The latter was shown to provide the best results. In general, the majority of the cultured parietal cells existed in the form of single cells or small cellular clumps, and they were shown to remain viable under the described conditions for up to 5 days with a purity of ~80%.

In the present study, histological analysis of the stomachs of *H. suis*-infected pigs at slaughter age, revealed that *H. suis* bacteria are often observed in close vicinity of parietal cells and they even can be observed inside the canaliculi of parietal cells, which reveals a

direct interaction of *H. suis* and parietal cells *in situ*. In addition, a considerable number of *H. suis* bacteria were found near or possibly inside the isolated parietal cells, which further confirmed the direct interplay between this bacterium and parietal cells *in vivo* and *in vitro*. Longer times of incubation of *H. suis* with isolated parietal cells showed similar results, and the most plausible explanation for this may be that a longer incubation time decreases the bacterial viability due to the improper medium and gas environment for this fastidious bacterium, requiring vigorous culture conditions. Future experiments should attempt to identify the involvement of possible adhesins.

Immunohistochemical and immunofluorescent analysis revealed that *H. suis* infection did not greatly affect parietal cell numbers in the stomach of naturally infected pigs. We were, nevertheless, able to show for the first time a direct effect of *H. suis* on the viability of cultured parietal cells. This confirms previous findings, showing that long-term *H. suis* infection can induce necrosis of parietal cells in the stomach of experimentally infected mice and Mongolian gerbils (Flahou *et al.*, 2010) and that swollen and degenerated parietal cells are found in NHPH-infected patients with chronic gastritis (Joo *et al.*, 2007). Additional experiments, using wild-type and Δ*ggt* strains of *H. suis*, showed that the γ-glutamyl transpeptidase of *H. suis*, which has been described to cause death of human gastric epithelial cells *in vitro*, does not seem to play an important role in affecting the viability of pig parietal cells (data not shown). Future experiments should aim to characterize the mechanisms involved. For *H. pylori*, it has been shown that infection can induce apoptosis of cultured rat parietal cells in a nuclear factor-κB and nitric oxide dependent manner (Neu *et al.*, 2002).

Other gastric *Helicobacter* species, including *H. pylori* and *H. felis*, have been described to cause massive parietal cell loss in rodent models, leading to the deregulation of gastric morphology and the development of intestinal metaplasia (De Bock *et al.*, 2006, Murakami *et al.*, 2013). Most likely, the development of gastritis in the corpus region, which is more pronounced compared to *H. suis* infection in these same animal models, contributes largely to this massive loss of parietal cells. Indeed, Feldman et al., have demonstrated a positive correlation between the severity of *H. pylori*-related corpus gastritis and the degree of reduction in acid secretion function of parietal cells (Feldman *et al.*, 1996), and other reports have shown that the development of chronic gastritis in patients with *H. pylori* infection is associated with or causes the loss of parietal cells (El-Omar *et al.*, 2000, Correa, 2005, Oh *et al.*, 2006).

Besides an effect on the viability of parietal cells, *H. suis* may also affect the normal function and homeostasis of parietal cells in particular and the gastric epithelium in general.

In the present study, mRNA expression levels of *Atp4a*, part of the proton pump, showed a trend towards being higher in *H. suis* positive pigs, which may be somewhat surprising, since other studies have shown that *H. pylori* infection can inhibit acid secretion through down-regulation of the expression of H^+/K^+ ATPase, resulting in hypochlorhydria (Saha *et al.*, 2008, Saha *et al.*, 2010, Smolka *et al.*, 2012). However, yet another group of studies have described that *H. pylori* infection can in fact also cause hyperchlorhydria (Smith *et al.*, 1990, Malfertheiner, 2011), depending on the distribution of bacteria within the stomach, the infection stage, the profile of cytokines produced by the local epithelial cells or immune cells, and the pattern of gastritis (Jaup, 2001, Chu *et al.*, 2012). Therefore, the effect of *H. suis* infection on the dynamic changes of expression of H^+/K^+ ATPase as well as the function of parietal cells in the pig stomach needs to be further explored in future experimental studies.

Interestingly, significantly elevated expression levels of *Shh* were demonstrated in *H. suis* positive animals compared to animals free of *H. suis*, suggesting that *H. suis* infection affects the *Shh* signalling pathway. Sonic, India, and Desert hedgehog are important members of the Hedgehog family, playing an essential role during regulation of differentiation and growth of many tissues and cells (Hui *et al.*, 2011). In the stomach of mammals, and especially in the stomachs of mice and humans, Shh has been described to serve as an important regulator in the differentiation of gastric epithelium and immune cells as well as gastric gland morphogenesis (van den Brink *et al.*, 2001, Lowrey *et al.*, 2002). An exclusive expression of Shh is detected in the parietal cells located at the gland-pit boundary in the human stomach, which has been proven to be co-localized with ATPase (van den Brink *et al.*, 2001, Zavros *et al.*, 2008). *H. pylori* infection has been described to induce an overexpression of Shh in mice during the early stage of infection and Shh may have a progressive role in the development of gastric cancer (Shiotani *et al.*, 2008, Schumacher *et al.*, 2012, Marwaha *et al.*, 2014). In addition, other studies have provided evidence that gastrin and gastric acid can stimulate the expression of *Shh*, while *Shh* in turn is also important for maintaining acid secretion, suggesting a feedback mechanism between gastric acid and *Shh* expression (Zavros *et al.*, 2007, El-Zaatari *et al.*, 2010). It is also worth noting that Shh signalling is crucial for macrophage infiltration in the stomach (Schumacher *et al.*, 2012). Indeed, higher numbers of macrophages have been detected in the fundic region of the stomach from BALB/c mice during the initial stages of *H. suis* infection (Flahou *et al.*, 2010).

In summary, an effective method for the isolation and culture of pig parietal cells was established. Direct interactions between *H. suis* and parietal cells were investigated using this *in vitro* cell model as well as *in vivo* in the stomach of pigs at slaughter age. *H. suis* was

shown to interfere with parietal cells, by directly affecting their viability *in vitro*. *H. suis* infection may induce abnormal mRNA expression levels of *Atp4a*, responsible for acid production and regulation. In addition, *H. suis* infection was shown to induce a marked up-regulation of transcriptional levels of *Shh*, a critical factor involved in gastric organogenesis, glandular differentiation, and gastric homeostasis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GZG, BM and BF participated in the design of the study, carried out the experiments, analysed the data and drafted the manuscript. RD participated in the design of this study and drafting of the manuscript. FH and RD coordinatd the study and participated in the design of the study, analysing the data and drafting the manuscript. All authors read and approved the final manuscript.

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Chapter 4
General Discussion

Helicobacter (*H.*) *suis*, a zoonotic microorganism generally colonizing the stomach of more than 60% of the pigs in most herds, causes various gastric diseases in pigs and humans (Haesebrouck *et al.*, 2009, Liu *et al.*, 2014). For a long time, the fastidious nature and unavailability of *in vitro* isolated strains have hampered the study of the pathogenesis of *H. suis* infection. Our research group was the first to successfully isolate this bacterium from the stomach of pigs, eliminating the main obstacle and opening new doors to perform research on this bacterium (Baele *et al.*, 2008). To our knowledge, our research group is currently the only one world-wide performing studies using *in vitro* isolated strains of *H. suis*. For the moment, all available isolates originate from pigs and as far as we know, no pure culture of *H. suis* are available from humans (Baele *et al.*, 2008, Yamamoto *et al.*, 2011, Liu *et al.*, 2014, Yamamoto *et al.*, 2014).

Several studies have shown that *H. suis* can persistently inhabit the host stomach causing chronic inflammation and other gastric diseases. The infection is often not cleared, despite vigorous innate and adaptive immune responses (Flahou *et al.*, 2010). In experimental study 1, we described the inhibitory effect of a *H. suis* virulence factor, γ-glutamyl transpeptidase (GGT), on the proliferation and normal function of lymphocytes from humans and mice, and we further demonstrated that glutamine (Gln) and glutathione (GSH) play an active and important role in the regulation of this effect on T lymphocytes *in vitro*. *H. suis* GGT was shown to impair T lymphocyte proliferation through deprivation of extracellular Gln, and hydrolysis of GSH (an important antioxidant) by the *H. suis* GGT resulted in the enhancement of the inhibitory effect of the enzyme. Besides glucose, certain amino acids (AA) are primary nutrients, important for the proper function and maintenance of eukaryotic cells. These amino acids include Gln, the most abundant free AA in the blood, and also an abundant AA in gut, skeletal muscle, kidney, fetal fluid and milk (Goldberg *et al.*, 1978, Wu *et al.*, 1994, Stumvoll *et al.*, 1999, Kwon *et al.*, 2003). Gln is considered a conditionally essential AA, playing an important role in the normal function and cell cycle of several cell types, including lymphocytes (Yaqoob *et al.*, 1997, Newsholme, 2001). GSH is an important antioxidant for cells in the body of humans and animals, and also in plants (Wu *et al.*, 2004). It is generally accepted that Gln and GSH have significant beneficial roles on the metabolism, function, and health of cells of the gastrointestinal (GI) tract as well as the regulation of cellular events (Wu *et al.*, 2004, Wustner *et al.*, 2014). Our *in vitro* data show that Gln can restore lymphocyte proliferation impaired by *H. suis* GGT, with the production of Glutamate (Glu). Glu can be taken up by *H. pylori*, playing an important role in maintaining the biomass

and function of *H. pylori* (Schilling *et al.*, 2002), which may also be true for *H. suis* (Vermoote *et al.*, 2011). Possibly, the conversion of Gln to Glu, followed by the consumption of Glu by the bacteria may contribute to the aggravation of gastric pathology in hosts infected with *Helicobacter*, since Glu can be taken up and oxidized by epithelial cells and is of great importance for the metabolism of the epithelium in the GI tract (Wu, 2009).

Although in the current studies, we did not investigate in detail the modulatory effect of Gln on the function of lymphocytes in the stomach of hosts infected with *H. suis*, it was shown by others that dietary Gln supplementation can temper gastric inflammation in the stomach of *H. suis*-infected animals (De Bruyne *et al.*, 2013), indicating the potential application of Gln supplementation in clinical patients with *Helicobacter* infection. Besides GGT from *H. pylori* and *H. suis*, the *H. bilis* GGT has also been shown to mediate Gln depletion, leading to reactive oxygen species (ROS)-mediated activation of several inflammatory pathways and subsequent IL-8 secretion in colon cancer cells upon *H. bilis* infection (Javed *et al.*, 2013). Very recently, beneficial effects of Gln as well as GSH were observed in human PBMC treated by GGT from *H. pylori* (Wustner *et al.*, 2014), which partly differs from our study in which Gln and GSH supplementation were shown to have a different modulatory effect on lymphocyte proliferation inhibition caused by *H. suis* GGT. Indeed, we observed that Gln restored normal proliferation of the lymphocytes, whereas supplementation with GSH (2mM) strengthened the *H. suis* GGT-mediated inhibition of proliferation. Wustner and colleagues further demonstrated that *H. pylori* GGT-mediated Gln deprivation in the gastric mucosa may suppress the function of T cells infiltrating the stomach of infected individuals through down-regulation of interferon regulatory factor 4 (IRF4), an important transcription factor for metabolic adaptation of T-lymphocytes, thereby favouring the bacterial persistence at the site of infection in the stomach (Wustner *et al.*, 2014).

H. pylori infection has been shown to trigger miRNA-155 expression in a GGT dependent manner *in vivo* and *in vitro* (Fehri *et al.*, 2010, Oertli *et al.*, 2011). miRNAs are non-protein coding, ~22 nucleotide RNAs that induce translational repression and/or degradation of their mRNA targets (Lim *et al.*, 2005). Accumulating evidence indicates that miRNAs are involved in important biological processes related to apoptosis, proliferation, differentiation, metastasis, angiogenesis and the immune response, with a special role in the development of various cancers (Lu *et al.*, 2005, Spizzo *et al.*, 2009, Wang *et al.*, 2010). In addition, the induction of miRNAs has been observed in a variety of lymphomas, including gastric MALT lymphoma in *Helicobacter* infected patients, which highlights their potential role in gastric tumorigenesis (Garzon *et al.*, 2008, Spizzo *et al.*, 2009, Liu *et al.*, 2010, Craig

et al., 2011). To date, no report is available on the possible regulation of the immune response by *H. suis* through miRNAs. Further experiments should be performed to investigate/determine this.

Results described in experimental study 1 showed that *H. suis* efficiently sheds outer membrane vesicles (OMV) harbouring the GGT. Some factors of these OMV, including GGT, were shown to be able to traverse a tight epithelial cell monolayer, without apparently affecting its integrity, providing a novel route through which *H. suis* may reach and affect cells residing in the lamina propria of the host stomach, including lymphocytes. OMV produced by Gram-negative bacteria contain numerous bacterial wall components and periplasmic constituents, including potential virulence factors (Mashburn-Warren *et al.*, 2008). Series of reports have indicated that OMV can be internalized by cells through endocytosis or membrane fusion (Amano *et al.*, 2010, Ellis *et al.*, 2010a). In our study, the other components of *H. suis* OMV, besides GGT, were not examined. Studies have demonstrated that *H. pylori* OMV harbour urease, GGT, cholesterol, the vacuolating cytotoxin (VacA), the cytotoxin-associated gene A (CagA), the blood group antigen-binding adhesin (BabA), the sialic acid–binding adhesin (SabA), and several other proteins/molecules (Mashburn-Warren *et al.*, 2008, Parker *et al.*, 2012). Based on the available genome information of *H. suis*, urease subunits and cholesterol are most likely also constituents of *H. suis* OMV (Vermoote *et al.*, 2011). In view of the fact that they act as long-distance delivery vehicles and contain potential bacterial surface products and antigens (e.g. GGT and urease), *H. suis* OMV may have an attractive potential for further application in vaccination studies. Indeed, vaccination with a combination of GGT and UreB has been shown to have a protective effect on subsequent *H. suis* challenge in mice (Vermoote *et al.*, 2013). For a number of pathogens, including enterohemorrhagic *E. coli*, *Vibrio cholerae*, and *H. pylori*, it has in fact been shown that immunization with OMV can elicit a significant systemic and mucosal immune response, and confer protection against bacterial challenge in experimental animal models as well as in humans, as shown by preclinical studies (Keenan *et al.*, 2003, Oster *et al.*, 2007, Chen *et al.*, 2010, Bishop *et al.*, 2012, Choi *et al.*, 2014).

Besides its clear role in the pathogenesis of *H. suis* infection, the *H. suis* GGT may also prove to be useful for certain clinical applications. Besides its potential use in vaccination, as described above, preclinical data described by Oertli and colleagues have shown that *H. pylori* infection reprograms dendritic cells towards a tolerogenic phenotype, inducing regulatory T cells (Tregs) with highly suppressive activity in models of allergen-induced asthma (Oertli *et al.*, 2013). They further demonstrated that *H. pylori* GGT is one of the most important factors

involved, further confirmed by the fact that a purified form of GGT can be administered to prevent asthma (Engler *et al.*, 2014). These intriguing data provide the first report about the exploitation of this immunomodulatory factor from helicobacters as a potential therapeutic approach in asthma prevention.

In addition to the *in vitro* studies described in experimental study 1, we investigated, in experimental study 2, the role of *H. suis* GGT in the pathogenesis of infection in rodent models, using an isogenic *ggt* mutant strain of *H. suis*. In this same study, *H. pylori* and corresponding isogenic *ggt* mutant strains were included as a reference. It should be pointed out that the screening of a suitable *Helicobacter* strain, successfully and persistently colonizing the stomach of a certain host, is of great importance for a correct scientific understanding of its pathogenesis (Wirth *et al.*, 1998). Therefore, a preliminary experiment was performed to select a suitable *H. pylori* strain for use in the Mongolian gerbil model. Although Wiedemann et al. showed that *H. pylori* strain B8 successfully adapted to life in the gerbil stomach, by several subculturing steps *in vivo* starting from the B128 parent strain (Wiedemann *et al.*, 2009), our preliminary experiment revealed that this strain was not capable of colonizing the animals used in our study. This also highlights the importance of animal selection, since the animals we used were obtained from a commercial breeder, whereas Wiedemann and colleagues used their own breed of animals.

The isogenic *ggt* mutant of *H. suis* we created is, to our knowledge, the first gene knockout mutant strain worldwide for this fastidious microorganism. For construction of a recombinant suicide plasmid, conventional gene cloning methods sometimes are time-consuming with a low efficiency, high costs, and other disadvantages. These obstacles were also encountered at the beginning of our studies. Therefore, we used a modified and efficient PCR-based strategy for generation of the plasmid construct. This method only requires conventional PCR, inverse PCR, and fusion PCR. Several recombinant plasmids have been constructed successfully and rapidly using this technique described in experimental study 1, resulting in 3 *ggt* isogenic mutants, including a *H. suis* isogenic *ggt* mutant strain and two *H. pylori* isogenic *ggt* mutant strains. Gene disruption or deletion requires homologous recombination at the target locus of the genome in microorganisms, and the size of the homologous arms can influence the efficiency of homologous recombination. The size of the homologous arms of a suicide plasmid for successful gene disruption in microorganisms varies from ~40 to ~1000 bp, depending on the specific microbe (Bahler *et al.*, 1998, Nelson *et al.*, 2003, Gong *et al.*, 2010a, Rossi *et al.*, 2012). For most reported *Helicobacter* isogenic

mutants, homologous arms of these suicide plasmids were mainly between 400 and 1000 bp, which is also the case for our study. Collectively, this PCR-based cloning strategy can greatly facilitate the generation of recombinant suicide plasmids and other kinds of recombinant plasmids with less expense within a minimal period of time, without using traditional restriction enzymes and ligases, and with an unrestricted choice of the insertion site.

For the creation of the *H. suis* mutant, three *H. suis* strains isolated and kept in our lab were used. Only for strain HS5cLP, however, we successfully obtained an isogenic *ggt* mutant. Most likely, the biphasic culture conditions play a role, since we previously showed that a high density of viable bacteria (> 1-5 x 10^7 viable bacteria/ml) is necessary to maintain the cultures and to enable subculture. Possibly, strain HS5cLP is more resistant to the shock induced by electroporation, enabling sufficient bacteria to survive, take up the plasmid and start growing. Recently, however, we developed a method to purify single *H. suis* colonies from solid 1% agar plates (Liang et al., 2015). This may facilitate the generation of *H. suis* mutants in the future.

In Mongolian gerbils used for the experiments described in experimental study 2, increased expression levels of IFN-γ, a T helper (Th) 1 cytokine, were detected and this was shown not to depend on the presence of GGT. This involvement of IFN-γ clearly differs from the immune response evoked in mice infected with pure *in vitro* isolated strains of *H. suis*. Both in BALB/c and C57BL/6 mice, *H. suis* infection has indeed been shown to trigger a Th2-directed response, rather than a Th1 cytokine response (Flahou *et al.*, 2012a). This in turn contrasts with several other studies (Cinque *et al.*, 2006, Mimura *et al.*, 2011), showing an up-regulation of IFN-γ expression in *H. suis*-infected mice. The most likely reasons for the seemingly contradictory results from our group and others, may be that the latter inoculate the animals with stomach homogenate obtained from pigs or cynomolgus monkeys, which not only contains *H. suis*, but also other microorganisms and molecules present in tissue, which may also affect the ultimate immune response of the host. Another possible reason may be that *H. suis* strains associated with monkeys may differ from that in pigs.

The fact that MALT lymphoma(-like lesions) are observed in *H. suis*-infected Mongolian gerbils showing a Th1-predominant immune response in part contrasts with the point of view generally held by many researchers that a Th2 response, rather than a Th1-predominant response, is associated with the development of low grade B-cell MALT lymphoma (Greiner *et al.*, 1997, Knorr *et al.*, 1999, Flahou *et al.*, 2012a, Joosten *et al.*, 2013a). Recently, Yang et al. reported that infection with mucosal homogenate from a *H. suis*-infected cynomolgus monkey induced a significant up-regulation of IFN-γ expression in

mice, and IFN-γ-producing follicular B cells were shown to trigger the expression of CXCL13, contributing to the formation of gastric lymphoid follicles, which may further evolve to the development of gastric MALT lymphoma (Yang *et al.*, 2014). In line with these findings, our present study revealed that *H. suis* infection in mice and Mongolian gerbils induces a substantial up-regulation of CXCL13, an identified B lymphocyte chemo-attractant crucial for the formation of lymphoid follicles and the development of gastric MALT lymphoma (Mazzucchelli *et al.*, 1999, Ansel *et al.*, 2000, Yamamoto *et al.*, 2014). As mentioned above, only in Mongolian gerbils, this was accompanied by increased expression of IFN-γ. Evidence provided by other researchers has shown that several signalling pathways, including those involving Nuclear Factor-κB (NF-κB), tumor necrosis factors-α (TNF- α), and protein kinase C, may contribute to the final induction of CXCL13 (Suto *et al.*, 2009). It is worth noting that, in our study, *H. pylori* infection did not show a significant up-regulation of CXCL13, neither in mice nor gerbils. Therefore, we conclude that experimental *H. suis* infection studies performed in Mongolian gerbils are a good model to investigate the formation of gastric MALT lymphoma and the underlying molecular mechanisms.

Previous studies have revealed that experimental *H. suis* infection in mice and Mongolian gerbils can induce necrosis of parietal cells (Flahou *et al.*, 2010). In order to investigate the interactions between *H. suis* and parietal cells in the natural host, the pig, we developed a method for parietal cell isolation and culture, which is described in experimental study 3. A preliminary exploration was done determining the direct effects of *H. suis* on the function and viability of this important cell type in the stomach. Cultured parietal cells, responsive to secretagogue, could be maintained *in vitro* in good health for at least 3 days. Further immunohistochemical and immunofluorescent analysis showed that *H. suis* is frequently observed in the vicinity of or inside of canaliculi of parietal cell in the fundic region of the stomach from slaughterhouse pigs and also in cultured parietal cells *in vitro*, providing evidence for a direct interaction between *H. suis* and parietal cells. Residing in the deep gastric gland or canaliculi of parietal cells is supposed to be a potential mechanism through which gastric helicobacters evade the immune response or eradication therapy (Taniguchi *et al.*, 1995, Tagkalidis *et al.*, 2002). This remarkable co-localization may, however, affect parietal cell function on the short term and alter gastric physiology on the long term.

Currently, the exact mechanisms are still unknown on how *H. suis* can target parietal cells after having moved through a thick and viscous mucus layer. The mucus layer in the

stomach is the first line of defense against pathogenic microorganisms (Atuma *et al.*, 2001). *H. suis* is tightly coiled with up to 6 turns, and as many as 10 flagella are present at both ends of the cell. Most *H. pylori* bacteria have been described to inhabit the mucus layer, mainly through adherence to mucins using bacterial adhesins (Linden *et al.*, 2002). Adhesins similar to porins related gene B (HorB) and *H. pylori* adhesin A (HpaA) are also encoded by *H. suis* (Vermoote *et al.*, 2011). These flagella and potential adhesins may endow this bacterium with the possibility to move through the thick mucus layer to reach the epithelium (Salama *et al.*, 2013).

Besides the mucus layer, the integrity of the lining epithelium is another vital line of defense against pathogenic microorganisms in the gastric lumen (Laine *et al.*, 2008). Some of the main components of the epithelial paracellular barrier are tight junctions (TJ) and adherens junctions (Farquhar *et al.*, 1963). Claudins are required to form the TJ strands (Powell, 1981, Tsukita *et al.*, 2001) and E-cadherin is one of the main components of adherens junctions. The destruction of cellular junctions can promote the development of gastric disease, including gastritis and gastric cancer (Srivastava *et al.*, 2007, Laine *et al.*, 2008). Several studies have demonstrated that *H. pylori* infection or oral administration of hydrochloric acid can depress or disrupt the expression of several claudin members of epithelial TJ, e.g. claudin 2, 4, 5, 7 and 18, and increase the gastric permeability *in vitro* and *in vivo* (Fedwick *et al.*, 2005, Lapointe *et al.*, 2010, Hayashi *et al.*, 2012, Song *et al.*, 2013, Wroblewski *et al.*, 2014). It is likely that *H. suis* infection in pigs and humans may also affect tight and adherens junctions in the stomach, for instance by inducing loss of epithelial cells, as demonstrated in experimental study 3, or by directly targeting junctional proteins. Indeed, *H. suis* possesses a homologue of the *H. pylori* HtrA, a serine protease which has been shown to degrade E-cadherin (Weydig *et al.*, 2007). These potential mechanisms need to be investigated in the future.

As described in experimental study 3, a significant up-regulation of sonic hedgehog (Shh) was observed in the stomach of *H. suis* positive pigs. The Hedgehog signaling pathways play a crucial role during the embryogenesis, maintenance of adult tissue homeostasis, stem cell differentiation, tissue repair during chronic inflammation, and carcinogenesis (Pasca di Magliano *et al.*, 2003, Katoh *et al.*, 2005, Lees *et al.*, 2005). In the stomach, Shh is expressed by the epithelium and mainly by parietal cells (van den Brink *et al.*, 2001), but the expression of Shh can also be observed in cells at the level of the gastric pit (Suzuki *et al.*, 2005), suggesting that it is not only a short-, but also a long-distance regulator. Studies revealed that Shh is closely associated with the pathogenesis of *H. pylori* infection *in vivo* and *in vitro* (Kim

et al., 2010, Schumacher *et al.*, 2012). Furthermore, it has been shown that frequent activation of the Shh pathway is consistently observed in gastric adenocarcinoma, but also other types of GI cancer (Katoh *et al.*, 2005, Ma *et al.*, 2005, El-Zaatari *et al.*, 2013), and may contribute to the survival and proliferation of tumor cells (Ma *et al.*, 2005, Singh *et al.*, 2010, Yoo *et al.*, 2011). Inhibition of the Shh pathway, for instance through blocking of patched family proteins PTCH1 and PTCH2, the receptors of Shh, or through other mechanisms, resulted in depressed cell proliferation and increased cell apoptosis of gastric cancer cells (Ma *et al.*, 2005, Yan *et al.*, 2013), predicting that the Shh signalling cascade may serve as a therapeutic target for gastric cancer in humans, one of the most common forms of human cancer worldwide.

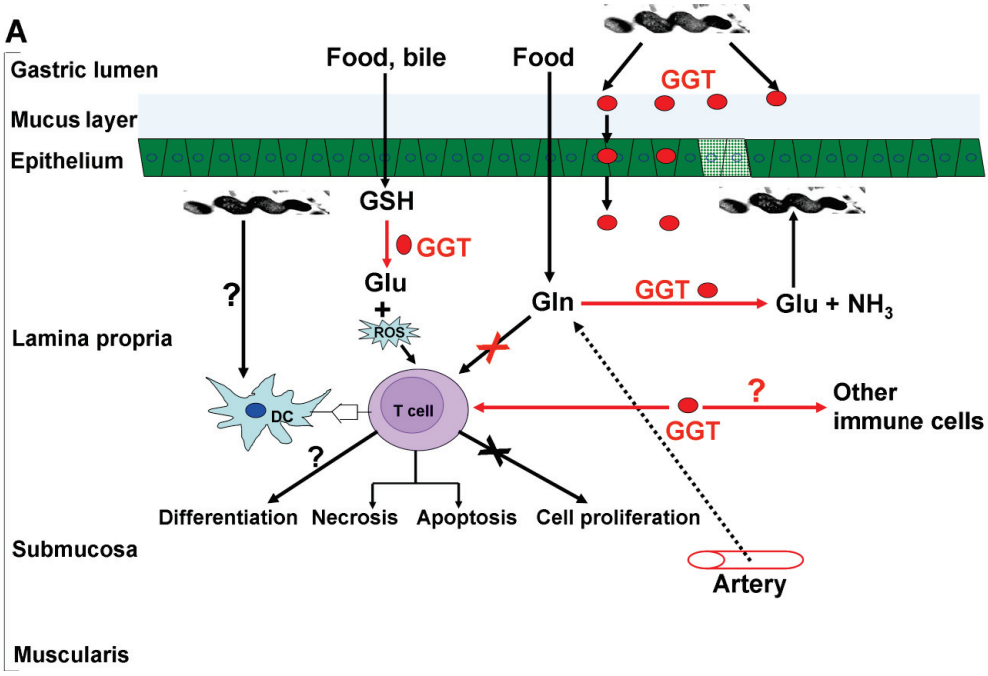
In this thesis, the precise mechanism behind the regulation of parietal cell expression levels of Shh and Atp4A (the α-subunit of H^+/K^+-ATPase responsible for gastric acid secretion) during *H. suis* infection was not investigated. EI-Zaatari and colleagues showed that *H. felis* infection in mice can activate the expression of Shh and also its target factor Gli-1, an anti-apoptotic factor possessing the ability to potentiate the malignant phenotype (El-Zaatari *et al.*, 2007). Very recently, another research group provided evidence that Shh is a target gene of transcription factor NF-κB, and *H. pylori* was shown to induce Shh expression in parietal cells via activation of NF-κB signaling (Schumacher *et al.*, 2014). Indeed, it was shown that NF-κB contributes to the constitutive expression of Shh mRNA in pancreatic cancer cells, and the same group further showed that inflammatory cytokines, e.g. interleukin-1β, TNF-α, and lipopolysaccharide can activate the Shh pathway using the same cell model (Nakashima *et al.*, 2006). Possibly, these pathways also mediate the regulation of Shh during *H. suis* infection, which needs to be explored in future experiments. Since, in parietal cells, Shh co-localizes with H^+/K^+-ATPase and can enhance the expression of Atp4A (El-Zaatari *et al.*, 2010), it is possible that the increased levels of Atp4A in *H. suis* positive slaughterhouse pigs (approximately 6 months old) are a direct consequence of the elevated level of Shh. On the other hand, however, a significant downregulation of Atp4A was demonstrated in mice infected with *H. suis* for 9 weeks. We therefore hypothesize that the effects may depend on the animal model or alternatively, that *H. suis* infection exhibits dynamic effects on the function of parietal cells at different stages of the infection.

In future studies, the effects of GGT on the function of immune cells (T lymphocytes, but also, for instance, DC) residing in the gastric mucosa, as well as the causal relationship between the levels of GGT expression and the immune response *in vivo* should be explored.

Investigating the relevant pathways involved in the induction of CXCL13 during *H. suis* infection can be of great importance for understanding the molecular mechanisms behind the development of MALT lymphoma. It may also be worth investigating the possible involvement of and modulation of the immune response by miRNAs in hosts suffering from *H. suis* infection. This future research may, in the end, also identify potential novel therapeutic targets.

In addition, the in-depth molecular mechanisms illustrating the effect of *H. suis* infection on the function of parietal cells should be further investigated at the level of mRNA and proteins using several approaches, including microarray or proteomics analysis. A better insight into the pathways and mechanisms regulating the acid secretion by parietal cells during *Helicbacter* infection should lead to an improved management of humans and animals with gastric disease and disorders.

The main findings of this thesis, as well as some hypotheses and future perspectives of our studies are summarized in Figure 1.



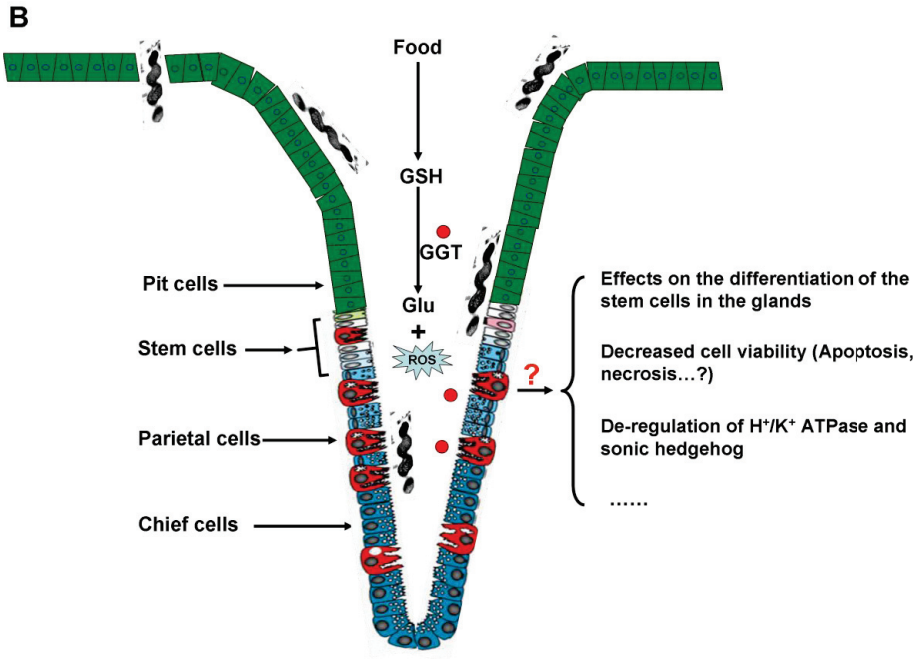


Figure 1. Schematic representation of the identified and possible roles of *H. suis* and its GGT in T cell dysfunction and impaired epithelial health.
(A) *H. suis* GGT was shown to inhibit the proliferation of T lymphocytes, and cell death was involved. Two main substrates of this enzyme, Gln and GSH, were shown to be involved in the modulation of T lymphocyte proliferation, however in an opposite way. Gln and Glu deprivation by GGT affects the normal T cell function, whereas degradation of GSH by GGT affects the function of lymphocytes through the production of ROS. (B) *H. suis* and its GGT were shown to affect proliferation of the gastric epithelium, a close interaction between *H. suis* and parietal cells was demonstrated in the fundic region of pigs. Degradation of GSH by GGT impairs the gastric epithelium through the production of ROS. *H. suis* was shown to be able to directly interfere with cultured pig parietal cells, causing a significant impairment in cell viability. In addition, transcriptional levels of Atp4A and sonic hedgehog were shown to be affected by *H. suis* infection in slaughterhouse pigs. Clearly, more work has to be done to further elucidate the pathogenesis of *H. suis* infection and the role *H. suis* GGT plays.
● GGT/outer membrane vesicles; GGT: gamma-glutamyl transpeptidase; DC: dendritic cell; Gln: glutamine; Glu: glutamate.

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Chapter 5
Summary

Summary

Gastric helicobacters can cause severe gastric diseases, both in humans and animals. In humans, the most prevalent and best-studied gastric *Helicobacter* species is *H. pylori*. Besides *H. pylori*, human patients suffering from gastric disease can be infected with non-*H. pylori Helicobacter* species, of which *H. suis* has been described to be the most prevalent one. *H. suis* is a zoonotic microorganism that is mainly found in pigs, with a prevalence of more than 60% of pigs in most herds and causing reduction in body weight gain, chronic gastritis and other gastric pathologies including gastric ulceration and mucosa-associated lymphoid tissue (MALT) lymphoma. Initially, little information was available on this microorganism, mainly due to its fastidious nature, hampering isolation and cultivation under laboratory conditions. The first successful *in vitro* isolation of *H. suis* from porcine stomachs was done in 2008, opening new doors for in-depth studies on the bacteria-host interactions and pathogenesis of *H. suis* infection in pigs and humans.

Similar to *H. pylori*, *H. suis* often persistently colonizes the stomach of its hosts, eliciting chronic inflammation, development of gastric MALT lymphoma and disruption of gastric epithelial cell homeostasis. The pronounced host immune response is not capable of clearing the infection. At the onset of these studies, no information was available on possible mechanisms through which *H. suis* interferes with the host immune response, leading to persistent infection in the stomach. In addition, little was known on the interactions between *H. suis* and the gastric acid-secreting epithelium and the role certain bacterial virulence determinants play in this process.

In our first study, we describe the effect of γ-glutamyl transpeptidase (GGT), a crucial virulence factor from *H. suis*, on various lymphocyte subsets, including human Jurkat T cells, as well as primary murine T ($CD4^+$ and $CD8^+$) and B ($CD20^+$) lymphocytes. The *H. suis* GGT was shown to inhibit the proliferation of these lymphocytes and eventually cause cell death. Two main substrates of this enzyme, glutamine (Gln) and reduced glutathione (GSH), were shown to be involved in the modulation of T lymphocyte proliferation, however in an opposite way. Gln supplementation restored normal proliferation rates of the lymphocytes, whereas supplementation with GSH aggravated the inhibition of cell proliferation induced by pure *H. suis* GGT. Furthermore, lysate from a wild-type (WT) *H. suis* strain exhibited a stronger inhibitory effect on Jurkat T cell proliferation compared to lysate from an isogenic *H. suis ggt* mutant strain. Finally, *H. suis* outer membrane vesicles, capable of being internalized by cells of a confluent epithelial monolayer, were identified as a possible delivery route for *H. suis* GGT to lymphocytes residing in the deeper mucosal layers. In conclusion, this study

described the effects of *H. suis* GGT on the function of lymphocytes as well as the modulatory actions of Gln and GSH during this process, providing new insights into the pathogenic mechanisms of *H. suis* infection in particular and infection with gastric helicobacters in general.

In order to extend our previous *in vitro* findings and to gain more insight into the role of the GGT in the pathogenesis of *H. suis* infection, we performed experimental infection studies in rodent models of gastric disease using WT and *H. suis* isogenic *ggt* mutant strains. A comparison between the pathogenic role of GGT from *H. suis* and *H. pylori* was also performed in the same study. The results are described in **our second experimental study.** *H. pylori* strains SS1Δ*ggt* (for mice) and PMSS1Δ*ggt* (for Mongolian gerbils) were obtained from WT *H. pylori* strain SS1 or strain PMSS1 using similar techniques as were used for creation of the *H. suis* isogenic *ggt* mutant strain (HS5cLPΔ*ggt*). Mice and Mongolian gerbils were inoculated with *H. suis*, *H. pylori* and their corresponding isogenic *ggt* mutants. At 4 weeks, 9 weeks, and 6 months post infection (pi), four mice from each group were euthanized, and the stomachs were processed for further qRT-PCR analysis and histopathological examination. At 9 weeks pi, gerbils were euthanized and gastric tissues were analyzed in a similar way.

All uninfected mice and gerbils were negative for *Helicobacter* colonization, and showed normal gastric histomorphology at all time-points. In contrast to *H. pylori*Δ*ggt* strains, showing a severely impaired colonization capacity, *H. suis*Δ*ggt* strains were capable of colonizing the stomach at levels comparable to WT strains, although they induced significantly less overall gastric inflammation in mice. Similarly, infection with isogenic *H. pylori ggt* mutant strains induced a significantly lower level of inflammation, compared to infection with WT *H. pylori* strains. Immunohistochemical staining revealed lower numbers of T and B lymphocytes, and generally a lower level of epithelial cell proliferation in HS5cLPΔ*ggt* and SS1Δ*ggt* infected animals, compared to WT infected animals. Furthermore, compared to uninfected gerbils, a clear loss of parietal cells was only observed in the transition zone between corpus and antrum of the stomach from animals with *H. suis* infection.

In contrast to mice, increased IFN-γ expression levels were observed in *H. suis*-infected gerbils. In general, compared to WT strain infection, *ggt* mutant strains of *H. suis* triggered lower levels of Th1, Th2 and Th17 signature cytokine expression. In contrast to *H. pylori* infection, a pronounced up-regulation of B-lymphocyte chemoattractant CXCL13 was observed, both in animals infected with WT and *ggt* mutant strains of *H. suis*. Interestingly,

the *H. suis* GGT was shown to affect the function of the epithelium, as shown by reduced expression levels of Atp4A and Atp4B, responsible for gastric acid secretion, only in WT *Helicobacter*-infected animals. In addition, *H. suis* GGT was associated with down-regulation of the glutamine transporter ASCT2.

Parietal cells in the fundic region of the stomach are highly specialized and programmed to secrete large amounts of hydrochloric acid into the gastric lumen. Gastric *Helicobacter* infection can cause dysfunction of parietal cells and imbalance of acid secretion, and it was previously shown that *H. suis* infection in mice and Mongolian gerbils induced necrosis of parietal cells. At the onset of this thesis, no reports had been published that illustrate the interactions between *H. suis* and parietal cells in pigs, the natural host of this bacterium. Therefore, the aim of **our third experimental study** was to examine the direct effects of *H. suis* on pig parietal cells, both using a newly developed *in vitro* parietal cell culture method and tissues from *H. suis*-infected pigs.

A method for isolation and culture of parietal cells from pig stomach was established, paving the way for *in vitro* experimental studies on the interaction between parietal cells and *H. suis*. A close interaction between *H. suis* and parietal cells was demonstrated using immunohistochemistry staining and immunofluorescent staining in the fundic region from *H. suis* positive pigs. *H. suis* was shown to be able to directly interfere with cultured pig parietal cells, causing a significant impairment in cell viability. Transcriptional levels of Atp4A, the proton pump in parietal cells essential for gastric acid secretion, showed a trend towards an up-regulation in *H. suis* positive pigs compared to *H. suis* negative pigs. In addition, sonic hedgehog, an important factor involved in gastric epithelial differentiation, gastric mucosal repair, and stomach homeostasis, was significantly up-regulated in *H. suis* positive pigs. To our knowledge, this is the first description of the effect of *H. suis* on pig parietal cells *in vivo* and *in vitro*.

In conclusion, this thesis demonstrated the effects of *H. suis* GGT on the proliferation and function of lymphocytes *in vitro*. We further showed that the observed effects depend in part on and can be modulated by the interaction of the enzyme with 2 important substrates, Gln and GSH. Subsequently, experimental infection studies in mice and Mongolian gerbils using WT and isogenic *ggt* mutant strains of *H. suis* confirmed that this enzyme affects the host immune response as well as the health of the gastric epithelium. Finally, we demonstrated an association of *H. suis* for pig parietal cells, which are responsible for

secretion of gastric acid, and *H. suis* was shown to affect the viability and function of this cell type.

Chapter 6
Samenvatting

Gastrale helicobacters veroorzaken ernstige maagaandoeningen bij mensen en dieren. De best bestudeerde en meest voorkomende *Helicobacter* soort bij mensen is *Helicobacter pylori*. Mensen met maagaandoeningen kunnen echter ook geïnfecteerd zijn met andere helicobacters. Van deze zogenaamde "niet-*H. pylori* helicobacters", is *H. suis* de meest voorkomende soort bij mensen. *H. suis* is een zoönotisch micro-organisme dat voornamelijk teruggevonden wordt bij varkens. Bij deze diersoort is tot 90% van de dieren geïnfecteerd en een infectie kan leiden tot een daling van de gemiddelde gewichtsaanzet, chronische maagontsteking en andere maagaandoeningen. Doordat deze bacterie specifieke groeiomstandigheden vereist, vormden isolatie en cultivatie onder laboratoriumomstandigheden steeds een grote uitdaging. In 2008 werd *H. suis* voor de eerste keer succesvol geïsoleerd uit de maag van een varken. Dit maakte nieuwe studies mogelijk met als doel een beter inzicht te bekomen in de bacterie-gastheer interacties en pathogenese van *H. suis* infecties.

Zoals beschreven voor *H. pylori*, is *H. suis* ook in staat de maag blijvend te koloniseren. Vaak wordt een chronische ontstekingsreactie waargenomen, met een verstoorde homeostase van het maagepitheel en een mogelijke ontwikkeling van "Mucosa Associated Lymphoid Tissue (MALT)"-lymfomen tot gevolg. Het afweersysteem van de gastheer is vaak niet in staat om deze ziekteverwekker te elimineren. Bij de start van dit onderzoek was er nog niet veel informatie beschikbaar over de onderliggende mechanismen die *H. suis* in staat stellen om aan het afweersysteem van de gastheer te ontsnappen. Er was ook weinig gekend over de mogelijke interacties van deze bacterie met het maagepitheel, en in het bijzonder de zuur-secreterende epitheelcellen van de maag. Ook over de virulentiefactoren van *H. suis* die een rol in dit proces kunnen spelen, waren geen gegevens beschikbaar.

In een **eerste studie** werd het effect van ɣ-glutamyltranspeptidase (GGT), een belangrijke virulentiefactor van *H. suis*, nagegaan op humane Jurkat T-cellen en op primaire muis T- ($CD4^+$ en $CD8^+$) en B- ($CD20^+$) cellen. Dit *H. suis* GGT was in staat om de proliferatie van lymfocyten te inhiberen en celdood te veroorzaken. Twee belangrijke substraten voor dit enzym zijn glutamine (Gln) en gereduceerd glutathion (GSH). Beide substraten bleken betrokken te zijn in de modulatie van de proliferatie van T-cellen door het *H. suis* GGT. Door Gln te supplementeren aan het cultuurmedium werd de proliferatie van de lymfocyten hersteld. Wanneer echter GSH toegevoegd werd aan het cultuurmedium werd het inhiberend effect van het *H. suis* GGT nog versterkt. Bacterieel lysaat van een wild-type *H. suis* stam

vertoonde een groter inhiberend effect op Jurkat T-cellen in vergelijking met lysaat van een isogene *H. suis* GGT mutant.

H. suis produceert buitenste membraan vesikels, die opgenomen kunnen worden door epitheelcellen. Deze vesikels werden geïdentificeerd als een mogelijke aanleveringsroute om het *H. suis* GGT tot bij de lymfocyten te brengen in de diepere lagen van de mucosa.

De effecten van *H. suis* GGT op lymfocyten *in vitro* werden in hoofdstuk 1 beschreven. Gegevens ontbraken echter nog over het belang van het *H. suis* GGT tijdens een *in vivo H. suis* infectie. Om dit te onderzoeken, werd een experimentele studie uitgevoerd in een muis- en een Mongoolse gerbilmodel. Hierbij werd gebruik gemaakt van een wild-type *H. suis* stam en een deletiemutant waarin het *ggt* gen ontbreekt. In deze studie werd ook het relatieve belang van het GGT in de pathogenese van een *H. suis* en *H. pylori* infectie onderzocht. De resultaten zijn beschreven in **onze tweede studie**. *H. pylori* stam SS1Δ*ggt* (muismodel) en PMSS1Δ*ggt* (Mongoolse gerbilmodel) werden met succes aangemaakt vertrekkende van de wild-type *H. pylori* stammen SS1 en PMSS1. De techniek die hiervoor gebruikt werd, is heel gelijkaardig aan de techniek die gebruikt werd voor het aanmaken van een isogene *H. suis ggt* mutant (HS5cLPΔ*ggt*). Muizen en gerbils werden geïnoculeerd met *H. suis*, *H. pylori* of één van de isogene *ggt* mutanten. Vier weken, 9 weken en 6 maanden na de inoculatie werden 4 muizen van elke groep geëuthanaseerd. De stalen van de maag werden geanalyseerd door middel van qRT-PCR en histopathologisch onderzoek. Negen weken na inoculatie werden de gerbils geëuthanaseerd, waarbij de maagstalen op dezelfde manier werden verwerkt als de stalen van de muizen.

Op geen enkel tijdstip werd *Helicobacter* DNA aangetoond bij de controledieren en deze dieren vertoonden ook geen afwijkingen in de maag bij histopathologisch onderzoek. De *H. pylori*Δ*ggt* stammen vertoonden een sterk verminderde capaciteit om de maag van muizen te koloniseren en er waren, in vergelijking met dieren geïnfecteerd met de wild-type stam, duidelijk minder ontstekingscellen aanwezig. De *H. suis*Δ*ggt* stam daarentegen, was in staat de maag in dezelfde mate te koloniseren als de wild-type stam en de ontstekingsreactie veroorzaakt door deze mutant in het muismodel, was ook minder uitgesproken. Immunohistochemische kleuring toonde aan dat er naast een daling van het aantal T- en B-lymfocyten, ook minder profilerende epitheelcellen aanwezig waren bij deze dieren, in vergelijking met de dieren die geïnoculeerd waren met de wild-type stam. Een verlies aan

pariëtale cellen ter hoogte van de overgang tussen het antrum en het corpus van de maag werd enkel waargenomen bij gerbils die geïnoculeerd waren met de *H. suis* stammen.

Bij de met *H. suis* geïnfecteerde gerbils werd, in tegenstelling tot bij muizen, een verhoogde expressie van INF-ɣ waargenomen. Over het algemeen werd een lagere expressie van Th1, Th2 en Th17 cytokines waargenomen bij dieren geïnoculeerd met de *H. suis ggt* mutant, in vergelijking met de wild-type stam. In tegenstelling tot infectie met *H. pylori*, veroorzaakte *H. suis* infectie, zowel met de wild-type stam als de *ggt* mutant, een uitgesproken expressie van CXCL13, een chemoattractant voor B-lymfocyten. Er werd bovendien aangetoond dat het *H. suis* GGT een rol speelt bij de daling in expressie van Atp4a en Atp4b, verantwoordelijk voor de zuursecretie, en een daling van de expressie van glutamine transporter ASCT2. Dit wijst er duidelijk op dat het GGT van *H. suis* het glutamine metabolisme van het maagepitheel beïnvloedt.

De pariëtale cellen in de fundusklieren van de maag zijn gespecialiseerde cellen die geprogrammeerd zijn om grote hoeveelheden zoutzuur te secreteren in het maaglumen. Een gastrale *Helicobacter* infectie kan dysfunctie van deze pariëtale cellen en een verstoorde balans van de zuursecretie veroorzaken. Het is reeds vroeger aangetoond dat een *H. suis* infectie in muizen en Mongoolse gerbils necrose van pariëtale cellen induceert. Bij aanvang van dit doctoraatsonderzoek waren er nog geen studies gepubliceerd die een interactie beschrijven tussen *H. suis* en pariëtale cellen uit de maag van varkens, de natuurlijke gastheer van deze bacterie.

De doelstelling van de **derde studie** was om de directe effecten van *H. suis* op pariëtale cellen van varkens na te gaan. Eerst werd een methode ontwikkeld voor de isolatie en het in cultuur brengen van pariëtale cellen uit varkensmagen, wat het mogelijk maakt om deze cellen te gebruiken voor *in vitro* studies. Een interactie tussen *H. suis* en pariëtale cellen werd aangetoond door middel van immunohistochemie en immunofluorescente kleuring van de fundusklieren van met *H. suis*-geïnfecteerde varkens. Eenzelfde interactie werd gezien tussen *H. suis* en *in vitro* geïsoleerde pariëtalen cellen, die bovendien een verminderde leefbaarheid vertoonden. Het transcriptie niveau van ATP4a, de protonpomp van pariëtale cellen die essentieel is voor de maagzuursecretie, vertoonde verrassend genoeg een verhoogde expressie bij met *H. suis* geïnfecteerde varkens, net als het ‘Sonic hedgehog’ eiwit. Dit is een belangrijke factor die betrokken is in differentiatie van het maagepitheel en in het herstel en de homeostase van de maagmucosa.

Samengevat hebben we in deze thesis duidelijk de effecten van *H. suis* GGT aangetoond op de proliferatie en functie van lymfocyten *in vitro*. Verder konden we aantonen dat deze effecten gedeeltelijk afhankelijk zijn van en gestuurd kunnen worden door de interactie van dit enzym met 2 belangrijke substraten, Gln en GSH. Experimentele infectiestudies bij muizen en Mongoolse gerbils bevestigden dat het GGT de immuunrespons van de gastheer beïnvloedt, alsook de gezondheid van het maagepitheel. Tot slot toonden we een duidelijk tropisme van *H. suis* aan voor de pariëtale cellen uit de maag van varkens en we konden waarnemen dat *H. suis* de viabiliteit en functie van dit celtype aantast.

Curriculum Vitae

Curriculum Vitae

Guangzhi Zhang was born in the city of Hengshui, Hebei province, China, on 01st Aug, 1983. He finished his bachelor study with the major of veterinary medicine in the Agricultural University of Hebei on July, 2007. He started his master study with the major of preventive veterinary medicine in The National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University on Sep, 2007, and his research topic was the study of apoptosis of dendritic cells induced by *Mycobacterium bovis* strains with different virulence. This work was performed under the supervision of Prof. Aizhen Guo. He completed his master studies in July, 2010. After that he continued to pursue his doctoral studies in the lab of bacteriology from the faculty of veterinary medicine, Ghent University. This work was supported by a grant from the China Scholarship Council (Grant No. 2010676001; October 2010) and by grants from the Research Fund of Ghent University, Ghent, Belgium (Grant No. GOA01G00408 and 01SC2411). His doctoral research topic is the interaction of *H. suis* and its virulence factors with several hosts, with an emphasis on the effects on lymphocytes and the gastric epithelium. This was performed under the guidance of Prof. Freddy Haesebrouck and Dr. Bram Flahou. His research has resulted in several publications in leading journals and his results were also presented at several international academic conferences.

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List of Publications in National and International Journals

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* Shared the senior authorship.

List of Attended International Academic Conferences

1. **Guangzhi Zhang**, Richard Ducatelle, Frank Pasmans, Freddy Haesebrouck, Bram Flahou. Effects of *Helicobacter suis* γ-glutamyl transpeptidase and its substrates on lymphocytes. (2012) 25th International workshop on *Helicobacter* and Related Bacteria in Chronic Digestive Inflammation and Gastric Cancer. Ljubljana, Slovenia. 2012.09.13-2012.09.15.
2. **Guangzhi Zhang**, Ellen De Bruyne, Frank Pasmans, Koen Chiers, Richard Ducatelle, Freddy Haesebrouck, Bram Flahou. Modulation of *Helicobacter suis*-induced inflammation by glutamine and reduced glutathione. (2012) 10th International workshop on Pathogenesis and Host Response in *Helicobacter* Infections. Helsingor, Denmark. 2012.07.04-2012.07.07.
3. Bram Flahou, Richard Ducatelle, Frank Pasmans, Koen Chiers, Annemieke Smet, Miet Vermoote, Lien De Cooman, **Guangzhi Zhang**, Jungang Liang, Freddy

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广智

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