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Effect of *Helicobacter* Gamma-glutamyl transpeptidase on epithelial cells

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Declaration

I solemnly declare that I have written the dissertation entitled

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and submitted to the Faculty of Medicine of the Technical University Munich for doctoral examination at the

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Munich, 30th October 2012

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Signature

In memory of my beloved mother, Yasmin Javed who has been an inspiration to me…

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Abbreviations

Akt	Ak-thymoma a.k.a Protein Kinase B (PKB)
AP-1	Activator protein 1
ATP	Adenosine tri phosphate
BHI	Brain heart infusion
BSA	Bovine serum albumin
BrdU	5-Bromo-2´-deoxyuridine
cagA	Cytotixic associated gene A
cagPAI	Cytotoxicity associated gene pathogenicity island
Cdk	Cyclin dependent kinases
Cdt	Cytolethal distending toxin
COX-2	Cyclooxygenase 2
CRE	Cyclic AMP response element
CREB	Cyclic AMP response element binding protein
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco’s Eagle's minimal essential medium
DMSO	Dimethyl sufoxide
DTT	Dithiothreitol
DupA	Duaodenal ulcer promoting protein A
EBP	Enhancer binding protein
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immune assay
ELISA	Enzyme linked immunosorbent assay
ERK	Extra cellular signal regulated kinase
FACS	Florescence activated cell sorting
FCS	Fetal calf serum
FITC	Florescin isothiocynate
FlaA/B	Flagellin A/B
GSH	Glutathione
gGT	gamma-Glutamyl transpeptidase
HBgGT	*H. bilis* gamma-Glutamyl transpeptidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPgGT	*H. pylori* gamma-Glutamyl transpeptidase
HRP	Horse radish peroxidase
HtrA	High temperature requirement protein A
HSgGT	*Homo sapien* gamma-Glutamyl transpeptidase
IBD	Inflammatory bowel disease
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL-6/8	Interleukin 6/8
IFNγ	Interferon gamma
iNOS	Inducible nitric oxide synthase
ISRE	Interferon stimulated response element
JNK	c-Jun N-terminal kinases
kAC	Potassium acetate
KCl	Potassium chloride
KOH	Potassium hydroxide
LB	Lysogeny broth
LPS	Lipopolysaccharide
MALT	Mucosa associated lymphoid tissue
MAPK	Mitogen activated protein kinase
MEK	Mitogen-activated protein kinase/extracellular signal-regulated kinase
MEROPS	The database of proteolytic enzymes, their substrates and inhibitors.
MOI	Multiplicity of infection
NaCl	Sodium chloride
NBT	Nitroblue tetrazolium chloride
NCBI	National center for biotechnology information
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
OD	Optical density
OMV	Outer membrane vescicles
OipA	Outer inflammatory protein A
PBS	Phosphate buffered saline

PI3K	Phospho inositol 3 kinase
PI	Propidium Iodide
RAS	Rat sarcoma
RAF	Rapidly accelerated fibrosarcoma
RPMI	Roswell Park Memorial Institute medium
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
TAK	Transforming growth factor β–activated kinase
TRAF	TNF receptor associated factors
TLR	Toll like receptor
TCA cycle	Tricarboxylic acid cycle
TEMED	Tetramethylethylenediamine
TRIS	Tris (hydroxymethyl) aminomethane
TNFα	Tumor necrosis factor α
UreA/B	Urease A/B
VacA	Vacuolating cytotxin A
WC-Dent	Wilkins-Chalgren-*H. pylori* selection (Dent)
ΔgGT	Knockout gamma-Glutamyl transpeptidase

Abstract

Helicobacter pylori (H. pylori) is the best characterized human pathogen in the *Helicobacter* family. Other *Helicobacter* species have also been detected in human clinical specimens, including *Helicobacter bilis (H. bilis)* which is associated with a higher incidence of IBD, thyphlocolitis, hepatitis and cholecystitis. However, little is known about its virulence determinants.

Bacterial γ-glutamyltranspeptidase (gGT) enzyme plays a key role in synthesis and degradation of glutathione and enables the bacterium to utilize extracellular glutamine and glutathione as sources of glutamate. In *H. pylori*, gGT plays an essential role in the colonization of the gastric mucosa and development of peptic ulcer disease in *H. pylori* infected individuals. Both *H. pylori* (HPgGT) and *H. bilis* (HBgGT) gGT induce similar apoptosis-independent suppression of gastric and colon cancer epithelial cell proliferation, supporting a conserved function for gGT in the pathogenesis of *Helicobacter* genus.

Transcriptional dysregulation in host cells is a mechanism employed in pathogenesis induced by *H. pylori* and *H. bilis*. gGT mediated loss of cell viability has so far been linked to DNA damage via oxidative stress but the signaling cascades involved have not been described. In this study, HPgGT and HBgGT recombinant proteins were shown to activate the transcriptional activity of CREB, AP-1 and NF-κB. NFAT activation by HPgGT was also observed. Activation of these pathways was translated into an infection setup in case of *H. bilis*, whereas other more potent virulence factors of *H. pylori* are known to play prominent roles here. In *H. bilis,* infected epithelial cells stimulation of these pathways was accompanied by the protein expression of c-jun as well as phosphorylation and subsequent activation of CREB and IkBα in a gGT dependent manner. Together, these transcription factors might be important regulators in the induction of a pro-inflammatory environment. Therefore, regulation of IL-8, a common downstream target of these transcription factors was studied. Upregulation of IL-8 in *H. bilis* infected cells was observed and was found to be partly dependent on gGT.

The regulation of these host cell responses by HBgGT could be linked to a dual activation mechanism, glutamine deprivation and increased superoxide production. Taken together, these results indicate that *Helicobacter* gGT modulates the activation of certain oxidative stress response cascades culminating in increased IL-8 production by the epithelial cells,

thereby inducing a pro-inflammatory environment in the mucosal tissue. This study implicates gGT as an important regulator of inflammation in *H. bilis* infection induced colitis.

Zusammenfassung

Helicobacter pylori (*H. pylori*) ist zwar das am besten charakterisierte humane Pathogen der *Helicobacter* Familie, jedoch wurden auch andere *Helicobacter* Spezies in humanen Biopsieproben gefunden. Unter anderem wurde *Helicobacter bilis* (*H. bilis*) entdeckt, ein Bakterium, welches häufig mit einem Auftreten von chronisch entzündlicher Darmerkrankung, Thyphlocolitis, Hepatitis und Cholezystitis assoziiert ist. Jedoch ist wenig über die virulenzdeterminierenden Faktoren bekannt.

Das bakterielle γ-Glutamyltranspeptidase Enzym (gGT) spielt eine entscheidende Rolle im Glutathion Stoffwechsel und ermöglicht es dem Bakterium, extrazelluläres Glutamin und Glutathion als Quelle für Glutamat zu verwenden. Des Weiteren kommt diesem Enzym eine essentielle Rolle bei der Kolonisation der gastrischen Mukosa und bei der Entwicklung von Magengeschwüren in *H. pylori* infizierten Individuen zu. *H. pylori* gGT (HPgGT) und *H. bilis* gGT (HBgGT) verursachen beide eine Apoptose-unabhängige Hemmung der Proliferation von epithelialen Tumorzellen aus Magen und Darm, was auf eine konservierte Funktion der gGT verschiedener *Helicobacter* Gattungen in der Pathogenese hinweist.

In Wirtszellen kommt dem Mechanismus der transkriptionellen Deregulation in der Pathogenese eine wichtige Rolle zu. Der durch gGT verursachte Verlust der Lebensfähigkeit von Zellen wurde bisher nur auf Schäden in der DNA, verursacht durch oxidativen Stress, zurückgeführt, aber die involvierten Signalkaskaden blieben uncharakterisiert. In dieser Studie wurde gezeigt, dass die rekombinanten HPgGT und HBgGT Proteine die transkriptionelle Aktivität von CREB, AP-1 und NF-kB aktivieren können. Des Weiteren konnte eine Aktivierung von NFAT durch HPgGT beobachtet werden.

Da diese Steigerung der transkriptionellen Aktivität jedoch nur bei *H. bilis* zu einer entsprechend verstärkten Infektion führt und außerdem für *H. pylori* die beschriebene transkriptionelle Aktivierung durch verschiedene potente Virulenzfaktoren hervorgerufen werden, wurden die weiteren Analysen der nachgeschalteten Regulations prozesse nur mit *H.bilis* durchgeführt. In mit *H. bilis* infizierten epithelialen Zellen wurde die gGT abhängige Stimulation dieser Signalwege sowohl von Proteinexpression von c-jun, also auch von Phosphorylierung und Aktivierung von CREB und IkBα begleitet. Diese Transkriptionsfaktoren können wichtige Regulatoren bei der Induktion einer entzündlichen Antwort sein. Aus diesem Grund wurde die Regulation der IL-8 Expression genauer

untersucht. Die erhöhte Transkription von IL-8 in *H. bilis*-infizierten Zellen war teilweise von gGT abhängig.

Diese HBgGT induzierten Effekte in Tumorzellen konnte auf 2 Ursachen zurückgeführt werden: den Mangel an Glutamin und eine erhöhte Superoxid-Produktion in Gegenwart von gGT. Diese Resultate deuten darauf hin, dass die *Helicobacter* gGT die Aktivierung einiger oxidativer Stress-Antwort-Kaskaden moduliert, was zu einer erhöhten IL-8 Produktion führt und dadurch eine entzündungsfördernde Umgebung in Geweben induziert. Dementsprechend stellt die HBgGT einen wichtigen Entzündingsregulator bei der durch eine *H. bilis* induzierten Colitis dar.

Chapter 1: Introduction

1 *Helicobacter* genus

Helicobacter is a genus of Gram-negative bacteria possessing a characteristic helical shape. Initially classified as members of the *Campylobacter* genus, they have since 1989 been re-grouped as a separate genus. The *Helicobacter* genus belongs to class *Epsilonproteobacteria*, order *Campylobacterales*, family *Helicobacteraceae* and already includes more than 35 species (Boyanova, Mitov et al. 2011).

The key features ascribed to the bacteria belonging to this genus are:

i) Gram negative.
ii) Helical, curved or straight unbranched morphology.
iii) Rapid darting cell motility by means of sheathed flagella that may be uni polar or bipolar and lateral with terminal bulbs.
iv) An external glycocalyx produced *in vitro* in liquid culture.
v) Absence of hexadecanoic acids in the major fatty acid profiles.
vi) Optimal growth at 37˚C ; growth at 30˚C but not at 25˚C ; variable growth at 42˚C.
vii) Microaerophilic, variable growth in air enriched with 100mL/L -CO2 and anaerobically.
viii) Susceptibility to penicillin, ampicillin, amoxicillin, erythromycin, gentamicin, kanamycin, rifampin and tetracycline. Resistance to nalidixic acid, cephalothin, metronidazole and polymysin.
ix) 35-44% GC content of chromosomal DNA (Goodwin, Bell et al. 1989; Bronsdon, Goodwin et al. 1991; Vandamme, Falsen et al. 1991).

Bacteria belonging to the *Helicobacter* genus manifest themselves in a broad range of gastrointestinal niches, some species colonizing the upper gastrointestinal tract like *Helicobacter bizzozeronii (H. bizzozeronii), Helicobacter heilmannii (H. heilmannii)* (Paster, Lee et al. 1991; Kemper, Mickelsen et al. 1993; Schauer, Ghori et al. 1993; Baele, Decostere et al. 2008), as well as the liver of mammals and some birds e.g; *Helicobacter hepaticus (H. hepaticus)* and *Helicpobacter bilis (H. bilis)* (Fox, Dewhirst et al. 1994), while others are prevalent in the lower gastrointestinal tract, e.g; *Helicobacter muridarum (H. muridarum)*

(Stanley, Linton et al. 1993; Fox, Yan et al. 1995; Fox, Chien et al. 2000). The most investigated of the *Helicobacter genus* member is *Helicobacter pylori (H. pylori)*.

1.1 *Helicobacter pylori*

H. pylori infection is the most prevalent bacterial infection worldwide, affecting approximately 50% of the world`s population. (Lacy and Rosemore 2001). *H. pylori* was first described by Marshall and Warren in 1984. They described the bacterium to be present in almost all patients with active chronic gastritis, duodenal ulcer, or gastric ulcer and speculated that it might be an important perpetrating factor in these diseases (Marshall and Warren 1984). Due to the overwhelming consequences of *H. pylori* infection like higher risk for the development of gastric carcinoma and mucosa associated lymphoid tissue (MALT) lymphoma in infected individuals, it has been classified as a class 1 carcinogen by World Health Organization (WHO).

1.2 *Non-pylori Helicobacter* species

H. pylori remains the best characterized human pathogen in the *Helicobacter* family; however other *Helicobacter* species have also been detected in human clinical specimens (Boyanova, Mitov et al. 2011). Several non- pylori *Helicobacter* species (NPHS) have been isolated from diseased tissue. NPHS are further categorized into two sub groups based on different organ specificity, gastric *Helicobacter* species (GHS) and enterohepatic *Helicobacter* species (EHS).

1.2.1 Gastric *Helicobacter* species

GHS include *Helicobacter* species colonizing the stomach of a broad range of hosts. *Helicobacter suis (H. suis), Helicobacter felis (H. felis), H. bizzozeronii, H. heilmannii* and *Helicobacter salomonis (H. salmonis)* are some of the GHS associated with chronic gastritis and peptic ulcers in humans, with a higher risk for developing MALT lymphoma (Boyanova, Mitov et al. 2011). Aside from *H. pylori, H. heilmannii* is probably the most abundant GHS found in human clinical specimens (Kusters and Kuipers 1998).

1.2.2 Enterohepatic *Helicobacter* species

EHS are a phenotypically and genotypically heterogeneous phylogroup within the *Helicobacter* genus including species colonizing the intestinal tract and/or the liver of mammals and birds (On SL. et al; 2005). Although some EHS are present as part of the normal microbiota of rodents, others may cause disease in these animals (Solnick and Vandamme 2001).

EHS including *H. hepaticus* and *H. bilis* have been detected in hepatobiliary diseased patient specimens. *H. bilis*, *H. hepaticus*, and *Helicobacter pullorum (H. pullorum)* have been associated with the development of Crohn's disease, Inflammatory bowel disease (IBD) and ulcerative colitis (Stanley, Linton et al. 1994; Maggio-Price, Bielefeldt-Ohmann et al. 2005; Jergens, Wilson-Welder et al. 2007; Liu, Ramer-Tait et al. 2011). Importantly, some species such as *H. hepaticus*, *Helicobacter mustelae (H. mustelae)* and *H. bilis* exhibit carcinogenic potential in animals (Ward, Fox et al. 1994; Foltz, Fox et al. 1998; Fox, Dewhirst et al. 1998; Maggio-Price, Bielefeldt-Ohmann et al. 2005).

In summary, many NPHS, some gastric and others enterohepatic are increasingly being recognized for their role in veterinary and human diseases. Some commonly known NHPS associated with diseases are summarized in Table 1. Of the EHS, *H. bilis* presents the most interesting example because of its broad range of hosts, variable niche as well as its link to inflammatory diseases and carcinogenic potential. This versatile bacterium will be discussed in the following sections and was part of my investigations.

1.2.2.1 *Helicobacter bilis*

Recently, a relatively less characterized *Helicobacter* species, *H. bilis*, has come to the attention of researchers. *H. bilis* is endemic in most experimental mice facilities and may induce disease in susceptible animals (Fox 2007). The bacterium was isolated from the aborted fetus of sheep and pig and possesses one of the broadest host spectrums of the *Helicobacter* genus (Rossi, Zanoni et al. 2010). *H. bilis* infection has been associated with a higher incidence of typhlocolitis (Jergens, Wilson-Welder et al. 2007; Liu, Ramer-Tait et al. 2011), hepatitis (Shomer, Dangler et al. 1997), IBD (Fox, Dewhirst et al. 1994), and cholecystitis (Fox, Dewhirst et al. 1998) in animals. In humans, it has been associated with chronic liver diseases (Fox, Dewhirst et al. 1998; Vorobjova, Nilsson et al. 2006) and biliary

tract and gall bladder cancer (Matsukura, Yokomuro et al. 2002; Murata, Tsuji et al. 2004). *H. bilis* has also been isolated from diseased human patients with chronic diarrheoa (Romero, Archer et al. 1988) and pyoderma gangrenosum like ulcers (Murray, Jain et al. 2010). Despite its high prevalence and possible role in several diseases, limited data are available on virulence determinants of *H. bilis*.

Taxonomic analysis of *H. bilis* strains isolated from dogs and cats showed two different genomic groups to be present with a suggested independent evolution that might be referred to as two genomospecies, namely the *H. bilis sensu stricto* and *Helicobacter* sp. 'FL56' (Rossi, Zanoni et al. 2010).

A) **B)**

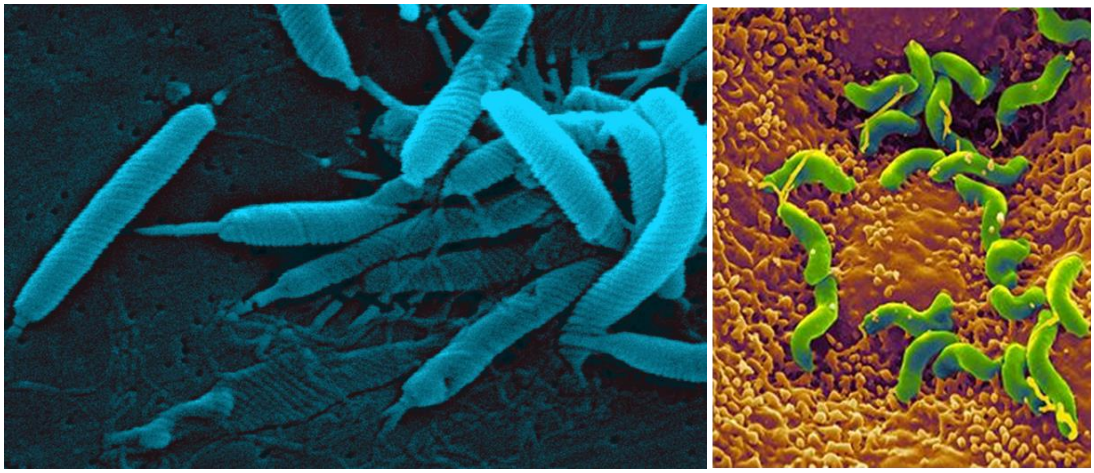


Figure 1: Scanning electron micrograph of *H. pylori* and *H. bilis*

Scanning electron micrograph of A) *H. bilis* (formerly classified in *Flexispira rappini*), an enterohepatic *Helicobacter specie* (image courtesy of Dr. Patricia Fields, Dr. Collette Fitzgerald. Public content CDC library). B) *H. pylori*, a gastric *Helicobacter specie* (image courtesy of Diasource).

Table 1: *Helicobacter* species isolated from diseased animals (GHS: Gastric *Helicobacter* spp., EHS: Enterohepatic *Helicobacter* spp.)

	***Helicobacter* species**	**Host/ reservoir**	**Disease**	**Frequency**	**Virulence factors**	**Reference**
GHS	*H. pylori*	Humans, primates, pigs	Gastritis, peptic ulcers, gastric adenocarcinomas	Common	Urease, catalase, CagPAI, VacA	(Marshall and Warren 1984)
	H. bezzozeroni	Dogs, humans	Gastric dyspepsia	Common	Urease, catalase, cdt	(Jalava, On et al. 2001)
	H. felis	Cats, dogs	Gastritis, Colitis	Common	Urease, catalase, cdt	(Paster, Lee et al. 1991)
	H. suis	Pigs, humans	Gastritis	Common	Urease, catalase	(Baele, Decostere et al. 2008)
EHS	*H. cinaedi*	Humans, hamsters	Gastroenteritis, septicemia, proctocolitis, cellulitis	Uncommon	Catalase, cdt	(Kemper, Mickelsen et al. 1993)
	H. fennelliae	Humans	Gastroenteritis, septicemia, proctocolitis	Uncommon	Catalase, cdt	(Totten, Fennell et al. 1985)
	H. rappini	Humans, sheep, mice	Gastroenteritis	Rare	Urease, catalase	(Schauer, Ghori et al. 1993)
	H. canadensis	Humans, pigs, geese	Diarrhea	Common	Catalase	(Fox, Chien et al. 2000)
	H. canis	Dogs, cats, humans	Crohn´s disease, diahrrea, hepatitis	Common	Cdt	(Stanley, Linton et al. 1993)
	H. pullorum	Chickens, humans, mice	Crohn´s disease, gastro-enteritis	Common	Catalase, cdt	(Stanley, Linton et al. 1994)
	H. muridarum	mice	Gastritis, hepatitis	Common	Urease, catalase,	(Hannula and Hanninen 2007)
	H. bilis	Humans, mice, pigs, hamsters, dogs, cats	Typhlocolitis, hepatitis, IBD, cholecystitis	Common	Urease, catalase, cdt	(Fox, Yan et al. 1995)
	H. hepaticus	Humans	Hepatitis, hepatocellular carcinoma, IBD	Common	Urease, catalase, cdt	(Fox, Dewhirst et al. 1994)

2 *Helicobacter* infections induce host cell pathogenesis

Helicobacter infection has long since been implicated in host pathology. The above mentioned associations between various *Helicobacter* infections and disease (Table 1) make it important to investigate the underlying host cell modifications like changes in transcriptional regulation of the host cell, especially those involved in inflammatory responses, leading to the development of gastritis, IBD as well as hepatobiliary disorders.

2.1 *Helicobacter* infections induce host cell transcriptional dysregulation

As mentioned previously, *H. pylori* is the most well known member of the *Helicobacter* genus with a high prevalence, therefore it has been the subject of many investigations. A large proportion of these studies focus on the alterations in host cells leading to ulceration and inflammation upon infection. Hence *H. pylori* presents an interesting example for future reference and comparison of *Helicobacter* induced infections. The host transcriptional changes caused by *H. pylori* infection may give useful hints as to which pathways might be also involved in other *Helicobacter* spp. induced infections.

Gastric mucosal transcription factors induced by *H. pylori* infection differ according to the phase and outcome of infection; where AP-1 and CREB levels are the early responders related to inflammation and ulceration, whereas NF-κB and ISRE are the late responders related to atrophy (Kudo, Lu et al. 2007). All these transcriptional regulators are regulated through the Mitogen activated protein (MAP) kinases, a key pathway controlling many homeostatic functions in the cell. Other reports have described extensive activation of mitogen activated protein kinases in *Helicobacter* infected epithelial cells.

Mitogen-activated protein kinases are proline-directed serine/threonine kinases that are activated by dual phosphorylation on threonine and tyrosine residues in response to a wide array of extracellular stimuli. Three distinct groups of MAP kinases have been identified in mammalian cells, namely the c-Jun N-terminal kinases JNK, p38 and/or extracellular regulated kinase (ERK). These MAP kinases are mediators of signal transduction from the cell surface to the nucleus. MAP kinases typically form multi-tiered pathways, receiving input several levels above the actual MAP kinase. These include MAP kinases which have a

phosphorylation-dependent activation mechanism and MAP2Ks, MAP3Ks which require multiple steps for their activation. One such MAP3K is c-Raf, which is involved upstream of the MEK and ERK1/2 pathway (Cargnello and Roux 2011). Constitutive activation of the Raf/MEK/ERK pathway is central to malignant transformations in many human tumors (Hoshino, Chatani et al. 1999; McCubrey, Steelman et al. 2007). JNK and p38 signaling pathways are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation and apoptosis. JNKs have a number of dedicated substrates that can phosphorylate (c-Jun, NFAT4, etc.), while p38 MAPK also has some unique targets ensuring the need for both in order to respond to stressful stimuli (Cargnello and Roux 2011). Another feature of the MAPK pathway is its activation and phosphorylation, including ERK, JNKs, p38 kinase, and the phosphoinositide 3 signaling protein (PI3K) via sensitive cysteine rich domains by reactive oxygen species, leading to increased gene transcription (Thannickal and Fanburg 2000). In addition, it has been reported that JNK is constitutively activated in several tumor cell lines and that the transforming actions of several oncogenes have been reported to be JNK dependent (Ip and Davis 1998). Activation of MAPK by *H. pylori* has been reported in several investigations and plays a central role in the subsequent activation of different pathways implicated in *H. pylori* induced pathology (Backert and Naumann 2010). Quite recently it was observed that *H. bilis* infected Huh7 cells showed increased c-met and KI-Ras expression both of which signal through the Ras/Raf/MEK/ERK cascade (Okoli, Sanchez-Dominguez et al. 2012).

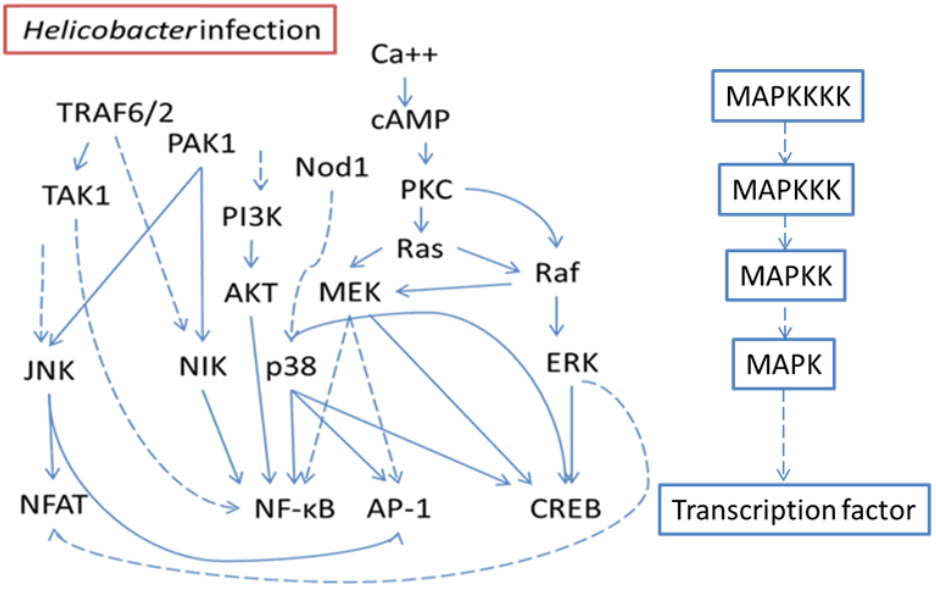


Figure 2: *Helicobacter* infection induced MAPK signaling cascades involved in transcription factor activation

Several transcription factors responsible for inducing cellular responses to stress are present downstream of the MAPKs. One of the most frequently associated transcriptional inducer activated by stress signals conducted by MAPKs is **Nuclear factor κB (NF-κB).** NF-κB is a protein complex found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation and bacterial antigens (Gilmore 2006). All proteins of the NF-κB family share a Rel homology domain in their N-terminus. NF-κB family of proteins include RelA, RelB, and c-Rel, which have a transactivation domain in their C-terminus, forming one sub-family (Karin and Delhase 2000). The second subset of NF-κB proteins include NF-κB1and NF-κB2, synthesized as precursors p105 and p100, which are processed to generate the mature NF-κB subunits, p50 and p52, respectively (Senftleben, Cao et al. 2001). NF-κB plays a key role in regulating immune response to infection. Consistent with this role, incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases. Many studies have implicated a constitutive activation of NF-κB in various malignant cells, such as lymphomas, leukemias, breast cancers, melanomas, pancreatic, and colorectal cancers (Nakshatri, Bhat-Nakshatri et al. 1997; Wang, Abbruzzese et al. 1999; Lind, Hochwald et al. 2001). The NF-κB signalling cascade involves several protein complexes which signal through the canonical (classical) or the non-canonical pathway. The canonical pathway is the major pathway involved in inflammation (Monaco, Andreakos et al. 2004). NF-κB subunit p65 is involved in the

canonical NF-κB pathway. p65 is retained in the cytoplasm by an inhibitory complex formed of IKK proteins. Phosphorylation of IKK proteins leads to release of p65, which can then translocate into the nucleus and initiate transcriptional activity of downstream target genes (Jacobs and Harrison 1998).

H. pylori infection leads to the activation of NF-κB through various signalling cascades and is central to the stimulation of a pro inflammatory environment. Several reports demonstrate activation of NF-κB via NIK involving TRAF6/2 and PAK1, others describe the activation to be mediated by MyD88. *H. pylori* dependent activation of NF-κB was also said to involve MEK, AKT and Nod1 (Lu, Wu et al. 2005; Choi, Park et al. 2007; Hisatsune, Nakayama et al. 2008; Backert and Naumann 2010). Other *Helicocobacter spp.* can also activate this pathway, for instance *H. muridarum* was also able to induce NF-κB via TLR2 and NOD1 in HEK293 and AGS cells (Chaouche-Drider, Kaparakis et al. 2009). In *H. bilis* infection NF-κB activation may play a vital role in the induction of hepatobiliary dieases, IBD and colitis by this species since, activation of this pathway is frequently associated with development of these pathological abnormalities. Increased NF-κB levels were also found in a *H. bilis*-infected bile duct cell line (Takayama, Takahashi et al. 2010).

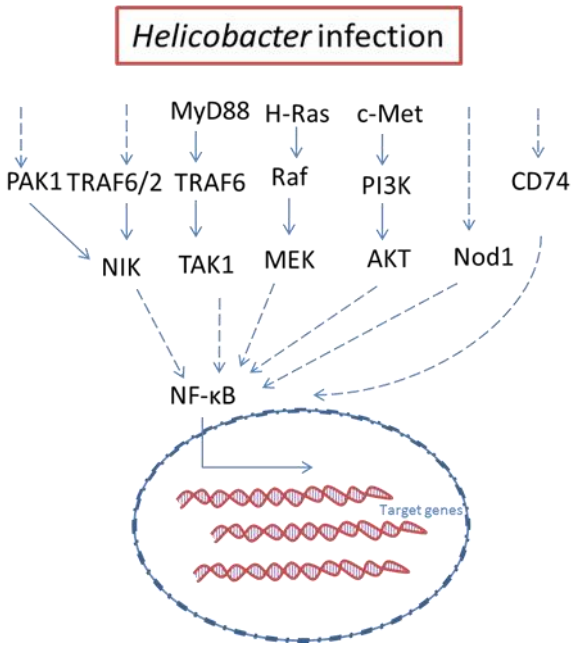


Figure 3: Signaling cascades leading to NF-κB activation in *Helicobacter* infected epithelial cells

Another transcription factor downstream of MAPK and found to be upregulated upon *H. pylori* infection is **Activator protein-1 (AP-1). AP-1** is a multipotent regulator of gene expression in response to a variety of stimuli, including cytokines, stress, and bacterial infections (Hess, Angel et al. 2004). AP-1 activation induces various cytokines and chemokines such as IL-2, IL-6, IL-8 and tumour necrosis factor α (TNFα) (Ameyar, Wisniewska et al. 2003). AP-1 thereby controls a number of cellular processes and therefore plays important role in infection biology. AP-1 is formed either as a homodimer of c-Jun or as a heterodimer of Jun (c-jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) proteins (Hess, Angel et al. 2004). Regulation of AP-1 activity is due to changes in transcription and mRNA stability of the individual AP-1 subunits as well as the specific interactions between AP-1 and other transcription factors or co-factors. In general, AP-1 activation involves the MAPK signalling cascade. MAPK activation leads to translocation of JNKs into the nucleus where they phosphorylate c-jun. p38 and ERK kinases are also involved in controlling c-jun and c-fos promoters (Karin, Liu et al. 1997). Oxidative stress also leads to the activation of AP-1, initiating the transcription of several genes encoding antioxidant enzymes, surfactant proteins, extracellular matrix metalloproteinases (MMPs), growth factors and receptors containing AP-1 binding sites in their promoter and/or enhancer regions (Reddy and Mossman 2002). Moreover, it is established that Ras-induced malignant transformation requires JNK induced phosphorylation of c-jun and thereby c-jun is found to be highly upregulated in many tumors (Johnson, Spiegelman et al. 1996; Behrens, Jochum et al. 2000).
H. pylori triggers the activation of AP-1 in gastric epithelial cells by inducing the proto-oncogenes c-fos and c-jun. This activation required an intact CagPAI but was independent of CagA (Meyer-ter-Vehn, Covacci et al. 2000). *H. pylori* AP-1 activation was also reported in AGS and MKN45 cells by Ding et al. This activation involved altered AP-1 subcomponent protein expression and AP-1 DNA-binding activity but no changes in overall subcomponent composition (Ding, Olekhnovich et al. 2008). Furthermore, a NOD-1 dependent activation of AP-1 involving the p38 MAPK pathway by *H. pylori* had been reported and implicated in COX-2 and iNOS production in epithelial cells (Allison, Kufer et al. 2009; Cho, Lim et al. 2010).

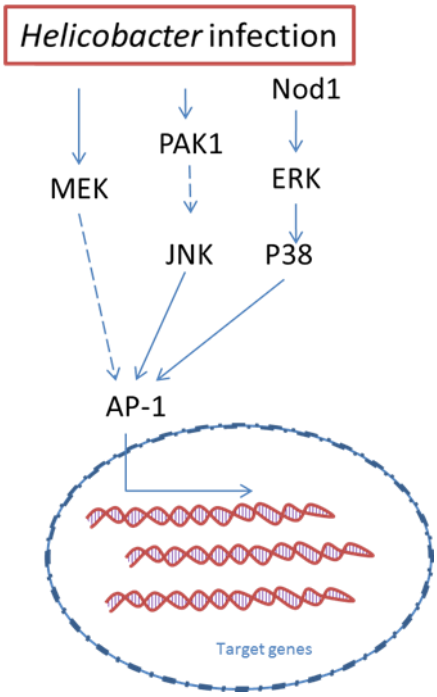


Figure 4: Signaling cascades leading to AP-1 activation in epithelial cells upon *Helicobacter* infection

The concomitant activation of AP-1 and NF-κB is essential in the development of certain chronic inflammatory diseases, where both transcription factors determine the cytokine gene activation profiles and disease progression (Karin, Liu et al. 1997). For instance, up-regulation of these transcription factors by *H. pylori* is central to the inflammation induced by the bacterium (Backert and Naumann 2010).

cAMP response element-binding (CREB) is a transcription factor which binds to cAMP response elements (CRE), thereby regulating the transcription of the downstream genes including c-fos, tyrosine hydroxylase, and many neuropeptides (Purves 2001). An activating signal binding to the corresponding receptor, leads to the production of a second messenger such as cAMP or Ca2+, which in turn activates a protein kinase. This protein kinase then is responsible for the phosphorylation and activation of the CREB protein which is then able to translocate into the nucleus. The activated CREB protein is able to bind a cAMP response element (CRE region), and then bound by a co-activator, CBP (CREB-binding protein), allowing it to switch certain genes on or off (Mayr and Montminy 2001). CREB was found to be constitutively active in human leukemia and plays a major role in growth and metastasis of some types of tumors (Jean and Bar-Eli 2000; Shankar, Cheng et al. 2005). Cre activation, as an early responder marking inflammatory responses to *H. pylori* infection was also observed

in infected mongolian gerbils (Kudo, Lu et al. 2007). CREB was also able to arrest the cells in G1/S phase by downregulation of miR-372 (Belair, Baud et al. 2011). In *H. pylori* infection, COX-2 mRNA and protein expression was enhanced in gastric epithelial cells *in vitro* and *in vivo* via induction of CREB transcription factors involving MEK/ERK1/2 pathways (Juttner, Cramer et al. 2003). TLR2 and TLR9, which activate MAPKs, especially p38, were also thought to be involved in *H. pylori* activation of CREB (Chang, Wu et al. 2005). Furthermore, Histatune et al observed that the virulence factor VacA of *H. pylori* was able to induce IL-8 secretion in epithelial cells through increased CREB binding which involved p38 MAPK (Hisatsune, Nakayama et al. 2008). CRE activity was also enhanced in *H. bilis*-infected cell lines (Takayama, Takahashi et al. 2010).

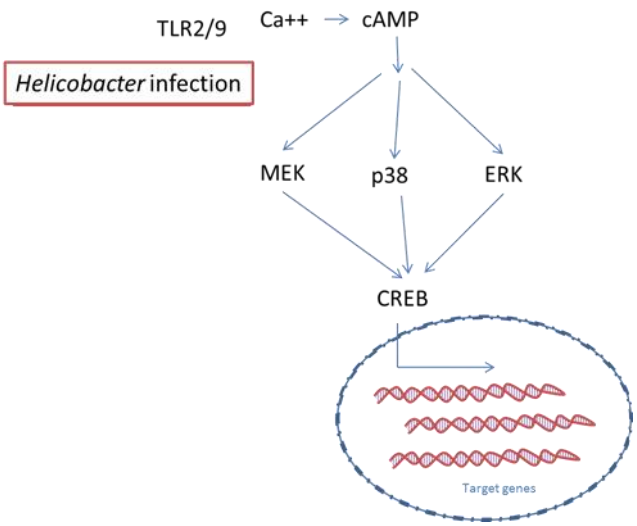


Figure 5: Signaling cascades leading to CREB activation in epithelial cells upon *Helicobacter* infection

activation by *H. pylori* also leads to the subsequent activation of **Nuclear factor of activated T-cells (NFAT)**, a family of transcription factors shown to be important in immune response. Although originally identified as a key regulator of cytokine expression in T lymphocytes, NFAT is expressed in most cells of the immune system as well as endothelial, myocardial and epithelial cells (Crabtree and Olson 2002). NFAT is regulated by calcium signaling via calmodulin (CaM), a calcium sensor protein, which activates the serine/threonine phosphatase calcineurin (CN). Activated CN rapidly dephosphorylates NFAT proteins resulting in a conformational change that exposes a nuclear localization signal resulting in NFAT nuclear import (Macian 2005). Ca^{2+} /CN-NFAT-mediated signaling pathways are involved in diverse

cellular reactions. Activating or deactivating function of NFAT is dependent on the binding partner involved. Interaction of NFAT with AP-1 turns on the genes involved in active immune responses, while NFAT without cooperative binding of AP-1 induces a T cell anergy program and blocks T cell activation and proliferation (Im and Rao 2004). Few NFAT target genes such as COX-2 have been identified in nonlymphoid cells (Duque, Fresno et al. 2005). Santini et al. demonstrated that NFAT transcriptionally activates p21 during keratinocyte differentiation causing a subsequent cell-cycle withdrawal (Santini, Talora et al. 2001). In addition it has been shown to be an important factor for cell migration, motility and intestinal cell differentiation via PTEN regulation in a cell signaling cascade mediated by AKT (Yoeli-Lerner, Chin et al. 2009; Wang, Zhou et al. 2011). The calcineurin-NFAT signaling pathway converges with the pathway to regulate Src (a proto-oncogenic tyrosine kinase) expression and promote Epithelial-to-Mesenchymal-Transition (EMT) (Li, Zhu et al. 2011). In keeping with this, it is not surprising that NFAT is upregulated in breast carcinoma and melanomas, promoting metastasis by increasing cell motility and invasiveness (Jauliac, Lopez-Rodriguez et al. 2002; Flockhart, Armstrong et al. 2009). In gastric epithelial cells, infection with *H. pylori* led to a CagA dependent activation of NFAT via pathway. This activation could be blocked by phospholipase C and CN inhibition (Yokoyama, Higashi et al. 2005).

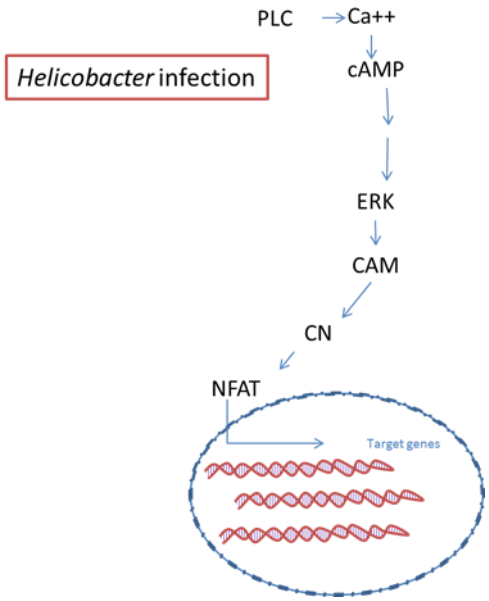


Figure 6: *Helicobacter* induces NFAT through ERK1/2 activation

2.2 *Helicobacter* infections trigger inflammatory responses

Chronic inflammation is the underlying cause in many hepatobiliary and gastroenteric disorders, predisposing the tissue to malignant changes. Activation of NF-κB, AP-1, NFAT and CREB in the host epithelium in response to *Helicobacter* infections may trigger a whole set of target genes many of which are cytokines and chemokines involved in inflammation. Target genes of NF-κB, AP-1, NFAT and CREB include pro-inflammatory chemokines and cytokines such as IL-8, IL-6 and COX-2 (Juttner, Cramer et al. 2003; Duque, Fresno et al. 2005; Lu, Wu et al. 2005; Hisatsune, Nakayama et al. 2008).

In a whole trascriptome analysis of the epithelial response to *H. pylori* exposure, **IL-8** was markedly up-regulated, and was involved in many of the most important cellular response processes to the infection (Eftang, Esbensen et al. 2012). IL-8 also known as CXCL8, is a member of the CXC chemokine family. This chemokine is one of the major mediators of the inflammatory response to infection and oxidant stress and can be secreted by several cell types. It functions as a chemoattractant, and is also a potent angiogenic factor (Modi, Dean et al. 1990). Neutrophil granulocytes are the primary target cells of IL-8, however a wide range of cells, including endothelial cells, macrophages and mast cells also respond to this chemokine (Kohidai and Csaba 1998). IL-8 is secreted in large amounts in response to oxidative stress, recruiting inflammatory cells. This in turn results in an added increase in oxidant stress mediators, making it a key player in localized inflammation (Vlahopoulos, Boldogh et al. 1999). Bezzerri et al showed an interaction of the transcription factors NF-κB, NF-IL6, AP-1, CREB, and CHOP with the corresponding consensus sequences in the IL-8 promoter, suggesting their participation in the transcriptional machinery (Bezzerri, Borgatti et al. 2011).
H. pylori VacA induced IL-8 production in U937 cells was by activation of the p38 MAPK via intracellular Ca^{2+} release, the activation was mainly attributed to ATF-2/CREB or NF-κB binding to IL-8 promoter regions (Hisatsune, Nakayama et al. 2008). Other *Helicobacter* infections like *H. muridarum* infection led to increased IL-8 production from epithelial cells (HEK293 and AGS) via NF-κB activation (Chaouche-Drider, Kaparakis et al. 2009).

H. pylori infection also induced the expression of the pro and anti- inflammatory cytokine, **IL-6** in gastric epithelial cells (Lu, Wu et al. 2005). IL-6 is extremely diverse in its functions.

It is secreted in response to tissue damage and during infection as host response to a foreign pathogen (vanderPoll, Keogh et al. 1997). Smooth muscle cells in blood vessels also produce IL-6 as a pro-inflammatory cytokine. IL-6 may also act as an anti-inflammatory cytokine through its inhibitory effects on TNFα and IL-1, and activation of IL-1ra and IL-10 (Smolen and Maini 2006).
H. pylori-induced IL-6 transcription required binding sites for NF-κB, cAMP response element (CRE), CCAAT/ enhancer binding protein (C/EBP), and AP-1 (Lu, Wu et al. 2005).

High **Cyclooxygenase-2 (COX-2, prostaglandin H synthase-2, PGHS-2)** mRNA and protein levels were found in *H. pylori* infected gastric epithelial cells *in vitro* and *in vivo* via binding of CREB transcription factors. COX-2 represents the inducible key enzyme of arachidonic acid metabolism and is not expressed under normal conditions in most cells, but elevated levels are found during inflammation (Kurumbail, Kiefer et al. 2001). Functional analysis of the COX-2 gene promoter mapped its *H. pylori*-responsive region to a proximal CRE/Ebox element at -56 to -48. USF1/-2 and CREB transcription factors binding to this site were identified to transmit *H. pylori*-dependent COX-2 transcription (Juttner, Cramer et al. 2003). This COX-2 activation by *H. pylori* involved TLR2/9 dependent activation of CREB through ERK1/2 and p38 MAPK (Chang, Wu et al. 2005). Others reported that COX-2 activation by *H. pylori* involved activation of CREB as well as AP-1 via p38, MEK and ERK1/2 MAPK pathways (Juttner, Cramer et al. 2003; Hisatsune, Nakayama et al. 2008).

Alteration of the transcriptional machinery of the host cells by *Helicobacter* involves the direct or indirect contribution of several factors in the *Helicobacter* repertoire that contribute to the virulence of a certain species. Some of these virulence factors are found recurring in many species and may hint to a common *modus operandi* in host cell colonization and pathology. A few of the better characterized virulence factors will be discussed in the following section.

3 *Helicobacter* virulence factors

Various bacterial factors are involved in aiding the bacteria to colonize and persist in the host. Many of these factors are also responsible for the severity of the disease thereby determining

the pathogenicity or virulence of the bacterium. Virulence determinants of *H. pylori* are the most studied, however some factors pertain to the *Helicobacter spp.* in general, both of which are discussed separately.

3.1 Virulence determinants of *H. pylori*

As previously described, *H. pylori* is the most well characterized of the *Helicobacter* genus due to its high prevalence and pathogenicity. Efforts to determine the bacterial components responsible for the persistence and virulence of the bacterium have yielded a long list of factors important for establishing infection and disease. A few of the more significant virulence factors are mentioned below:

Flagella enable the bacteria to move in their ecological niche represented by the mucous layer of the gastric epithelium. *H. pylori* possesses a unipolar bundle of two to six sheathed flagella (Suerbaum 1995). A correlation between the motility state of some *H. pylori* isolates and their ability to colonize the gastric epithelium has been established (Eaton, Morgan et al. 1992). Flagella are responsible for the chemotactic movement of the bacterium towards high urea, (Nakamura, Yoshiyama et al. 1998) and nutrient concentrations (Worku, Sidebotham et al. 1997).

H. pylori is able to adhere to the host cells via a group of unique **adhesins** which bind to Lewis B antigens of the host cell, namely BabA, and sialyl antigens, SabA. Other adhesins include HpaA, AlpA/B, NapA and HopZ proteins (Clyne, Dolan et al. 2007).

Urease A/B is able to neutralize the gastric acid in the stomach, locally increasing the pH making a habitable environment for the bacterium to live in (Eaton and Krakowka 1994). **UreB** was implicated in NF-κB activation in epithelial cells (reviewed in Backert and Naumann 2010). Urease is an essential factor for colonization of the gastric mucosa by the bacterium (Eaton, Brooks et al. 1991).

CagPAI are a group of genes coding for the bacterial cytotoxin **CagA** and a type IV secretion system for the translocation of CagA into the host cell (Backert and Naumann 2010; Tegtmeyer, Wessler et al. 2011). CagA is able to disrupt the epithelial barrier by breaking the

tight junctions and inducing cytoskeletal rearrangement of the cells (Saito, Murata-Kamiya et al. 2010)**.** In epithelial cells, *cag* PAI-positive *H. pylori* have been shown to induce NF-κB through direct cellular contact (Maeda, Mitsuno et al. 2001) and this activation was shown to be partly responsible for IL-8 induction (reviewed in Backert and Naumann 2010). Other studies showed that CagA is able to activate the ERK1/2 pathway leading to cellular transformation and immortalization (Zhu, Zhong et al. 2005). Patients infected with CagA positive strains of *H. pylori* are at a higher risk for developing gastric cancer (Parsonnet, Friedman et al. 1997).

Vacuolating cytotoxin A (VacA) is a protein secreted by *H. pylori* responsible for blocking the proliferation of T cells (Gebert, Fischer et al. 2003). It causes vacoulation and apoptosis in the gastric cancer cell line, AZ-521 (Radin, Gonzalez-Rivera et al. 2011). *H. pylori* strains with CagA and VacA are associated with severe disease outcomes (van Doorn, Figueiredo et al. 1998) and allelic polymorphisms within these genes correspond to virulence of the bacterium (Censini, Lange et al. 1996; Letley, Rhead et al. 2003; Jang, Jones et al. 2010). Histatune et al showed that *H. pylori* VacA induced activation of p38 MAPK lead to activation of the transcription factors, ATF-2, CREB, and NF-kB and increased IL-8 production (Hisatsune, Nakayama et al. 2008). Additionally functional antagonism between CagA mediated activation and VacA modulated inactivation of the NFAT pathway in epithelial cells was described by Yokohama and collaborators (Yokoyama, Higashi et al. 2005).

High temperature requirement protein A (HtrA) of *H. pylori* is able to cleave E-cadherin thereby disrupting the epithelial cell barrier. It is essential for the survival of the bacterium by allowing it to access the intercellular space (Hoy, Lower et al. 2010).

gamma-Glutamyl transpeptidase (gGT) is a heterodimeric enzyme that catalyzes the hydrolysis and transpeptidation of a gamma-glutamyl moiety of a suitable substrate and is essential for colonization of the gastric mucosa (Chevalier, Thiberge et al. 1999). It has an immunomodulatory effect by blocking the cell cycle progression of T cells (Schmees, Prinz et al. 2007), as well as causing epithelial cell death (Kim, Lee et al. 2010). The pathogenic potential of this important bacterial enzyme will be discussed in further sections.

Other virulence deteminants of *H. pylori* include **outer membrane vescicles (OMVs), outer inflammatory protein A (OipA) and duodenal ulcer promoting gene (DupA)**.
OMVs that continuously bud from the surface of *H. pylori* carry effector-promoting properties which may be important for disease development (Olofsson, Vallstrom et al. 2010). Oip A when expressed together with CagA, is associated with an enhanced inflammatory response in the gastric mucosa (Yamaoka, Kikuchi et al. 2002), while DupA is able to stimulate mononuclear inflammatory cells (Hussein, Argent et al. 2010).

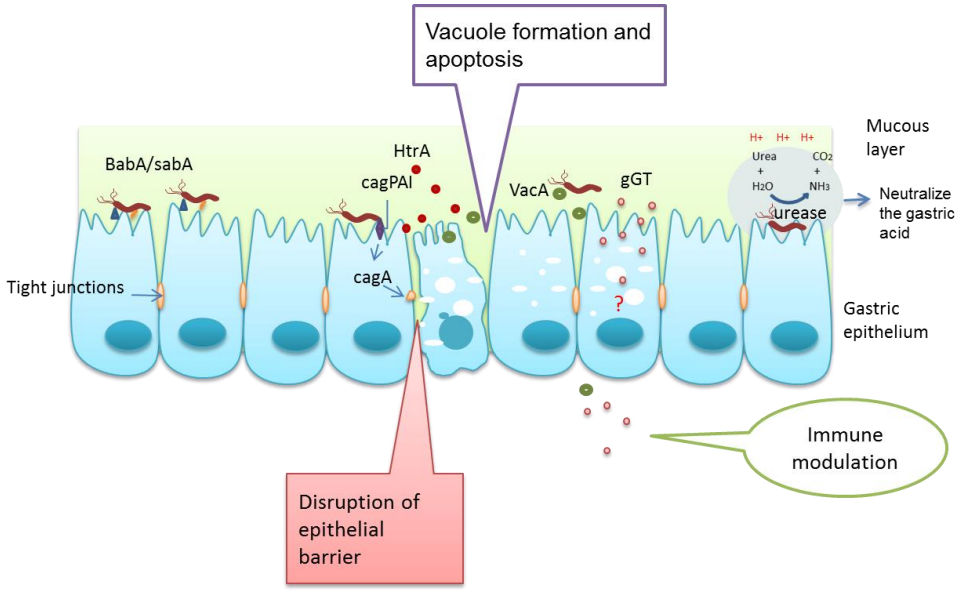


Figure 7: Important virulence determinants of *H. pylori.*

3.2 Common virulence determinants of Non-*pylori Helicobacter* species

NPHS harbour many virulence genes and may cause diseases not only in animals but also in humans. The known common virulence factors so far are flagella, urease, cytolethal distending cytotoxin and gamma-glutamyl transpeptidase.

All gastric and enterohepatic *Helicobacter* species are highly motile and **flagella** confer motility and aid in bacterial attachment to the host cells, preventing flushing of the bacteria through the gastrointestinal lumen. The characteristic sheathed flagellar filaments of *Helicobacter spp.* are composed of two copolymerized flagellins, FlaA and FlaB. It could be shown for *H. mustelae* and *H. felis* that flagellar motility is essential for these *Helicobacter* species to colonize the gastric mucus (Spohn and Scarlato 2001).

Helicobacter spp. are able to thrive in the very acidic mammalian stomach by producing large quantities of the enzyme **urease** which enables the bacterium to neutralize the gastric acid, locally raising the pH from ~2 to a more biocompatible range of 6 to 7 (Dunn, Vakil et al. 1997). All GHS present urease activity, however there are some EHS which have either none or low urease activity, e.g; *H. canis, H. cinaedi, H. fennelliae* and *H. rodentium.* Other EHS that possess urease activity include *H. bilis*, *H. hepaticus* and *H. trogontum*, (Solnick and Vandamme 2001). Urease activity is very important for colonization of the gastric mucosa by the *Helicobacters*, as urease negative mutants of both *H. mustelae* and *H. pylori* lost the ability to colonize the stomach (Eaton, Brooks et al. 1991; Andrutis, Fox et al. 1995).

Cytolethal distending toxin (cdt) is another commonly found pathogenic factor in the *Helicobacter* genus. Encoded by the cdtA/C, it enables the bacterium to disrupt the epithelial barrier. Bacterial cdts are a family of heat-labile proteins with the ability to block the mammalian cell cycle and cause progressive cellular distension. Three linked genes, cdtA, cdtB, and cdtC, encode proteins of similar molecular masses and all three genes must be expressed for cdt to initiate cellular toxicity. Cdt is a tripartite toxin in which cdtB is the active toxic unit; cdtA and cdtC are required for cdt binding to target cells and for delivery of cdtB into the cell interior (Lara-Tejero and Galan 2002). It is remarkably similar in *H. bilis*, *H. muridarum* and *H. canis* (Chien, Taylor et al. 2000). In *H. hepaticus*, cdt was found to induce cell cycle arrest in HeLa cells (Young, Knox et al. 2000).

gGT is present in all gastric *Helicobacter* species. However, only a few EHS express this enzyme (On SL. et al; 2010). gGT confers metabolic advantages to the organism and will be discussed shortly. Despite the importance of gGT for the colonitzation of the gastric mucosa and its immunomodulatory functions, not much effort has gone into elucidating the mechanism of action of this important bacterial enzyme. Thereby, gGT modulated changes in host cells leading to inflammation and disease remain elusive. Its presence in other *Helicobacter spp.* underlines its importance in bacterial metabolism and possible part in disease.

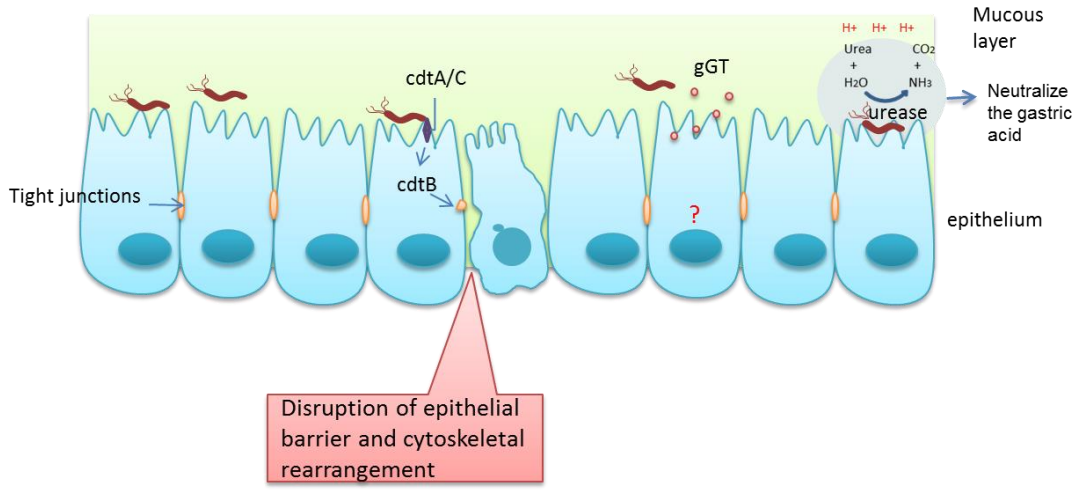


Figure 8: Common virulence determinants found in the *Helicobacter genus*

4 gamma-Glutamyl transpeptidase

gGT is a threonine N-terminal nucleophile (Ntn) hydrolase (Suzuki, Kumagai et al. 1986), which is widely distributed in living organisms, produced by both bacteria and eukaryotic cells and is highly conserved (Boanca, Sand et al. 2007). In bacteria this enzyme is secreted, whereas in mammalian cells it is integrated in the plasma membrane, its active site exposed to the outside where it is used in the γ-glutamyl cycle (Meister, Tate et al. 1981).

4.1 gGT is conserved within the *Helicobacter* genus

Mammalian and bacterial gGT homologues share more than 25% of sequence identity (Boanca, Sand et al. 2007). 540 (200 genera) of the 1000 of whole genome sequenced bacterial species available in MEROPS databases (Rawlings, Barrett et al. 2010) possess gGT-like proteins belonging to protease family T03. Many of these bacterial species possess multiple copies of genes annotated as gGT, but most of them lack functional verification. gGT is conserved in all GHS, however, only *H. aurati, H. bilis, H. canis, H. muridarum and H. trogontum* of the described EHS possess this enzyme (On SL. et al; 2005). *H. pylori* expresses gGT constitutively *in vivo* and *in vitro* (Wachino, Shibayama et al. 2010). The conservation

of gGT in various *Helicobacter spp*. indicates that this bacterial enzyme may confer metabolic advantages and possibly be involved in aiding bacterial colonization and pathogenesis.

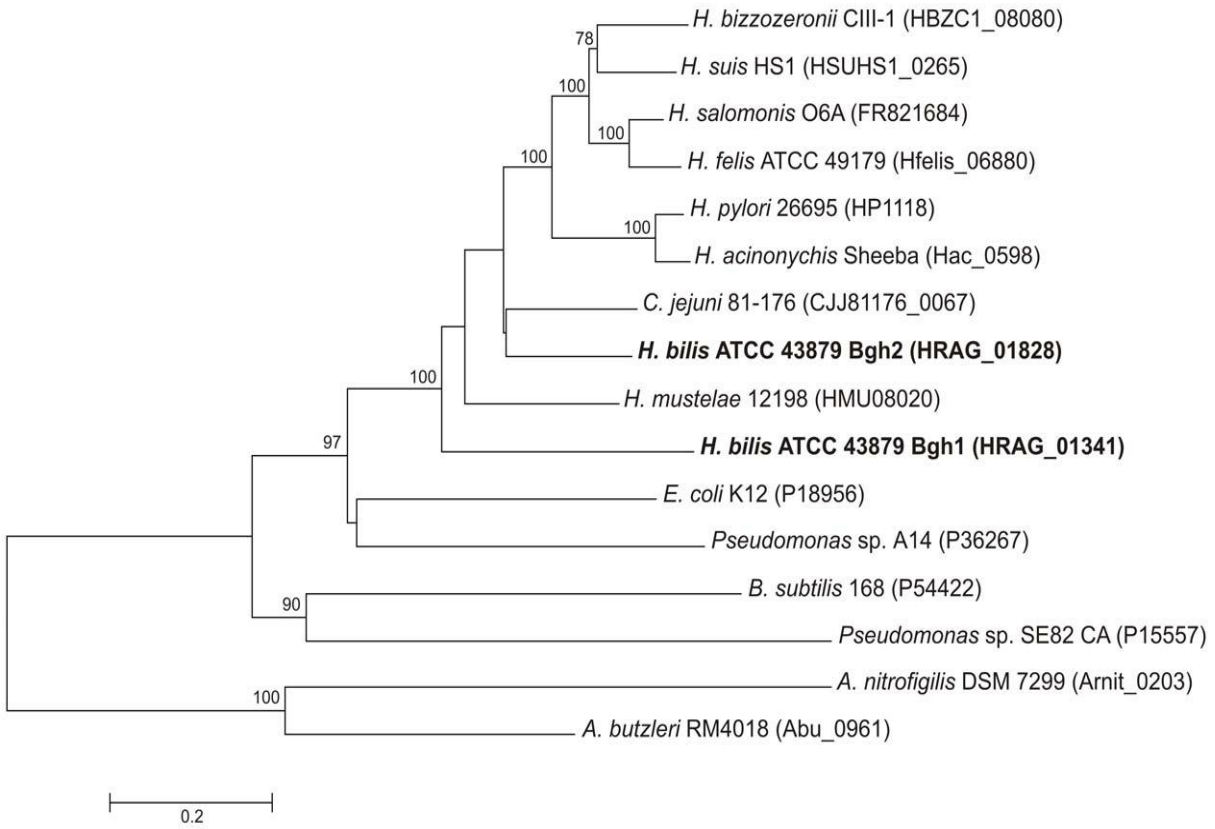


Figure 9: Unrooted tree based on complete amino acid sequences of different bacterial gGTs.
gGT is conserverd among the *Helicobacter* genus. Unrooted tree based on complete amino acid sequences of different bacterial gGTs (Rossi, Bolz et al. 2012).

4.2 Structure

Helicobacter spp. *H. canis, H. muridarum, H. aurati* as well as *H. pylori* possess a single copy of the gGT sequence. On the other hand, *H. bilis* genome contains two gGT genes, one of which codes for the functionaly active enzyme. *H. pylori* gGT (HPgGT) and *H. bilis* gGT (HBgGT) are both expressed as heterodimeric enzymes consisting of two molecular subunits. HPgGT is secreted as a pro-form of 60 kDa autocatalysis of which yields a 38 kDa and a 20 kDa (Schmees, Prinz et al. 2007). HBgGT is secreted as a soluble pro-form of approximately 60 kDa. Autocatalytic cleavage of the pro-enzyme generates the two molecular subunits, a 40 kDa large subunit and a 20 kDa small subunit (Rossi, Bolz et al. 2012).

Crystal structures of HPgGT in conjugate with glutamate reveal that residues that comprise the gamma-glutamyl binding site are primarily located in the 20 kDa subunit and make numerous hydrogen bonds with the alpha-amino and alpha-carboxylate groups of the substrate. A loop region covers the gamma-glutamyl binding site that could be accessed through conformational changes in the Tyr 433-containing loop. Formation of a hydrogen-bond network in HPgGT, involving side chains of Asn 400, Glu 419, and Asp 422 with the r-amino group of glutamate, as well as the co-ordination of the r-carboxylate group directly or indirectly via a bridging water molecule by Arg 103, Ser 451, and Ser 452 is the basis for the substrate binding. Site directed mutagenesis of these residues result in a functionaly inactive enzyme (Morrow, Williams et al. 2007).

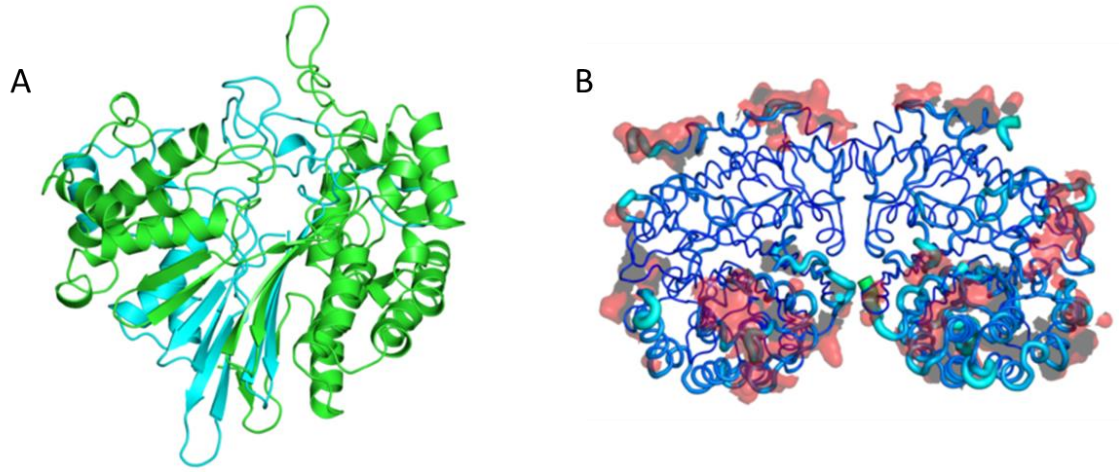


Figure 10: 3D crystallographic structure of *H. pylori* gGT (HPgGT).

A) Heterodimeric assembly as defined by the author (Williams, Cullati et al. 2009) and software (PISA). This assembly consists of 2 molecules: 1 copy of gGT large subunit and 1 copy of gGT small subunit. B) Backbone of the macromolecules comprising the gGT protein. The thickness reflects the B-values (thin = low, thick = high). The colour varies from blue to red corresponding to a B-factor range of 10 to 100 $Å^2$. The red surface patches indicate regions where symmetry-related crystal contacts occur (5Å cut-off) (Image downloaded from http://www.ebi.ac.uk/pdbe-apps/widgets/PDBimages/fn/3m/index.html).

4.3 Function and role of gGT in bacterial metabolism

As mentioned previously, gGT is a threonine N-terminal nucleophile hydrolase which catalyses the transpeptidation and hydrolysis of the γ-glutamyl moiety of glutathione and related compounds. The reaction involves the formation of a γ-glutamyl-enzyme intermediate followed by nucleophilic substitution by water, amino acids, or peptides (Figure 11) (Suzuki, Kumagai et al. 1986).

HBgGT and HPgGT exhibit similar affinity to γ-Glutamyl-p-nitroanilide and to L-Glutamine (Rossi, Bolz et al. 2012). Since this enzyme doesn't seem to be essential for colonization of the lower gastrointestinal tract (On SL. et al; 2005), its presence in EHS like *H. bilis* could provide metabolic advantages allowing the bacterium to adapt to other niches. HBgGT was found to be significantly less active than HPgGT with a reported KM value 20% lower than that of *H. pylori* gGT, while the k_{cat} value was 10-fold reduced with a pH optimum of 6-7 (Rossi, Bolz et al. 2012).

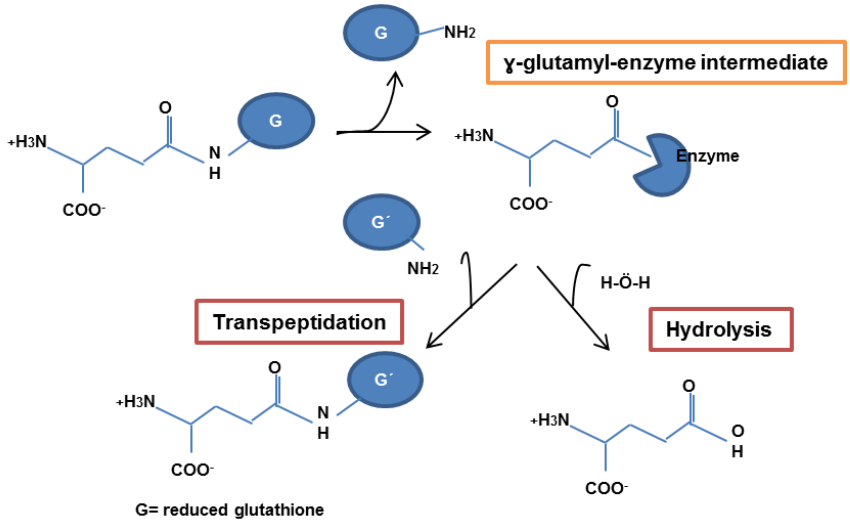


Figure 11: A typical gGT reaction

A typical gGT reaction involves the cleavage of the γ-glutamyl group of compounds, e.g., glutathione (GSH), and its transfer to other amino acids and peptides (G´). The reaction catalyzed by gGT has been thought to proceed via a γ-glutamyl-enzyme intermediate followed by nucleophilic substitution by water, amino acids, or peptides (Adapted from Okada T. et al; 2006).

H. pylori cells are unable to take up glutamine and glutathione and the conversion of these into glutamate is essential for the bacterium. gGT enables the bacterium to utilize extracellular glutamine and glutathione as a source of glutamate. Glutamate is then incorporated into the TCA cycle and used for glutamine synthesis in the cytosol of the bacterium (Shibayama, Wachino et al. 2007). In addition, the hydrolysis of extracellular glutamine by gGT is accompanied by ammonia production in the periplasm. Ammonia efflux from *H. pylori* supports the urease in increasing the pH of the surrounding environment and possibly promoting host cell damage by ammonia.

4.4 *Helicobacter* gGT in host cell pathogenesis

HPgGT represents an important virulence factor since it plays an essential role in the colonization of the gastric mucosa (Romano et al; 2006 and (Chevalier, Thiberge et al. 1999). A higher risk of developing peptic ulcer disease has been shown to correlate with increased enzymatic activity of gGT in *H. pylori* infected individuals (Gong, Ling et al. 2010). Moreover, infected individuals present antibodies against HPgGT, indicating that high levels of this virulence factor are produced during infection (Gerhard, Schmees et al. 2005; Schmees, Prinz et al. 2007). During *H. pylori* infection, gGT affects host cell viability and is one of the bacterial protagonists responsible for inducing inflammatory reponse. Meanwhile, the role of gGT of other *Helicobacter spp*. like *H. bilis* infections remains unknown.

4.4.1 *Helicobacter* gGT effects host cell viability

HPgGT was found to be the factor in *H. pylori* culture supernatants responsible for inhibiting the proliferation of T lymphocytes in a dose dependent manner. Moreover, it induced a G1 cycle cell arrest in these cells (Gerhard, Schmees et al. 2005; Schmees, Prinz et al. 2007). These findings indicate that HPgGT can effect cellular proliferation by modifying cell cycle pathways, as well as cell survival pathways. Ras/Raf activation has been suggested to be involved in HPgGT modulated cellular effects (Schmees, Prinz et al. 2007).

HPgGT induced apoptosis in epithelial cells has been reported by some groups (Shibayama, Kamachi et al. 2003; Kim, Lee et al. 2007). While it was indicated that HPgGT induced apoptosis via the mitochondrial pathway in the gastric cancer cell line, AGS (Kim, Lee et al. 2007), another report demonstrated that *H. pylori* gGT is able to induce cell cycle arrest in epithelial cells in the G1 phase of the cell cycle (Kim, Lee et al. 2010). A recent report suggested that HPgGT induced human biliary cell death which involved decreased levels of anti-apoptotic Bcl2 and increased pro- apoptotic Bax (Boonyanugomol, Chomvarin et al. 2012). A different study demonstrated that HPgGT and HBgGT induced decrease in cell viability was independent of apoptosis (Rossi, Bolz et al. 2012). These conflicting findings may be due to the different experimental set ups used in the studies. However further investigation of these inconsistencies is necessary.

Although it has been well established that HPgGT is able to reduce host cell viability, the exact mechanism behind this effect is not well understood. Shibayama et al. presented a novel mechanism of HPgGT induced epithelial cell death. They argued that HPgGT is able to

exhaust the glutamine and glutathione supply of the host cells due to its considerably low Km values as compared to the mammalian gGT (Shibayama, Wachino et al. 2007). Both glutamine and glutathione are important antioxidants in mammalian cells and maintain the redox balance of the cells (Kaplowitz, Aw et al. 1985; Dominici, Paolicchi et al. 2003; Njalsson and Norgren 2005). Thus, the depletion of these substances impairs the redox balance of the host cell thereby rendering it prone to reactive oxygen species (ROS) induced cell death by triggering a whole set of redox sensitive host cell signaling cascades. Indeed, it was shown that bacterial gGTs from *H. pylori* and *H. suis* induced ROS in epithelial cells in presence of glutathione leading to host epithelial cell death (Flahou, Haesebrouck et al. 2011).

4.4.2 gGT induces inflammatory stress responses in host epithelium

Increased oxidative stress as well as lowered levels of antioxidants in the cell surrounding contribute to a pro-inflammatory environment by induction of various oxidative stress-induced signaling cascades (Rahman, Gilmour et al. 2002; Bobrovnikova-Marjon, Marjon et al. 2004). Gong et al. reported that gGT induced IL-8 production via NF-κB activation which was in turn upregulated in response to oxidative stress induced by the enzyme (Gong, Ling et al. 2010). Futhermore, *H. pylori* gGT was implicated in COX-2 upregulation at mRNA and protein levels. Blockade of phosphatidylinositol-3 kinase and p38 kinase inhibited HPgGT induced upregulation of COX-2 (Busiello, Acquaviva et al. 2004). Recently, Boonyanugomol W. and colleagues found that HPgGT was able to induce iNOS and IL-8 production by human biliary cells (Boonyanugomol, Chomvarin et al. 2012). These findings further validate the importance of this enzyme in *Helicobacter* induced pathogenesis.

5 Aims of the present study

The main focus of the present study is on *Helicobacter* gamma glutamyl-transpeptidase (gGT) induced effects on epithelial cells, an important yet less characterized virulence factor in *Helicobacter* infections. Since its identification as an important immunomodulatory factor in *H. pylori* infection (Schmees, Prinz et al. 2007) and the fact that gGT seems to be essential for colonization of the gastric mucosa (Chevalier, Thiberge et al. 1999), efforts have been made in trying to elucidate the mechanism by which gGT is able to aid the bacterium in establishing infection in host cells.

The presence of gGT in several GHS and EHS prompted us to look at the preservation of its function in two different *Helicobacter* species. gGTs from *H. pylori*, a gastric *Helicobacter* specie and *H. bilis* as an example of an enterohepatic *Helicobacter* specie which is able to colonize a wide variety of hosts and niches, were studied. Hence, the first part of the thesis focuses on the conserved function of *Helicobacter* gGT on growth and viability of gastric and colon epithelial cell lines.

Limited data are available on the transcriptional alterations induced in NPHS infections. Moreover, gGT has been described to be a key factor involved in oxidative stress induction in *H. pylori* as well as *H. suis* infections (Flahou, Haesebrouck et al. 2011). The role of *H. pylori* gGT in development of peptic ulcer disease (Gong, Ling et al. 2010) prompted us to look at oxidative stress induced signalling cascades. So the second part encompasses the molecular signalling pathways involved in gGT mediated oxidative stress induction in epithelial cells and its possible role in inflammation.

Finally, the aim of this study was to identify the mechanism by which gGT is able to induce oxidative stress responses in epithelial cells.

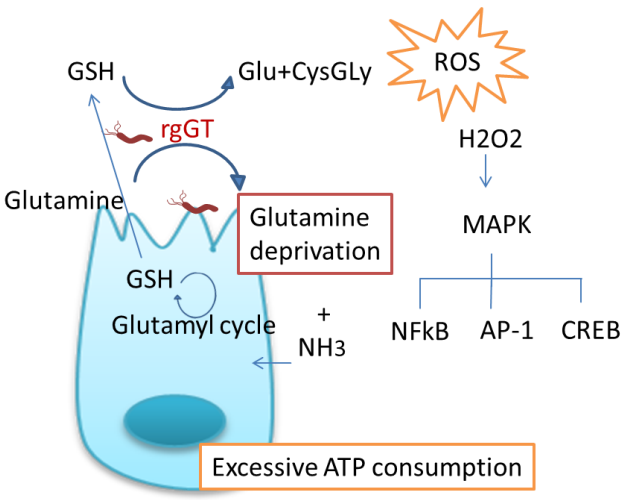


Figure 12: Hypothesis regarding mechanism of gGT action.

Glutamine deprivation and exhaustive consumption of Glutathione and subsequent generation of free radicals by helicobacter gGT induces several oxidative stess response transcriptional activation in host cells.

Chapter 2: Materials and Methods

2.1 Laboratory equipment

Table 2: Laboratory equipment

Instrument	Provider
Axiovert 200M Florescence Microscope	Zeiss
AxioVert 40 Florescence Microscope	Zeiss
Biofuge Pico Centrifuge	Heraeus, Thermo Scientific
Biofuge Primo R Centrifuge	Heraeus, Thermo Scientific
Centrifuge 5415D	Eppendorf
Centrifuge 5424	Eppendorf
Centrifuge Micro 200R	Hettich
Developing Machine (Curix60)	AGFA
Electrophoresis Power Supply PowerPac300	Bio-Rad
Electronic Pipet Filler, Easypet	Eppendorf
Flow cytometer CyAn ADP	Beckman Coulter
Freezer -80C	Thermo Scientific
Freezer -20C	Liebherr
Heatable Magnetic stirrer	IKAMAG RET-G
Hera cell incubator, 240	Heraeus, Thermo Scientific
Hera Cell incubator	Heraeus, Thermo Scientific
Ice maschine	Ziegra
Innova 2100 Platform Shaker	New Brunswick Scientific
Keyence BZ-9000 Microscope	Keyence
Laboratory Scale	Kern
Laminar flow sterile work bench	Heraeus, Thermo Scientific
Leica DMRB Microscope	Leica Microsystems
Luminometer ELISA	Tecan
Microwave	Panasonic
Mithras LB9440	Berthhold technologies
Multichannel Pipette Aid (5-50µl)	Corning

Multichannel Pipette Aid (50-300µl)	Corning
Multistep pipette	Eppendorf
Neubauer hemocytometer cell counting chamber	Marienfeld Superior
Nitrogen tank	Air Liquide
Orion Microplate Luminometer	Berthold
pH Meter	WTW inoLab
Power supply PowerPac300 Electrophoresis	Bio-Rad
Power Supply PowerPac Basic	Bio-Rad
Pipette Aid (0.5-10µl)	Corning
Pipette Aid (2-20µl)	Corning
Pipette Aid (20-200µl)	Corning
Pipette Aid (100-1000µl)	Corning
Precision weighing scale	Precisa
Refrigerator 4°C	Liebherr
Scanner	Epson perfection 4490 Photo
Shaker, Titramax 100	Heidolph
Spectrophotometer NanoDrop 1000	Thermo Scientific
Spectrophotometer	Thermo Scientific
Spectrophotometer UV-1602	Shimadzu
Sonoplus HD60	Bandelin
T3000 Thermocycler	Biometra
Thermomixer compact	Eppendorf
Waterbath Sonifier (Transsonic 460)	Elma
Waterbath Julabo	Julabo
Waterbath	GFL
Western Blot X cell blot module	Invitrogen
Western Blot X cell sure lock electrophoresis cell	Invitrogen
UV Transilluminator, Eagle Eye Gel Doc	Bio-Rad
Vortex Genie 2	Schultheiss

2.2 Consumables

Table 3: List of Consumable items

Plastic ware	**Description**
96-Well white polystyrene, round bottom	Corning
96-Well white polystyrene, flat bottom	Corning
Blotting paper	Hartenstein
Cell culture flasks 25 cm^2	VWR International
Cell culture flasks 75 cm^2	VWR International
Cell culture flasks 125 cm^2	VWR International
8 Chamber Culture Slides Polysterene vessel	BD falcon
8 Chamber Culture Slides	Thermofisher Scientific
Centrifuge tubes, 50 ml	Greiner Bio-one
Centrifuge tubes, 15 ml	Greiner Bio-one
Cell scraper 30 cm	TPP
Cover slips 30x26mm	Menzel-Gläser
Cover slips 18x18 mm	Marienfeld Superior
Cryotubes (1.8ml)	Alpha Laboratories
Drigalski spatulas	VWR
Feather scalpels	FDM medical
Filter Pipette tips, 100-1000µl	Starlab
Filter Pipette tips, 20-200µl	Starlab
Filter Pipette tips, 1-10µl	Starlab
Inoculation Spreader	VWR
Medical X-ray film (Super RX)	Fujifilm
Novax® Gel cassettes	Invitrogen
Novax® Gel combs 10-, 12-, 15 wells	Invitrogen
Pipette tips, 100-1000µl	VWR
Pipette tips, 20-200µl	VWR
Pipette tips, 1-10µl	VWR
Polysterene Round bottom tube, 5 ml	Becton Dickinson
Protran Nitrocellulose transfer membrane 0.45µm	Whatman
Quali-PCR tube strip	G. Kisler
Superfrost plus microscope slides	Thermoscientific
Safe lock Microcentrifuge Tubes, 1.5 ml	Eppendorf
Safe lock Microcentrifuge Tubes, 2 ml	Eppendorf
Serological pipette, 25 ml	Greiner Bio-one
Serological pipette, 10 ml	Greiner Bio-one
Serological pipette, 5 ml	Greiner Bio-one
Serological pipette, 2 ml	Greiner Bio-one
Tissue culture plate 6 well	BD Bioscience

Tissue culture plate 12 well	BD Bioscience
Tissue culture plate 24 well	BD Bioscience
Tissue culture plate 96 well	BD Bioscience

2.3 Chemical reagents

Table 4: Chemical reagents

Chemical	Catalogue number	Manufacturer
2-Propanol	67523	Roth
30 % Acrylamid/Bis Solution	161-0158	Bio-Rad
Agar-Agar	5210	Roth
Agarose	A9539-5009	Sigma-Aldrich
Albumin Fraction V (BSA)	A1391,0500	AppliChem
Ammoniumpersulfat, APS	A2941,0100	AppliChem
Ampicillin	K029.3	Roth
Aqua ad iniectabilia Diaco	2034374	Serag-Wiessner KG
Bench Top 1kb DNA Ladder	G7541	Promega
Beta-Mercaptoethanol	M3148	Sigma-Aldrich
BHI medium	CM1135	Oxoid
Bromophenol Blue	8122	Merck
BrdU	B5002	Sigma-Aldrich
Calcium chloride	2388.1000	Merck
Chloramphenicol	3886.2	Roth
Complete Protease Inhibitor Cocktail Tablets	04693159001	Roche
Cyclosporine A	30024	Sigma-Aldrich
DENT supplemet	SR147E	Oxoid
Defibrinated Horse blood	SR00S0C	Oxoid
Dibasic Na-phosphate	567550	Merck
Disodium hydrophosphate, $Na_2HPO_4.7H_2O$	119753	Merck
Dithiothreitol, DTT	A3668,0050	Applichem
DMSO	A3672,0250	AppliChem
Dulbecco´s modified Eeagle Medium, DMEM	41965	GIBCO®, Invitrogen
Dulbecco´s modified Eeagle Medium, DMEM, L-Glutamine free	11960-044	GIBCO®, Invitrogen
EDTA-free Protease Inhibitor tablets, Complete	4693159001	Roche
Ethanol, absolute	A3678,1000	AppliChem

Ethylenediaminetetraacetic acid, EDTA	A2937,0500	AppliChem
Fetal Calf Serum, FCS	F7524-500ML	Sigma-Aldrich
Foskolin	344270	Calbiochem
Glycerin	A2926,1000	AppliChem
Glycyl-glycine	108451-47-4	Sigma-Aldrich
Glycine	3790.3	Sigma-Aldrich
GoTaq® Green Master Mix (2X)	M7112	Promega
HEPES	9105.4	Roth
Hydrochloric acid, HCl	1090631000	Merck
Kanamycin sulphate	10106801001	Roche
Keratinocyte growth medium	10744019	GIBCO®, Invitrogen
LB Agar	75851	USB
LB Broth	75852	USB
Lipofectamine 2000	11668	Invitrogen
L- Glutamine	25030081	GIBCO®, Invitrogen
L-γ-glutamyl-p-nitroanilide	G1135-1G	Sigma-Aldrich
Manganese chloride, $MnCl_2$	T881.2	Roth
Methanol	4627.4	Roth
Milk Powder	T145.2	Roth
Monopotassium phosphate, KH_2PO_4	1048730250	Merck
Monobasic Na-phosphate	567545	Merck
Nitroblue tetrazolium chloride, NBT	N6639	Sigma-Aldrich
Opti-MEM	51985	GIBCO®, Invitrogen
Paraformaldehyde	1:04005:1000	Merck
Penicillin/Streptomycin (P/S) 100X Solution	15140	GIBCO®, Invitrogen
peqGOLD Protein Marker V	27-2210	PeqLab
Phosphate Buffered Saline, DPBS, 1X	14190	GIBCO®, Invitrogen
Pierce ECL Western Blotting substrate	32106	Thermo Scientific
Potassium acetate, KAc	T874.2	Roth
Potassium chloride, KCl	4936.1000	Merck
Potassium hydroxide, KOH	6751:1	Roth
Potassium nitrate KNO_3	812cc558661	Merck
Phosphoric acid H_3PO_4	P6560	Sigma-Aldrich
phorbol 12-myristate 13-	P1585	Sigma-Aldrich

acetate (PMA)		
Propidium iodide PI (PI)	556463	BD Pharmingen™
Restore Western Blot Stripping Buffer	21059	Thermo Scientific
RNaseA	R4642	Sigma-Aldrich
Rothi®-Safe	3865.1	Roth
RPMI	21875091	GIBCO®, Invitrogen
Safranin O	S2255	Sigma-Aldrich
Saponine	4185.1	Roth
SDS-Pellets	CN30.2	Roth
Sodium Citrate di hydrate	1.12005.5000	Merck
Sodium Chloride, NaCl	3957.1	Roth
Sodium deoxycholate, DOC	3484.2	Roth
Sodium hydroxide, NaOH	A4422,5000	AppliChem
Staurosporine	S-9300	LC laboratories
Super signal west Pico chemiluminescent substrate	34080	Thermo Scientific
Tetramethylethylenediamine, TEMED	A1148,0025	AppliChem
Tris	9090.3	Roth
Triton X-100	A1388,0500	AppliChem
Trypan Blue 0.4 %	15250	GIBCO®, Invitrogen
Trypsin/EDTA	41010	GIBCO®, Invitrogen
Tryptone	A1553,1000	AppliChem
Tween 20	A1389,0500	AppliChem
Vectashield mounting medium with DAPI	H-1200	Vector Laboratories
WC-agar	CM0619	Oxoid
Yeast extract	212750	BD Bioscience

2.4 Buffers, media and solutions

LB (Luria-Broth) medium:

1 % (w/v) NaCl

0.5 % (w/v) Yeast extract

1 % (w/v) Tryptone

pH 7.4

Before addition of antibiotics (Ampicillin, 100 µg/ml), LB medium was autoclaved and cooled to 50-60 °C. LB medium was stored at 4 °C.

LB agar:

0.5 % (w/v) NaCl
0.5 % (w/v) Yeast Extract
1 % (w/v) Tryptone
1.5 % (w/v) Agar
pH 7.4

LB agar was autoclaved and cooled to 50-60 °C before addition of antibiotics (Ampicillin, 100 µg/ml) and transfer to plates. After cooling to room temperature, LB agar plates were stored at 4 °C.

BHI- DENT:

18.5g BHI (Brain heart infusion) broth
1 tube DENT supplement
500 ml H_2O

WC- DENT agar:

21.5g WC-agar
0.2 g KNO_3
50ml Defibrinated horse blood
1 tube DENT supplement
500ml H_2O

WC agar was autoclaved and cooled to 50-60 °C before addition of DENT supplement, horse blood and transfer to plates. After cooling to room temperature, plates were stored at 4 °C.

Cell culture medium:

500 ml DMEM
50 ml FBS
5 ml Penicillin/Streptomycin (100x solution)

500 ml RPMI

50 ml FBS

5 ml Penicillin/Streptomycin (100x solution)

Keratinocyte growth medium

Agarose gel electrophoresis

1x TAE:

40 mM Tris

20 mM Acetic acid

1 mM EDTA

Immuno blotting

1x PBS:

137 mM NaCl

2.7 mM KCl

4.3 mM Na_2HPO_4 x $2H_2O$

1.4 mM KH_2PO_4

pH 7.4

1x TBS:

50 mM Tris

150 mM NaCl

pH 7.5

1x TBS-T:

1x TBS

0.1 % Tween 20

Running Buffer:

25 mM Tris

0.2 M Glycin

0.1 % (w/v) SDS

Transfer Buffer:

190 mM Glycin

25 mM Tris

0.01 % (w/v) SDS

20 % ethanol

1x SDS-PAGE Stacking gel buffer:

0.4 % (w/v) SDS

0.5 M Tris

pH 6.8

1x SDS-PAGE Separation gel buffer:

0.4 % (w/v) SDS

1.5 M Tris

pH 8.8

Stacking gel (1 cassette):

1.06 ml H_2O

0.39 ml 30 % Acrylamide/Bis Solution

0.49 ml Stacking gel buffer

0.01 ml 10 % APS

0.01 ml TEMED

Separation gel (1 cassette):

2.43 ml H_2O

2.02 ml 30 % Acrylamide/Bis Solution

1.5 ml Separation gel buffer

0.02 ml 10 % APS

0.01 ml TEMED

4x SDS sample buffer:

240 mM Tris/HCl pH6.8

40 % (v/v) Glycerol

8 % (w/v) SDS

5 % ß-Mercaptoethanol

0.004 % (w/v) Bromophenol Blue

Buffers for cell lysis

1x SDS lysis buffer:

62.4 mM Tris pH 6.8

2 % SDS

10 % Glycerol

50 mM DTT

0.01 % Bromphenol blue

RIPA buffer (for IP): 50 mM Tris-HCl pH 7.4

150 mM NaCl

1 % Triton X-100

1 % Sodium deoxycholate

0.1 % SDS

1 mM EDTA

Complete protease inhibitor cocktail tablet

Blocking solutions:

1. Milk :

5% (w/v) fat free milk

TBST

2. BSA:

5% (w/v) BSA

TBST

Anitibody dilution buffer:

5% (w/v)BSA

TBST

Immuno florescence staining

Fixation solution:

Mix 3 parts Solution A to one part solution B

Solution A:

2430µl of 1M dibasic Na-phosphate, pH 9

570µl of 1M monobasic Na-phosphate, pH 4.1

In 27 ml dH_2O

Solution B:

8g Paraformaldehyde

95ml 10mM NaOH

Paraformaldehyde was solubilized in 10mM NaOH at room temperature till pH reached 10. pH was adjusted to 7.4 with HCl. Aliquoted and frozen.

IF blocking and permeabilization:

3 % (w/v) BSA

1 % (w/v) Saponine

0.5 % (v/v) Triton X-100

in 1x PBS

Wash solution 1:

3 % (w/v) BSA

1 % (w/v) Saponine

in 1x PBS

Wash solution 2:

1 % (w/v) Saponine

in 1x PBS

Flow cytometry

Fixative:

70% ethanol

Chilled at -20℃ before use

FACS binding buffer:

10mM HEPES/NaOH, pH 7.4

140 mM NaCl

5 mM $CaCl_2$

In dH_2O

Wash solution 1:

1% (w/v) BSA

PBS

Wash solution 2:

1% (w/v) BSA

0.5% (v/v) Tween-20

In 1x PBS

Sodium borate solution

0.1M ($Na_2B4O_7H_2O$)

In dH_2O

pH 8.5

DNA Denaturing solution:

0.5% (v/v) Triton x100

In 2N HCL

Propidium Iodide staining solution:

3.8 mM NaCitrate

50µg/ ml PI

In 1x PBS

RNase solution:

10µg/ ml RNaseA

Boiled 5 minutes and frozen

ELISA

Wash solution:

0.05% (v/v) Tween-20

1x PBS

Stop Solution:

1M H_3PO_4

2.5 Antibodies

Table 5: Primary and Secondary antibodies

Antibody	Application & Concentration	Catalogue number	Manufacturer
p-Creb	IB 1:1000	9191	Cell signaling
Creb	IB 1:1000 IF: 1:1500	9197	Cell signaling
p-IκBα	IB 1:1000	9246	Cell signaling
IκBα	IB 1:1000	Sc-371	Santa Cruz
c-Jun	IB 1:1000	9165	Cell signaling
β-Actin	IB 1:5000	A5441	Sigma-Aldrich
NFATc3	IF: 1:100	Sc-8321	Santa Cruz
p-65	IF 1:100	Sc-8008	Santa Cruz
p-ERK1/2(Thr202/Tyr204)	IB 1:1000	9101	Cell signaling
ERK1/2	IB 1:1000	9102	Cell signaling
p-p38 MAPK	IB 1:1000	9211	Cell signaling
p38 MAPK	IB 1:1000	9291	Cell signaling
p-MEK1/2(ser217/221)	IB 1:1000	9121	Cell signaling
MEK1/2	IB 1:1000	4694	Cell signaling
HRP conjugated anti rabbit	IB 1:2500	W4011	Promega
HRP conjugated anti mouse	IB 1:2500	W4021	Promega
BrdU-FITC	FACS	347583(7583)	BD Pharmingen™
Annexin V-FITC	FACS	556419	BD Pharmingen™
Alexa Flour 488 chicken anti rabbit IgG	IF: 1:750	A21441	Invitrogen
Alexa Flour 488 goat anti	IF: 1:750	A11001	Invitrogen

mouse IgG			
Alexa Flour 594 chicken anti rabbit IgG	IF: 1:750	A21442	Invitrogen
Phalloidin	IF: 1: 100	505-33	Dyomics

2.6 Kits

Table 6: Kits used in the sudy

Kit	Description
Dual Luciferase Reporter Assay System®	Promega
DNeasy® DNA Purification Kit	Qiagen
Wizard® SV Minipreps Plus DNA Purification System A1460	Promega
PureYieldTM Plasmid Midiprep System	Promega
IL-8 ELISA	eBioscience
BCA Assay	Thermo Scientific
Caspase 3/7 Assay®	Promega
Cell Titer-Glo®	Promega
Mycoplasma Test PCR kit	Applichem

2.7 Software

Table 7: Softwares used for data analysis

Software	Description
Axio Vision Rel. 4.4	Zeiss
Photoshop CS	Adobe Systems, CA (USA)
IMAGE J software	National Institutes of Health, MD (USA)
Flow Jo	FlowJo, Ashland, Oregon, USA
Quantity one software.	BioRad
LAF-AS	Leica
NCBI/BLAST	National Center for Biotech
OligoCalc	Northwestern University Chicago

2.8 Cell lines

Table 8: Cell lines

Cell line	Cell type/ description	Purchased/ Reference
Jurkat	Human T lymphocytes (Acute Leukemia)	TIB: 152
GRANTA-519	B cell lymphoma	(Jadayel, Lukas et al. 1997)
KATOIII	Human gastric adenocarcinoma	HTB-103
AZ-521	Human gastric adenocarcinoma	JCRB0061, (Taki, Ishikawa et al. 1985)
AGS	Human gastric adenocarcinoma	CRL-1739
MKN45	Human gastric undifferentiated carcinoma	RCB:1001
HCT116	Human colorectal carcinoma	CCL-247
HEK293	Human embyonic kidney epithelial cells	CRL-1573
DLD1	Human colorectal adenocarcinoma	CCL-221
LS174T	Human colorectal adenocarcinoma	CCL-188
Caco-2	Human colorectal adenocarcinoma	HTB-37
COS-7	Transformed monkey kidney fibroblasts	CRL-1651
HaCaT	Human immortalized keratinocytes	(Boukamp, Petrussevska et al. 1988)
Huvec	Human umbilical vein endothelial cells	Biederstein

2.9 Bacteria

Table 9: Bacterial strains

Bacterium	**ATCC number**	**Description**	**Provided by/ reference**
H. pylori	G27	Human clinical isolate	(Baltrus, Amieva et al. 2009)
*H. pylori*ΔgGT	-	gGT sequence disrupted by Kanamycin resistance cassette	(Schmees, Prinz et al. 2007)
H. bilis	43879	Human clinical isolate	(Romero, Archer et al. 1988)
H. bilis ΔgGT	-	gGT sequence disrupted by Chloramphenicol resistance cassette	(Rossi, Bolz et al. 2012)
DH5α E.coli		Transformation competent cells	(Hanahan 1983)

2.10 Recombinant proteins

Table 10: List of recombinant proteins.

Recombinant proteins	**k_{cat} [10^2 min^{-1}]**	**K_M^{gGpNA} [µM]**	**Provided by/ reference/ description**
HPgGT	17.7±0.5	9.8±1.5	Christian Bolz (Schmees, Prinz et al. 2007)
HPΔgGT	-	-	Christian Bolz (Schmees, Prinz et al. 2007)
HBgGT	1.12±0.03	7.7±1.2	Christian Bolz (Rossi, Bolz et al. 2012)
IFNγ	-	-	Human, purchased from Preprotech (cat no: 100–21C)
TNFα	-	-	Human, purchased from Preprotech (cat no: 300-01A)

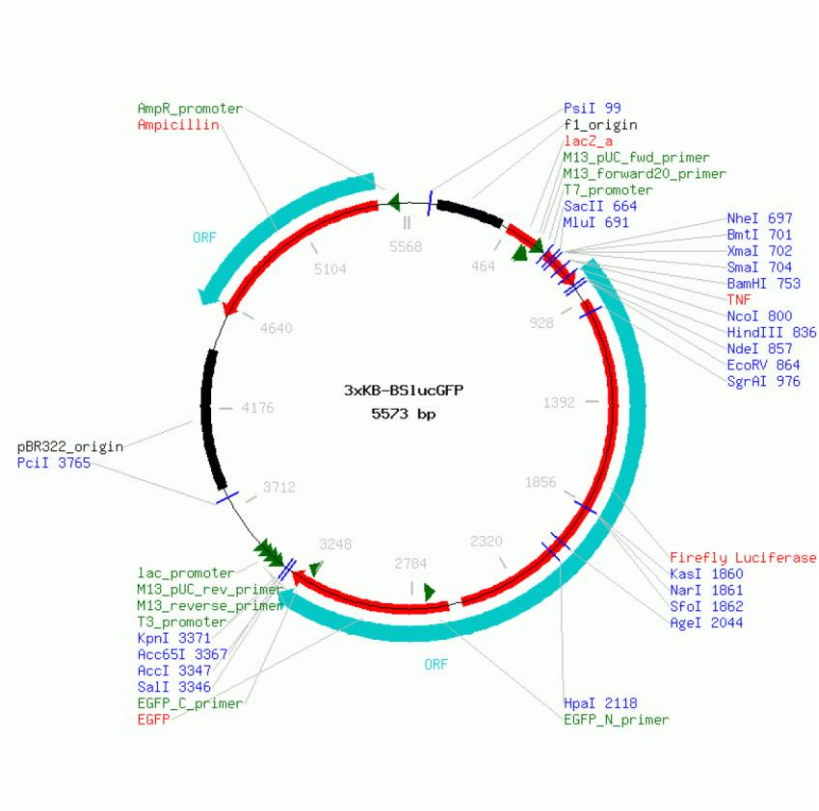
2.11 Primer sequences

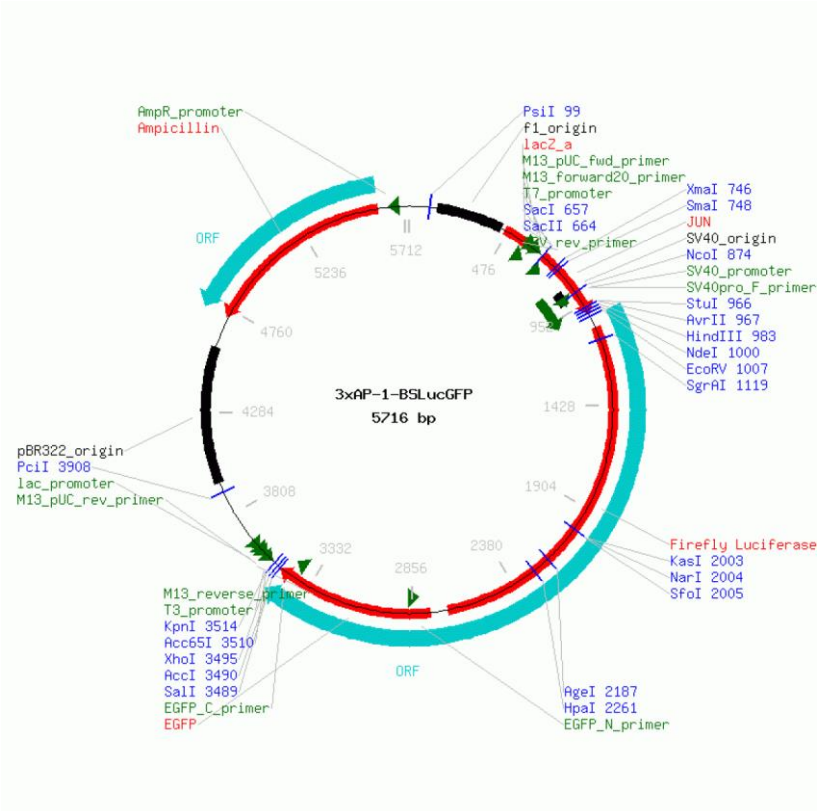
Table 11: List of Primer sequences.

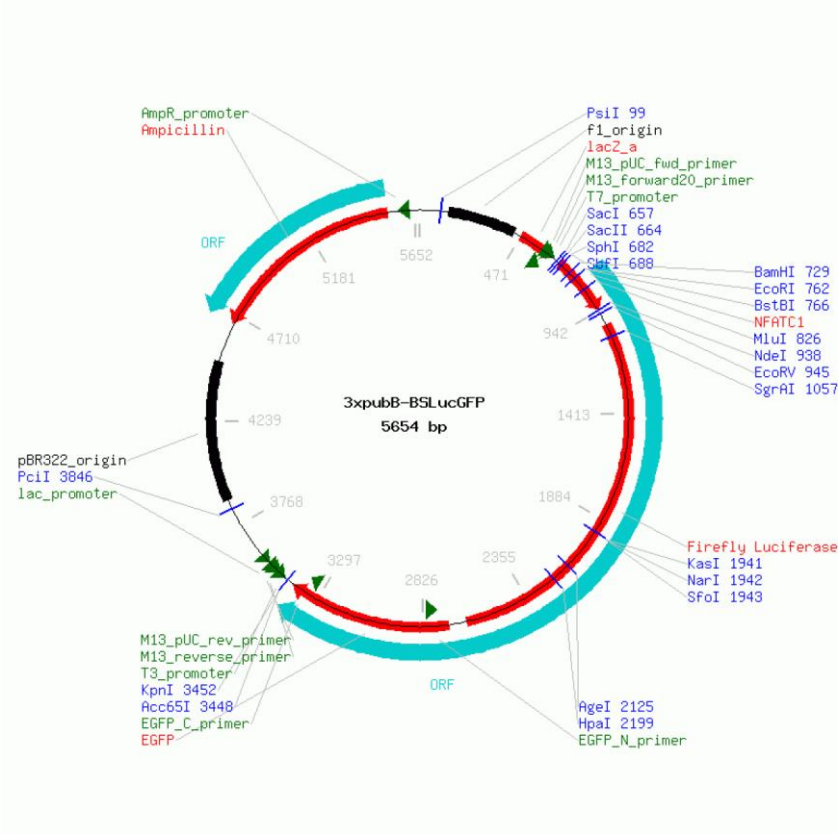
Primer	Primer sequence 5´-3´	Annealing temperature	Amount for sequencing
Promoter screening (luciferase reverse primer)	**5′-CTTGCCGGTGGTGCAGATGA-3′**	60°C	7.5pmol
HPgGT screening-forward	**5′-AAACGATTGGCTTGGGTGTGATAG-3′**	60°C	5pmol
HPgGT screening-reverse	**5′-GACCGGCTTAGTAACGATTTGATAG-3′**	60°C	5pmol
HBgGT screening-forward	**5′- TGC AAA GGG CAA GGC AGT C-3′**	60°C	5pmol
HBgGT screening-reverse	**5′- ATC CTT GCA CCA CCC GGA CT -3′**	60°C	5pmol

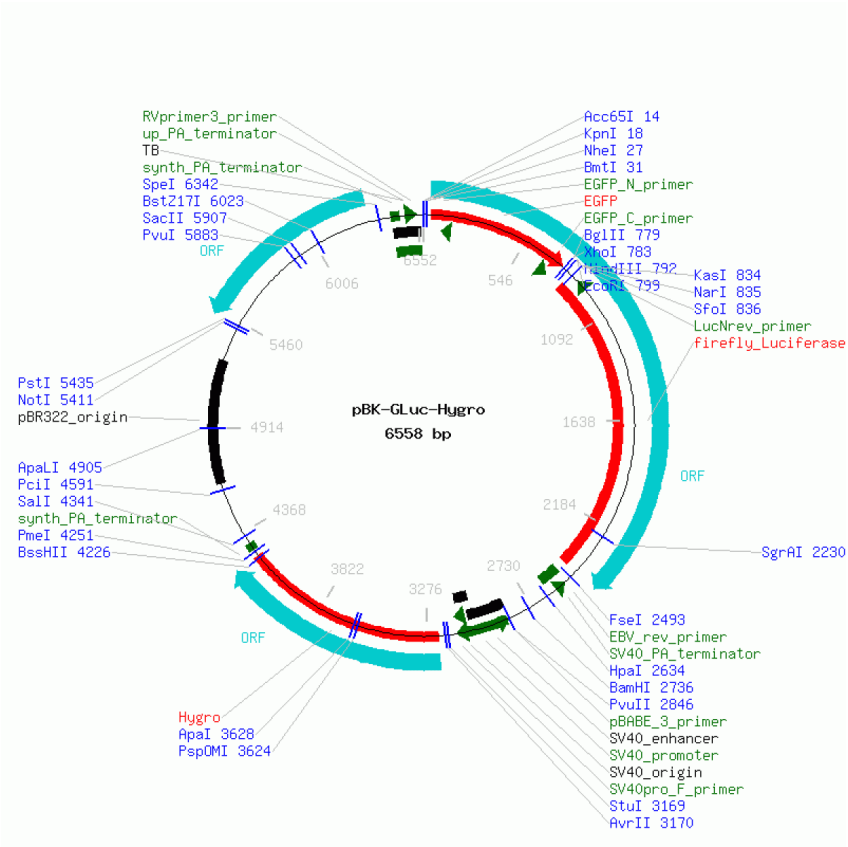
2.11 Plasmids

2.11.1 Luciferase reporter plasmid maps






3xpubB-BSLucGFP
5654 bp
AmpR_promoter
Ampicillin
ORF
5181
5652
PsiI 99
f1_origin
lacZ_a
M13_pUC_fwd_primer
M13_forward20_primer
T7_promoter
SacI 657
SacII 664
SphI 682
SbfI 688
471
BamHI 729
EcoRI 762
BstBI 766
NFATC1
MluI 826
NdeI 938
EcoRV 945
SgrAI 1057
942
1413
1884
Firefly Luciferase
KasI 1941
NarI 1942
SfoI 1943
2355
2826
AgeI 2125
HpaI 2199
EGFP_N_primer
ORF
3297
M13_pUC_rev_primer
M13_reverse_primer
T3_promoter
KpnI 3452
Acc65I 3448
EGFP_C_primer
EGFP
3768
pBR322_origin
PciI 3846
lac_promoter
4239
4710


pBK-GLuc-Hygro
6558 bp
RVprimer3_primer
up_PA_terminator
TB
synth_PA_terminator
SpeI 6342
Bst2I7I 6023
SacII 5907
PvuI 5883
ORF
6006
6552
Acc65I 14
KpnI 18
NheI 27
BmtI 31
EGFP_N_primer
EGFP
EGFP_C_primer
BglII 779
XhoI 783
HindIII 792
EcoRI 799
546
KasI 834
NarI 835
SfoI 836
LucNrev_primer
firefly_Luciferase
1092
5460
PstI 5435
NotI 5411
pBR322_origin
4914
1638
ApaLI 4905
PciI 4591
SalI 4341
synth_PA_terminator
PmeI 4251
BssHII 4226
4368
ORF
2184
SgrAI 2230
3822
3276
2730
FseI 2493
EBV_rev_primer
SV40_PA_terminator
HpaI 2634
BamHI 2736
PvuII 2846
pBABE_3_primer
SV40_enhancer
SV40_promoter
SV40_origin
SV40pro_F_primer
StuI 3169
AvrII 3170
ORF
Hygro
ApaI 3628
PspOMI 3624

Renilla luciferase repoter vector (Simian Virus SV 40) was purchased from promega.

1. Cell culture methods

1.1 Maintainance of cell cultures

All cell lines were maintained at standard conditions (37°C / 5% CO_2).

Non-adherent NCEB, Granta and Jurkat cell lines were maintained in RPMI containing L-Glutamine (except where indicated) supplemented with 10% FBS and 1% Penicillin/ Streptomycin. Cells were passaged twice a week by disrupting the cell clumps mechanically via pipetting and resuspending single cell suspension in fresh medium.

Adherent AZ-521, AGS, MKN45, KATOIII, COS-7, HEK, HCT116, DLD-1 and LS174T cell lines were maintained in DMEM containing L-glutamine (except where indicated) supplemented with 10% FBS and 1% Penicillin/Streptomycin. HaCaT and HUVEC cells were maintained in serum free medium (KSF) supplemented with growth factors.

Confluent cells were passaged by washing once with PBS and trypsinizing with 0.05 % Trypsin-EDTA solution until cells detached from culture dish. Trypsinization was blocked by adding culture medium containing FBS. Cells were collected, centrifuged for 5 minutes at 1000 rpm at room temperature, resupended in fresh culture medium and seeded 1:10 aliquot onto a new tissue culture flask.

1.1.1 Freezing / thawing of cells

Cells were suspended at 1-2x10^6 cells per ml of culture medium with 10% DMSO. The cells were passively frozen in cryovials first at -80°C and long term stored in liquid nitrogen.

Frozen cells were thawed and immediately resuspended in culture medium, washed once to remove any residual DMSO and seeded into tissue culture flasks with fresh medium. The culture medium was changed after 1 day of culture.

1.1.2 Mycoplasma Contamination check

Regular mycoplasma contamination tests were performed using a commercially available PCR Kit.

Cells were cultured for 3 days without antibiotics. The supernatant was removed and centrifuged to remove any detached cells or debris. Supernatant was stored at -20°C till tested.

1.2 Bacterial culture

Bacterial cultures were maintained on blood agar plates supplemented with WC-DENT. *H. bilis* and *H. pylori* could be maintained with minimum viability loss for up to 3 days in culture after which the bacteria were sub-cultured onto fresh agar plates. Bacterial cells were only sub-cultured up to 3 times to minimize genotypic and/or phenotypic changes.

1.2.1 Bacterial stocks (freezing/ thawing)

Bacterial stocks were made by harvesting a full grown plate in 1 ml of BHI dent medium. The cell suspension was centrifuged at 4000 rpm for 10 minutes at 4°C. The cells were resuspended in 1ml of BHI dent with 20% glycerol and aliquoted and flash frozen in liquid nitrogen. Bacterial stocks were stored at -80°C.

H. bilis or *H. pylori* from a frozen stock was streaked onto a blood agar plate with WC-DENT. Bacteria were sub-cultured once and incubated for 1 to 2 days before the bacteria were used for inoculation. The gGT deficient bacteria were cultured onto WC-DENT agar plates with the respective selection antibiotic (chloramphenicol for *H. bilis* and kanamycin for *H. pylori*).

1.2.2 Isolation of bacterial genomic DNA

The gGT knock out bacteria were confirmed via PCR using whole genomic DNA from the bacteria. A few loopfulls of bacterial culture from the WC-DENT plates was resuspended in PBS and spun down at 5000rpm for 5 minutes. DNA was isolated according to the manufacturer´s instructions using the Qiagen DNeasy kit.

1.3 Cell and Bacterial co-culture

H. bilis or *H. pylori* from a frozen stock was streaked onto a blood agar plate with WC-DENT. Plates were incubated for 1 day before the bacteria were used for inoculation of 80 % confluent cells. Bacteria were suspended in DMEM and adjusted OD to 1.0 ($2x10^8$ CFUs/ml). Cells out of one well of a pre-seeded cell culture plate (all wells with the same number of cells seeded) was counted after trypsinization. Bacteria were added accordingly to an MOI of 5-50 per cell.

1.4 Cell viability assay

CellTiter-Glo Luminescent Cell Viability Assay system was used to determine cell viability. This is a robust assay which relies on the generation of a luminescent signal when the assay substrate (Beetle luciferin) is converted to Oxylluciferin by thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) in the presence of ATP. The luciferase reaction for this assay is shown in Figure 13. High ATP levels are present in viable cells. The luminescent signal generated by this method is directly proportional to the number of cells.

Briefly, 5000 cells were seeded per well in a 96 flat bottom plate. Adherent cells were allowed to attach before treatment with recombinant gGT protein for 48 hours. At the end of treatment cells were lysed by adding 100 µl/well of the lysis reagent with the substrate provided in the kit. 150 µl of the lysate was transferred to an opaque microplate to minimize background and luminescence was recorded at 4 nm in the Mitras plate reader. Luminescence was measured in triplicates and expressed as arbitrary units.

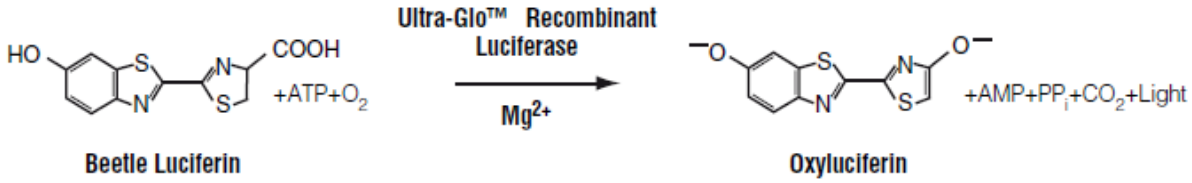


Figure 13: The luciferase reaction.

Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg2+, ATP and molecular oxygen (Picture coutesy of Promega Cell Titer Glo Assay guide).

1.5 Caspase 3/7 assay

Caspase-Glo Assay uses a proluminescent caspse-3/7 substrate containing the signature DEVD peptide sequence. Following the addition of Caspase-Glo3/7 reagent Caspase-3 or -7 was released by cell lysis, which cleave the DEVD substrate from the aminoluciferin. Aminoluciferin is then oxidized by luciferase producing light. The amount of light produced is proportional to the amount of caspase-3 or -7 activitiy.

1x 10^4 cells were seeded per well in a 96 flat bottom plate. Adherent cells were allowed to attach before treatment with recombinant gGT protein for 6-24 hours. At the end of treatment culture plates were brought to room temperature prior to cell lysis by addition of 100µl/ well of the reagent provided in the kit. Plates were then transferred to a shaker to mix the contents of the well briefly before incubation for 30 minutes at room temperature. 150µl of the lysate

was transferred to an opaque microplate to minimize background and luminescence recorded at 450 nm in the Mitras plate reader. Background luminescence was determined from wells containing only medium and sustracted from the final reading. Luminescence from samples was measured in triplicates and expressed as arbitrary units.

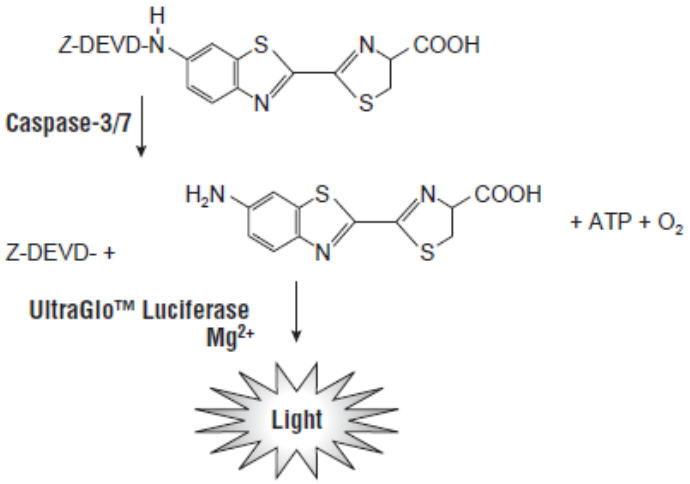


Figure 14: Reaction quantified in the caspase 3/7 assay

Caspase-3/7 cleavage of the luminogenic substrate containing the DEVD sequence. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and the production of light (Picture coutesy of Promega Capase 3/7 assay guide).

2. Flow cytometry

2.1 Apoptosis assay

Dying cells can be detected with annexin V. Labeling of annexin V with fluorescent or radioactive molecules makes it possible to detect binding of labeled annexin V to the cell surface of apoptotic cells. Annexin V binds to the phospholipid surface of the cells where it assembles into a trimeric cluster. This clustering of annexin V on the membrane greatly increases the intensity of annexin V signal when labeled with a fluorescent probe. Two-dimensional crystal formation is believed to cause internalization of annexin V via endocytosis which occurs on cells which are in the early phase of cell death. Internalization additionally amplifies the intensity of the annexin V signal on stained cell.

Propidium iodide (PI) is an intercalating agent and a fluorescent molecule that can be used to stain DNA. PI is membrane impermeable and generally excluded from viable cells. In flow

cytometry, PI is commonly used to identify dead cells or DNA content during cell cycle analysis.

1x 10^5 cells (HCT116, DLD-1, MKN-45 and AGS) were seeded in a 6 well tissue culture plate. Cells were allowed to adhere and then treated with different amounts of recombinant gGT protein for 24 and 48 hours. 2x 10^5 cells were harvested by trypsinization and washed once with PBS by spinning at 2000 rpm for 5 minutes. Cell pellets were resuspended in FITC-Annexin V antibody at a concentration of 10 µg/ml in binding buffer and incubated in the dark for 20 minutes at room temperature. Following incubation, the mixture was diluted in 0.1 ml of binding buffer. 10 µl of a 50 µg/ml PI stock sloution was added to the cells directly before flow cytometric data acquisition in p5 cyan BD flow cytometer. 10000 events per sample were recorded and cell debris excluded via forward vs. side scatter. Staurosporine, a potent protein kinase inhibitor was used to induce apoptosis in the cells. Unstained and single stained cells were used for compensation of spillover of florescence from the two channels.

2.2 Cell cycle analysis by Flow cytometry

2.2.1 BrdU PI cell cycle staining (2 dimensional cell cycle analyses)

BrdU can be substituted for thymidine in the DNA of replicating cells (during the S phase of the cell cycle). Antibody (BrdU-FITC) specific for BrdU can then be used to detect the incorporated chemical into the DNA of actively replicating cells. Propidium iodide (PI) on the other hand could be used to stain the DNA thus enabling the quantification of the DNA content of a cell.

Cells in G1 phase of the cell cycle have less DNA and don't incorporate BrdU. Cells in G2 phase have no or relatively less BrdU incorporation and more DNA as compared to G1 cells. Early S phase have less DNA content and start to incorporate BrdU in the DNA, whereas in late S phase the cells have BrdU and more DNA.

1x10^6 Cells were cultured in 6 well tissue culture plates and let to adhere. Cells were serum starved for 24 hours prior to treatment with recombinant gGT for 24 hours. 20µM of BrdU was added for upto 1x $10^{6\text{-}8}$ cells and incubated for 2 hours for maximum incorporation into the cells. Cells were harvested by trypsinization and washed twice with PBS and pelleted at 500xg for 15 minutes at room temperature. The cells were resuspended in 100µl PBS and fixed in 3ml of 70% Ethanol on ice for 30 minutes. Cells were pelleted and resuspended in 1ml HCl-Triton X 100 solution dropwise while maintain a vortex to denature the DNA. The cells were then incubated 30 minutes at room temperature. The cells were centrifuged and

resuspended in neutralizing sodium borate solution. The cells were washed once with wash solution 2, quantified and stained by incubating 1 hour at room temperature with anti-BrdU-FITC antibody (1:8 in wash buffer 2 per 1x106 cell count). Cells were washed again and resuspended in 500µl PBS containing 0.5µg/ml PI.

The cells were acquired with cyan cytometer and data analysed by FlowJo. Unstained and single stained cells were used for compensation of spillover of florescence from the two channels. Serum starved cells were used as a G1 arrest control.

2.2.2 PI staining (1 dimensional cell cycle analysis)

PI staining was used to determine the DNA content of a cell for cycle analysis. PI alone could be used to separate cells in different phases of the cell cycle on the basis of their DNA content on a logarithmic scale. Cells in G1 phase of the cell cycle have less DNA; cells in G2 phase have more DNA. S phase cells have less DNA content than G2 cells but more than cells in G1.

Cells were cultured and treated as described in 2.2.1. Harvested cells were washed in PBS/BSA and spun down at 500xg for 10 minutes. After washing, cells were suspended in ice cold PBS and fixed in 3ml of 70% Ethanol for 30 minutes on ice. Cells were washed twice in PBS/BSA and the pellet was resuspended in 1ml of PI staining solution. RNAse digestion was done for 3hours at 4°C to get rid of unspecific PI binding to RNA in the cells. Samples were acquired with cyan cytometer and data analysed by FlowJo.

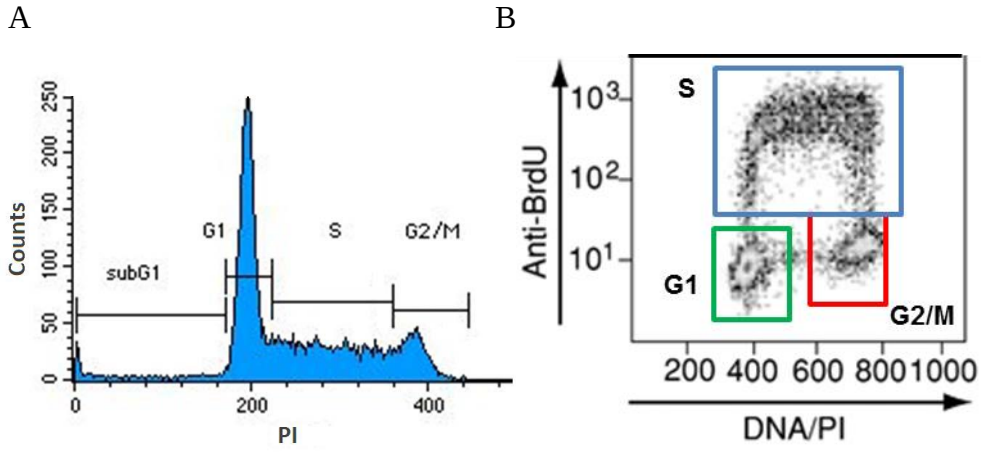


Figure 15: Flow cytometric analysis of the cell cycle.

Different phases of cell cycle as observed with flow cytometry using A) PI (Propidium iodite) staining and B) BrdU and PI staining.

3. Functional assays

3.1 Luciferase reporter assays

Transcriptional activation of different pathways can be measured in a precise manner using the luciferase reporter system. Therein reporter plasmid contructs were transfected into the cells by a cationic lipid lisosome, Lipofectamine. A typical reporter plasmid contruct for investigation of transcriptional activity is shown in figure 16, where the Firefly luciferase gene is under the regulation of a promoter containing binding sites of a certain transcription factor.

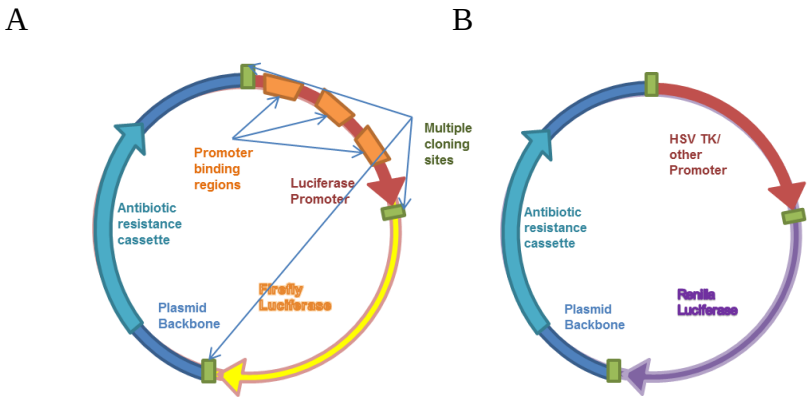


Figure 16: Typical plasmid contstructs used for dual luciferase reporter assay system

A) a typical luciferase reporter plasmid contstruct B) Renilla contruct

Firefly and Renilla luciferases, because of their distinct evolutionary origins, have different enzyme structures and substrates, making it possible to discriminate between their bioluminescent reactions.

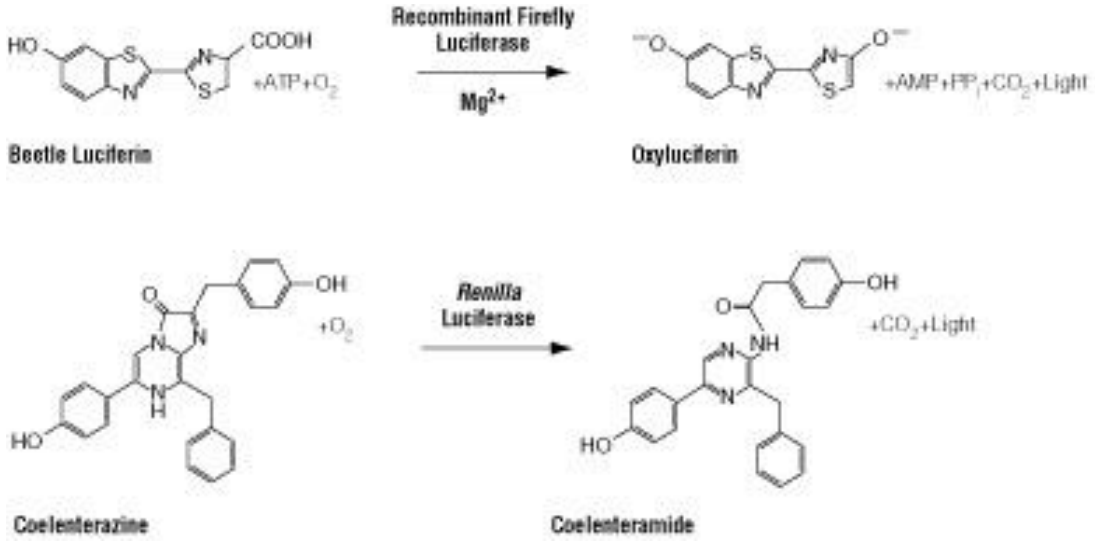


Figure 17: Firefly and Renilla luciferase reactions

Firefly and Renilla luciferase reactions with their respective substrates, beetle luciferin and coelenterazine, to generate bioluminescence (Picture coutesy of Promega Dual luciferase reporter assay guide).

A dual luciferase reporter assay system allows for the measurement of the two separate luminescent signals generated by firefly and renilla luciferase enzymes expressed simultaneously from transfected cells. In a transient transfection system, although absolute quantification of the luciferin signal is possible, however it is necessary to perform a comparative analysis as the tranfection efficiency may vary between different treatments. For this purpose an internal control is used to determine the transfection efficiency. Therefore, target cells are transfected in parallel with a renilla plasmid. So, while cells with varying transcriptional activation of the reporter plasmid may have different firefly luciferase values, all successfully transfected cells would have the same renilla value.

3.1.1 Transient transfections

Cells were transiently transfected with the AP-1, NFkB, NFAT and CREB luciferase reporter plasmids. The luciferase activity as normalized with renilla directly correlates to the respective transcription factor activity.

$1x10^5$ cells were seeded per well of a 24 well tissue culture plate, twenty-four hours prior to transfection, the cells were serum starved for an additional 24 hours. Serum supplemented medium was added before starting the transfection. 1 µl Lipofectamine was diluted in 100 µl Opti-MEM, and then 750 ng of plasmid DNA added and incubated for 30 minutes at room

temperature. Culture medium was replaced with 0.4 ml of fresh culture medium with 10% serum and 100µl of the transfection mixture was added to the cells. The cells were transfected for 20 hours prior to experimental treatment or infection.

3.1.2 Preparation of lysates for Luciferase assay

Culture media from cells after a certain treatment period was removed and the cells washed 1x with PBS. 100µl per well of passive cell lysis buffer was added and the cells incubated at room temperature on a shaker for maximum cell lysis. Plates were stored at -20C until needed. 20µl from each well was used for the quantification of luciferase activity.

3.2 Plasmid preparation

3.2.1 Transforming competent cells DH5

2 µl of plasmid DNA was added to a 50µl aliquot of competent bacteria and mixed carefully. The bacteria were incubated on ice for 10 minutes; heat shocked at 42°C for 45 seconds and then put on ice for 10 minutes. 900µl LB-medium was added and incubated at 37°C with shacking for 45 minutes. Bacteria were centrifuged at 400rpm for 5 minutes and resuspended in 100µl LB medium. Next the bacteria were plated onto LB agar plates with ampicillin and incubated over night at 37°C.

3.2.2 Plasmid isolation

Competent DH5α *E.coli* was transformed with plasmid DNA and plasmids were isolated from overnight LB medium cultures with Promega Midi prep kit according to the manufacturers' instructions. Amount and quality of plasmid DNA was quantified by nanodrop spectrophotometer.

3.2.3 Plasmid promoter sequence confirmation

The promoter sequence of the plasmids was confirmed by sequencing. Sequencing was performed by MWG eurofins using primers (sequneces indicated in Table: 11) pre-mixed with 800 ng of plasmid DNA preparation.

3.3 Immune florescent staining

Cells were grown on cover slips or in chamber slides. Following treatment, the cells were washed once with PBS and fixed with Fixative for 15 minutes. Fixative was removed and

cells were washed 3 times with PBS. Cover slips and chamber slides were transferred to a humidified chamber (dark box with parafilm and wet paper towels) and permeabilized in Permeabilization buffer for 15 min at room temperature. Permeabilization buffer was removed and cells incubated with primary antibodies (diluted in washing buffer) overnight at 4°C. Incubation with secondary antibodies was done for 4 hours at 4°C. After incubation, antibody solution was removed and cells were washed 3-5 times for 5 minutes with washing buffer. Before mounting, cells were washed once with PBS. Cells were mounted with Vectashield and sealed with nail varnish.

Samples were imaged with a florescence microscope, arranged by using Volocity software and assembled with Photoshop.

3.4 gGT activity assay

gGT activity was measured using the artificial substrate l-γ-glutamyl-p-nitroanilide. Briefly, a reaction buffer consisting of 20 mmol/L glycyl-glycine as acceptor, 2.5 mmol/L L-γ-glutamyl-p-nitroanilide as donor substrate, and 100 mmol/L Tris-HCl (pH 8.0) was prepared. Purified recombinant HPgGT, mutant HPgGT, HBgGT or the heat inactivated HBgGT were added to the reaction mixture and incubated at 37°C for 30 minutes. The release of p-nitroanilide was monitored by spectrophotometry at 405 nm. One unit of activity was defined as the quantity of enzyme that released 1 μmol of p-nitroanilide per minute and per milligram of protein at 37°C.

4. Biochemical methods

4.1 gGT PCR screening

A gGT screening PCR was performed to confirm for successful disruption of the gGT sequence in case of both *H. pylori* and *H. bilis*. 50ng of the genomic DNA isolated from the bacteria was amplified using specific gGT screening primers. The following PCR protocol was used:

PCR Reaction:

10X High Fidelity Buffer 5μl

dNTP mix 2,5mM each 4μl

50 mM MgSO4 2µl

Forward Primer 0.5µl

Backward Primer 0.5µl

Template DNA 1µl

Taq DNA Polymerase HF 0.2µl

dH2O 36.8µl

Cycling Parameters:

Denaturation 94°C 2 min

Denaturation 94°C 30sec
Annealing 60°C 30 sec } 35 Cycles
Extension 72°C 30 sec

Final Extension 72°C 10 min

4.2 Agarose gel electrophoresis

1% agarose gels were made by boiling 1g of agarose in 100ml of TAE buffer and 5µl of ROTI safe was added for DNA detection. Amplified DNA was separated by northern blot on a 1% agarose gel at 95 volts for 45 minutes. The agarose gels were imaged using the gel doc XR+ documentation system from BioRad.

4.3 Immunoblotting

Proteins were probed using western blot.

4.3.1 Preparation of whole cell protein lysates

Whole cell lysates were obtained after 1x wash PBS and cells were scraped and collected in 75µl of SDS lysis buffer or 1x RIPA buffer. Cells were lysed after 15 min, 30min, 1hr, 2hrs, 6hrs, 8hrs, 10hrs and 24hrs of bacterial co-culture. Lysates were sonicated in the sonic water bath for 3min, the protein content quantified and boiled at 95°C (4x SDS sample buffer was added to lysates in RIPA buffer). The lysates were briefly centrifuged to collect any condensation in the lid and stored at -20°C.

4.3.2 Determination of protein concentration

Protein concentration of the samples before addition of SDS sample buffer was determined using a colorimetric assay. The BCA assay kit was used for this purpose according to the manufacturer's instructions. Color development was measured by a spectrophotometer.

4.3.3 Gel electrophoresis:

4-5µl of each lysate was loaded onto a 1 mm 10% Acrylamide gel. Gel electrophoresis was performed with the BioRad electrophoresis system containing SDS-Buffer at 150 Volts for 90 minutes at room temperature.

4.3.4 Protein Transfer

Following electrophoresis protein transfer was done using the wet transfer method and ethanol transfer buffer. The acrylamide gels were sandwiched between a nitrocellulose membrane and several sheets of whattman paper and sponges, soaked in transfer buffer. Proteins were transferred to a nitrocellulose membrane at 230mA for 2 hours. The membrane was briefly stained with Ponceau.

4.3.5 Blocking and antigen detection

Following transfer, the membranes were blocked at room temperature for 1 hour in 5% Milk. TBST. After blocking the membranes were briefly washed in TBST before incubating in the Primary antibody diluted in 5% BSA.TBST overnight at 4°C:

Primary antibody was removed after incubation and washed four times TBST (10 min wash each). Membranes were incubated with secondary antibody diluted in 5% Milk. TBST at room temperature for 1 hour.

Membranes were washed at least six times with TBST (10 min wash each). Membranes were removed from the last wash, put in a dark chamber and overlaid with 1:1 prepared solution of ECL developing solution for 1 minute in dark. The developing solution was removed and the membranes arranged in the developing cassette. Negatives were developed after exposure timings with the membranes from 15 seconds ranging up to 15 minutes.

Negatives were scanned and assembled with Photoshop. Relative quantification was done with the Quantity one software.

4.4 Enzyme linked Immunosorbant assay

Cells were grown in 12 well tissue culture plates. Following treatment, supernatants from the cells were collected and centrifuged briefly at 13000 rpm to remove non-adheret cells, bacteria and deris. Supernatants were stored at -20°C. Il-8 ELISA was performed according to the manufacturer's instructions.

ELISA plates were coated with 50 µl/well of capture antibody in Coating Buffer and incubated overnight at 4°C. After overnight incubation plates were washed 5 times with ELISA Wash Buffer and blotted on absorbent paper to remove any residual buffer. Wells were blocked with 100µl/well of 1X Assay diluent at room temperature for 1 hour. After a second wash step the wells were loaded with 50µl of the appropriate dilutions of the standards and sample supernatants and incubated overnight at 4°C for maximal sensitivity. Wells were aspirated and washed 5x to remove any unbound antigens. 50 µl/well of detection antibody was added to each well and incubated at room temperature for 1 hour. Following incubation the wells were washed 5x with the wash buffer. 50 µl/well of Avidin-HRP solution was added and plates incubated at room temperature for 30 minutes. Wells were washed 7X with wash buffer and 50 µl/well of Substrate Solution was added to each well for detection. Plates were incubated at room temperature for 15 minutes before stopping the reaction with 25 µl of Stop Solution.

Color development was recorded at 450 nm and values of 570 nm were subtracted. Each sample and standard was run in duplicates and analyzed with the TECAN ELISA plate reader.

4.5 Superoxide anion Quantification

Production of intracellular superoxide anion (O_2^-) can be measured using the NBT assay, whereby Nitroblue tetrazolium salt is reduced by O_2^- resulting in accumulation of dark blue Formazan crystals in the cells. The reaction is summarized in figure 18.

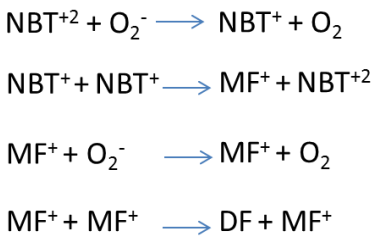


Figure 18: Superoxide reaction with NBT to form formazan crystals involved in the NBT assay.
NBT is present as a di-cation NBT+2 at normal physiological pH. It is reduced by a superoxide anion to NBT+, which reacts with itself to produce monoformazan (MF). Buildup of MF leads to a further reduction producing diformazan (DF), both of which are dark blue in color with absorption maxima of 530nm and 560nm respectively.

Cells were grown in 24 well plates and treated as previously. Medium was removed after treatment period and cells were washed once with DMEM. 250µl of 0.2% NBT solution was added per well and the cells incubated for 1 hour.
For visualization of the formazan crystals the cells were fixed with 4% PFA for 15 minutes on ice. After washing thrice with PBS, a 0.1% solution of safranin O was added for 30 seconds to stain the nuclei. Cells were washed three times with water and mounted with Flouromount.
Quantification was done by fixing the cells after NBT incubation with 100% methanol for 15 minutes. The cells were washed twice with 70% methanol and left to dry overnight in a fume hood. Cells were lysed by addition of 62.5µl per well of KOH and 75µl per well DMSO and homogenized by shaking the plate. 100µl of the lysate was transferred to a 96 well plate. Color development was recorded at 650 nm. Each sample and controls were run in duplicates and analyzed with the TECAN ELISA plate reader.

4. Statistics

Mean values and SEMs were calculated from at least three independent experiments. Statistical analysis was performed using the Student's T-Test, as indicated on the figures. Statistical significance denoted with asterisk, with *=p value of <0.05, **=p value <0.005, ***=p value <0.0005. The software GraphPad Prism (Graph Pad Software) was used.

Chapter 3: Results

3. Effect of HPgGT on cell viability

As previously discussed, HPgGT has been described to be an important bacterial virulence factor. It is essential for the colonization of the gastric mucosa by *H. pylori* (Chevalier, Thiberge et al. 1999), however, the mechanisms by which gGT is able to aid the bacterium in the infection process have not been fully characterised.

Previous work in our lab described HPgGT to inhibit proliferation of T lymphocytes (Gerhard, Schmees et al. 2005; Schmees, Prinz et al. 2007). Since the epithelial cells lining the gastric lumen are also exposed/encountered to with this bacterial protein upon infection, the effect of HPgGT on epithelial cells was analyzed. For this purpose, cells were treated with the recombinant protein and viability was examined.

3.1 *H. pylori* rgGT effects cell proliferation

To test the effect of HPgGT on cell viability, cell lines of different lineages including a human embryonic kidney epithelial cell line (HEK), human endothelial cells (HUVEC), human keratinocytes (HaCaT) and transformed monkey kidney fibroblasts (COS-7) as well as several gastric cancer epithelial cell lines (MKN45, AGS, AZ-521, KATOIII), were used. T and B cell lines Jurkat and GRANTA-519 were included in the assay as a comparative control as effect of HPgGT on the reduction of proliferation in these cells was previously demonstrated. Cells were treated with different concentrations of the recombinant HPgGT protein (0.1-10µg/ml) or the mutant HPgGT (10µg/mL) for 48 hours and number of viable cells determined by measuring the ATP content of the cell lysates (Figure 19), as measure of the ATP content directly corresponds with cell numbers. Mutant HPgGT was generated by introducing mutations in the serine 451 and 452 by replacing them with alanine residues of the active site rendering it enzymatically inactive.The loss of enzymatic activity was tested in an activity assay and the mutant protein was used as a control (Schmees, Prinz et al. 2007). The enzymatic activity of the recombinant HPgGT was also determined in the activity assay.

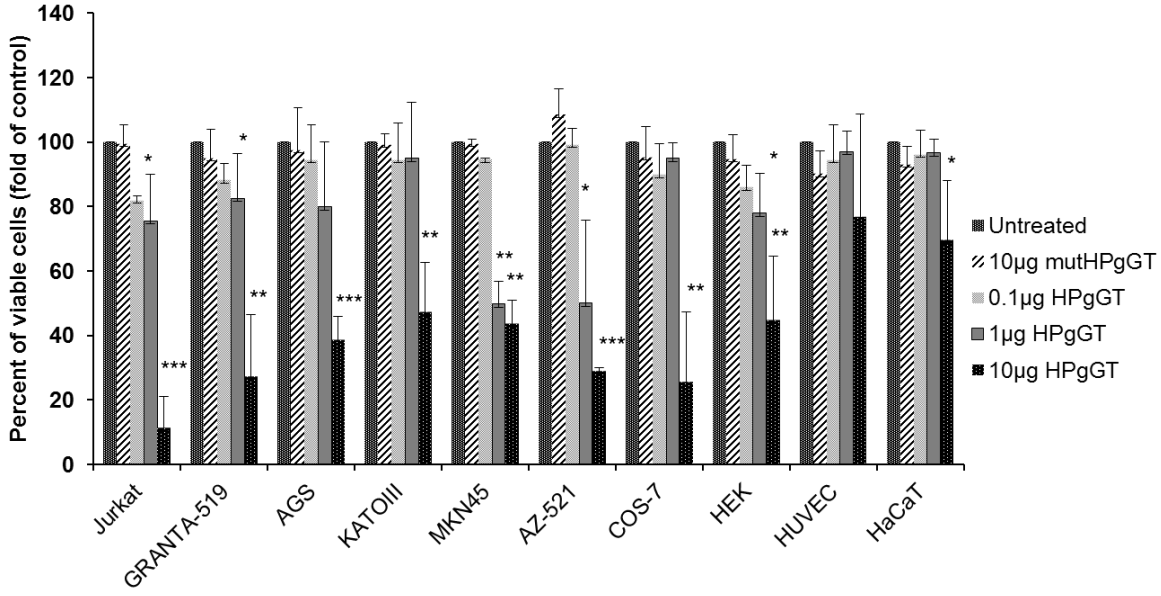


Figure 19: Cell viability after recombinant HPgGT treatment for 48 hours in different cell lines.
HPgGT in doses of 0.1-10µg/ml was used and mutant HPgGT (mutHPgGT) at a concentration of 10µg/ml was used as a control. Results are expressed as % of mean values normalized to the untreated control from three independent experiments. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

A dose dependent decrease in the number of viable cells could be observed upon treatment of cells with increasing amounts of the recombinant HPgGT (Figure 19). The T lymphocyte cell line Jurkat was the most susceptible to HPgGT, with a decreased cell viability of about 80% with 10µg of HPgGT per ml. Some other cell lines responded in a similar manner after exposure to 10µg/ml per ml of HPgGT, such as the gastric cancer epithelial cell lines AGS showing a decrease in cell growth of 62%), AZ-521 (71% decrease in cell growth), KATOIII (53% decrease in cell growth) and MKN45 (57% decrease in cell growth). Overall the gastric epithelial cancer cells exhibited 60-80% decreased cell growth. HEK and COS-7 cells showed also a significant reduction in proliferation of up to 56% and 75% respectively. The endothelial cells (HUVEC) and keratinocytes (HaCaT) showed the least susceptibility to HPgGT. The enzymatically inactive mutant HPgGT at a concentration of 10µg/ml on the other hand was not able to affect the viability of the cells.

3.2 Effect of HPgGT on cell growth is independent of apoptosis

In order to determine whether the reduced cell viability after exposure of cells to HPgGT was due to apoptotic changes in the cells, apoptosis assays were performed. Apoptosis was analysed by annexin V and PI staining.

Annexin V is an early marker for apoptosis since it intercalates into the membrane of early apoptotic cells, while PI binds to the DNA of apoptotic cells and is usually excluded from living cells. Early apoptotic cell populations incorporate only annexinV, and mark cells in early stages of apoptosis, whereas cells which are already perforated are positive for both annexinV and PI, representating cells undergoing apoptosis. Viable cells, on the other hand can exclude both dyes and hence are tested negative. Gastric epithelial cells AGS were treated with HPgGT at various doses (0.1, 1 and 10µg/ml) for 24 hours and then stained with PI and annexin V (Figure 23). Mutant HPgGT was used as an enzymatically inactive control at a concentration of 10µg/ml and staurosporine, a known apoptosis inducer in cells, was used as a positive control. Cells were FACS sorted to separate the apoptotic cell populations. Jurkat cells were used as a control, as the apoptosis independent growth inhibition in these cells has been well documented (Gerhard, Schmees et al. 2005; Schmees, Prinz et al. 2007).

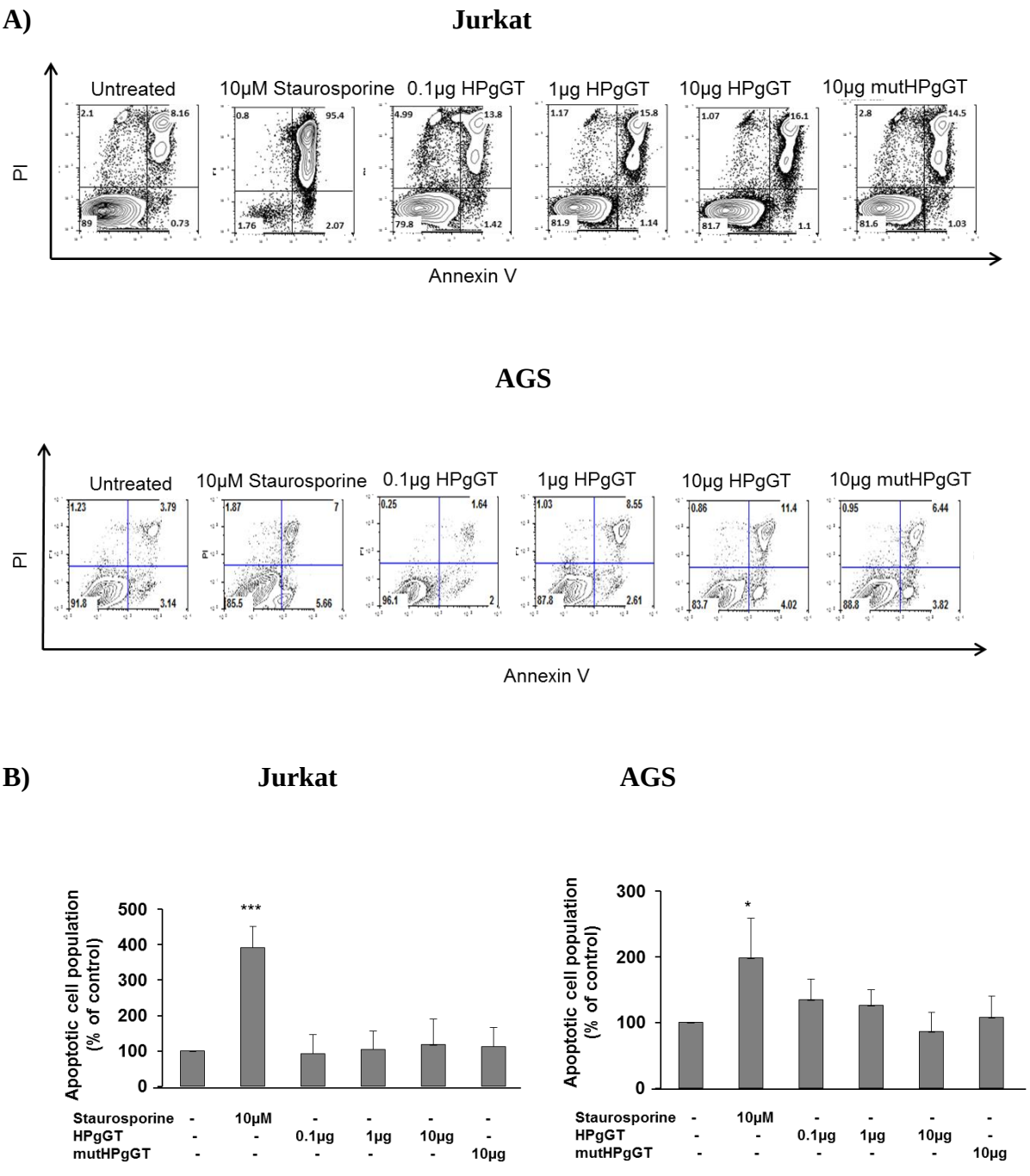


Figure 20: Apoptosis analysis of Jurkat and AGS cells after HPgGT treatment.

A) Representative zebra plot FACS analysis of annexin V-PI stained Jurkat and AGS cells after 24 hours of treatment with the recombinant HPgGT (amounts indicated were added per ml of cell culture). B) Comparison of annexinV and PI positive cell populations in both Jurkat and AGS cells normalized to untreated controls in three independent experiments. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

A bar graph representative of the percentages of the cell populations undergoing apoptosis (annexin V+PI positive) and the ones in early stages of the apoptosis (annexin V positive) was

plotted. A significant induction of apoptosis could be observed when the cells were treated with staurosporine, as expected. However, no significant differences in the apoptotic cell populations for both AGS and Jurkat cells could be observed after HPgGT treatment indicating apoptosis independent gGT effects on cell viability.

3. 3 HPgGT induces cell cycle arrest

Previous studies in our lab showed that HPgGT was able to induce a cell cycle arrest in T cells (Schmees, Prinz et al. 2007). To check if HPgGT was also able to arrest the epithelial cells in their cell cycle, AGS and MKN45 cells were treated with HPgGT at different concentrations (1µg and 5µg/ml) and mutant HPgGT (5µg/ml) for 24 hours. Cyclosporine A, which is a known inhibitor of the cell cycle and arrests the cells in the G1 phase of the cell cycle, was used as a positive control at a concentration of 10µg/ml.

The cells were serum starved overnight prior to treatment in order to synchronize them. After treatment the cells were incubated with BrdU to enable the replicating cells to incorporate it into the DNA. BrdU was stained with an anti BrdU FITC antibody and PI was used as a counter stain to account for cell numbers. The cell populations in different phases of the cell cycle were sorted via FACS analysis.

Both epithelial cell lines exhibited an increase in the cell population in the G1 phase of the cell cycle upon HPgGT treatment (Figure 21). AGS cells showed a significantly increased G1 cell population, from 46% in the untreated control to 59% in the HPgGT 5µg/ml treated cells. HPgGT dose of 1µg was however not so effective in inducing this arrest and only an increase of 3.2% in the G1 cell population could be observed. MKN45 cells showed an increase of approximately 14% from 33% in the untreated control to 47% and 49% in the G1 phase of the 1µg HPgGT and 5µg HPgGT treated cells, respectively. This result indicates that HPgGT is able to arrest the cells in the G1 phase of the cell cycle not only lymphocytes but also epithelial cells. This cell cycle arrest depends on the enzymatic activity of HPgGT since the mutant enzyme was not able to arrest the cell cycle.

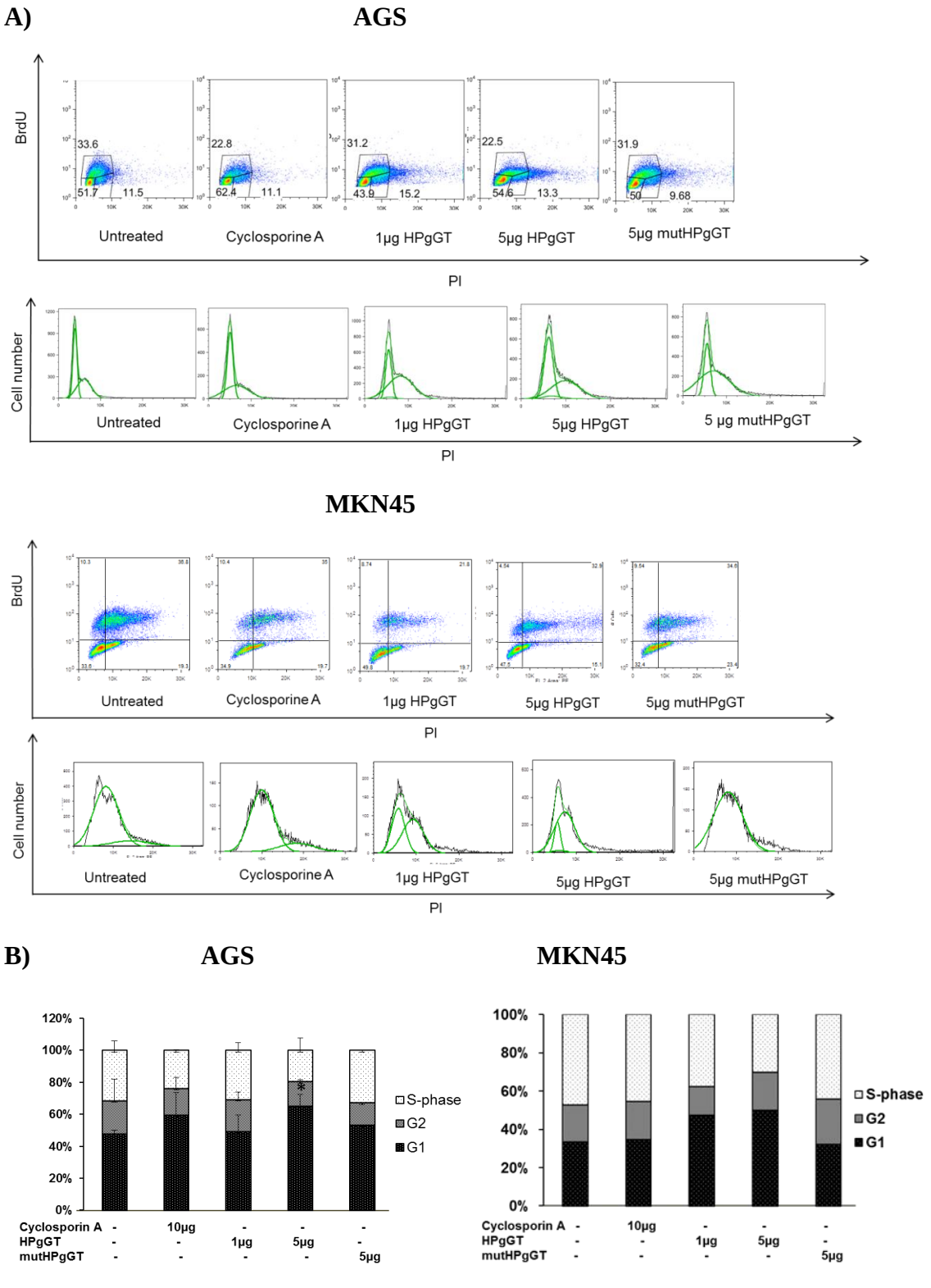


Figure 21: Cell cycle analysis of AGS and MKN45 cells after 24 hour of HPgGT treatment.

A) Representative BrdU-PI cell cycle plot B) Analysis of cell populations in different phases of the cell cycle upon HPgGT (1-5µg) treatment of AGS (n=3) and MKN45 (n=2) cells. mutant HPgGT (mutHPgGT) at 5µg and Cyclosporin A (10µg) were used as controls. All amounts were added per ml of cell culture. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

4. HPgGT alters host cell transcription

The molecular mechanisms involved in HPgGT mediated reduction in cell growth needed to be explored in order to define its role in bacterial pathogenesis. Hence, identification and functional characterization of cellular signalling pathways by which HPgGT affected cellular proliferation as well as other cellular responses related to the pathogenicity of the bacterium was an important aspect of the project.

H. pylori infection has been associated with several transcriptional changes in the host cell. A few of the key transcription factors involved in inducing transforming changes in the host cells include NF-κB (Lu, Wu et al. 2005; Choi, Park et al. 2007; Hisatsune, Nakayama et al. 2008; Backert and Naumann 2010); AP-1 (Ding, Olekhnovich et al. 2008; Allison, Kufer et al. 2009; Backert and Naumann 2010; Cho, Lim et al. 2010); CREB (Juttner, Cramer et al. 2003; Chang, Wu et al. 2005; Kudo, Lu et al. 2007; Hisatsune, Nakayama et al. 2008) and NFAT (Yokoyama, Higashi et al. 2005). Changes in the regulation of these transcription factors can also lead to proliferative changes in the epithelium. Hence, it was studied whether these transcriptional regulators could be responsible for the the HPgGT induced effects.

4.1 HPgGT activates NFkB

Activation of NF-κB is one of the late response mechanisms activated in *H. pylori* infected mucosa involved in tissue atrophy (Kudo, Lu et al. 2007). NF-κB is also the main mediator of IL-8 secretion in response to *H. pylori* infection resulting in inflammation (Backert and Naumann 2010). Since this transcriptional regulator plays such a major role in inflammation and disease, it was important to see whether gGT could affect this pathway.

NF-κB transcriptional activity was measured after HPgGT exposure in a transient transfection system. AGS and MKN45 cells were transfected with a NF-κB reporter plasmid containing three binding sites for NF-κB and treated with the recombinant HPgGT. The resulting luciferase activity directly corresponded with the NF-κB transcriptional activity. A significantly increased NF-κB transcriptional activation in both cell lines tested could be observed after 24 hours of treatment with HPgGT. TNFα, a known inducer of NF-κB transcriptional activity, was used as a positive control (Figure 22). A further increase in transcriptional activity could be observed after 48 hours of HPgGT treatment. In addition, there was no change in the NF-κB transcriptional activity as compared to the untreated control

when the gGT was mutated, which shows that the increased transcriptional activity observed was a direct result of the HPgGT enzymatic activity.

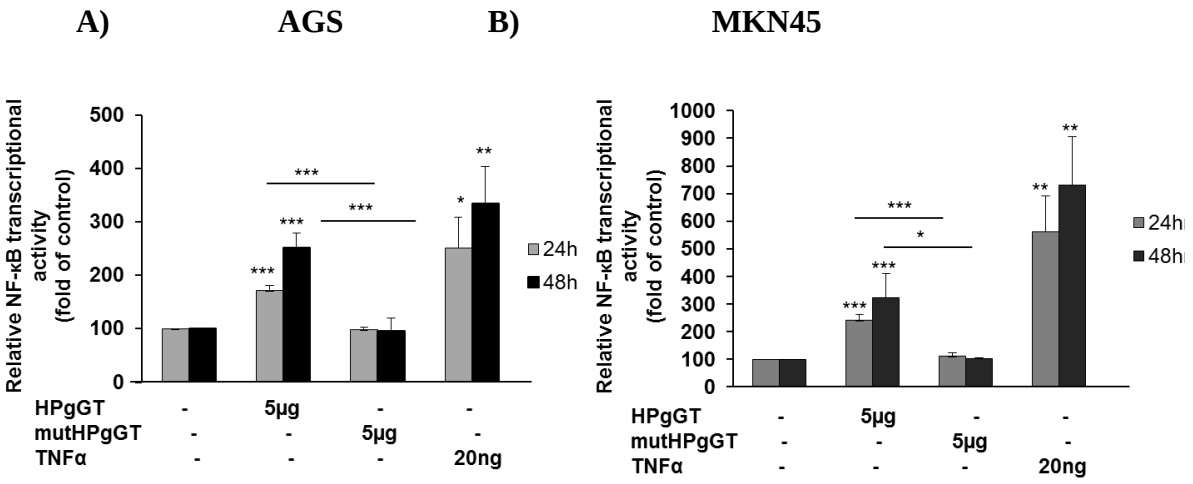


Figure 22: NF-κB transcriptional activity in gastric cancer epithelial cells.

NF-κB transcriptional activation in A) AGS and B) MKN45 cells transiently transfected with a NF-κB reporter plasmid and treated with HPgGT (amounts added/ml of cell culture) for 24 and 48 hours. TNFα (20ng/ml) was used to induce the transcriptional activity as a positive control. Results from three independent experiments are shown. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

To confirm activation of the signalling pathway, nuclear translocation of p65 subunit of NF-κB was analyzed after exposure to HPgGT (Figure 23).

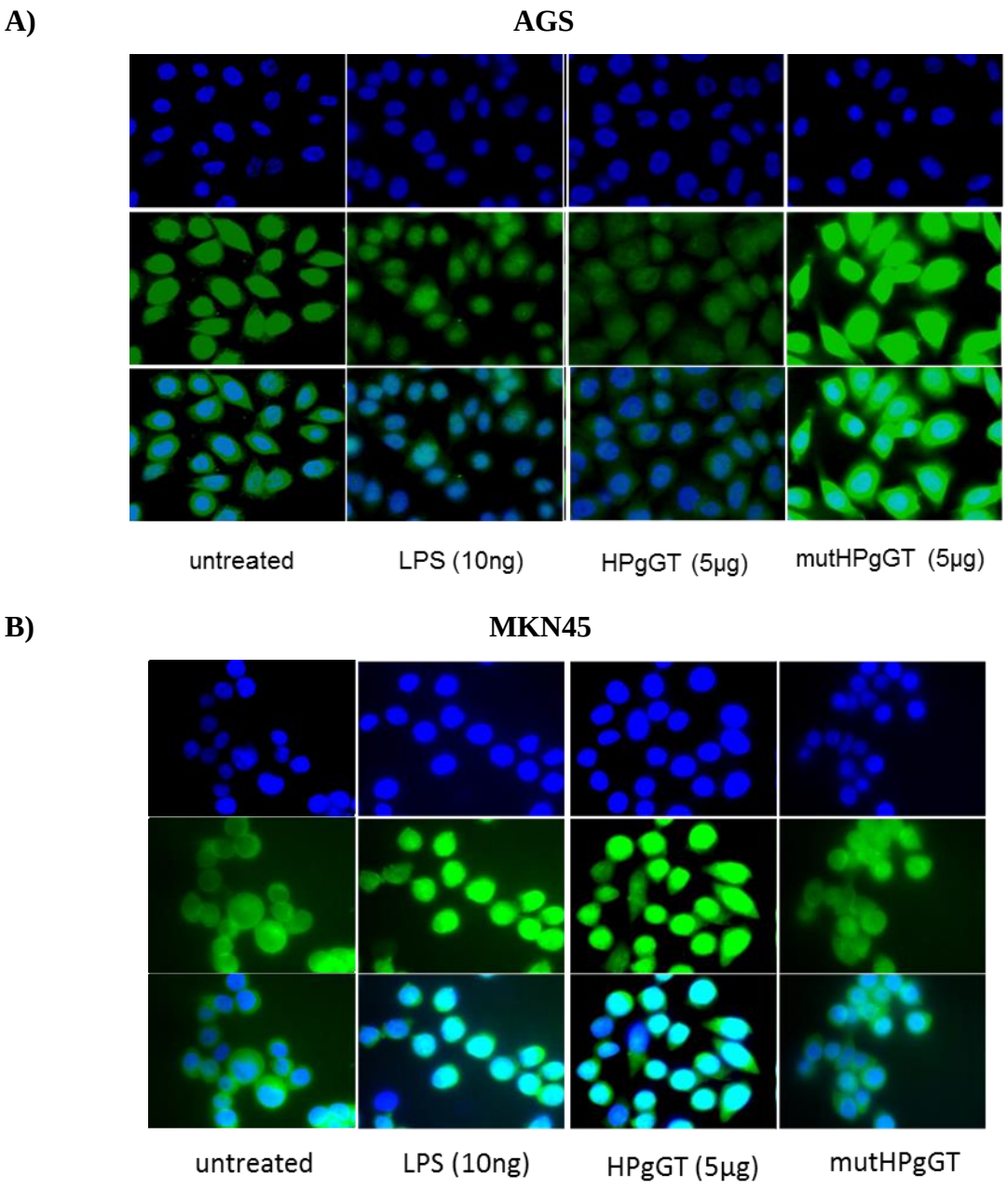


Figure 23: Nuclear translocation of fluorescent labeled p65 after 24 hours of HPgGT treatment

Nuclear translocation of fluorescently labeled p65 after 24 hours of HPgGT (amounts shown were added per ml of cell culture) treatment in A) AGS B) and MKN-45 cells. LPS (10ng/ml) was used as a positive control. p65 was labelled with Alexa 488 (green) and the cell nuclei were stained with DAPI (blue). Figures are representative of three independent experiments.

After 24 hours of HPgGT (5µg/ml) treatment, nuclear translocation of p65 was observed in both MKN45 and AGS cells indicating activation of the canonical NF-κB signalling pathway.

No changes were observed when using the mutated HPgGT at the same concentration. *E. coli* LPS (10ng/ml), a known NF-κB inducer was used as a control
to induce the activation of the pathway.

Taken together these results indicate that HPgGT is able to activate NF-κB signalling in gastric epithelial cells.

4.2 HPgGT activates AP-1

Another signalling cascade activated in *H. pylori* infections is AP-1 which has been shown to be activated during infection and co-related with enhanced ulceration and inflammation in Mongolian gerbil gastric mucosa (Kudo, Lu et al. 2007). To see whether this activation could be dependent on gGT, transcriptional activity of AP-1 was tested after HPgGT treatment of AGS and MKN45 cells. Transiently transfected cells with an AP-1 reporter plasmid with three binding sites for AP-1 were treated with HPgGT for 24 and 48 hours. Significantly increased transcriptional activity could be observed after 24 hours of HPgGT (5µg/ ml) exposure in both cell lines (Figure 24). A further increase in the transcriptional activity could be shown in AGS cells after 48 hours of HPgGT treatment, but in MKN45 cells a decline in the transcriptional activation after 48 hours was observed. IFNγ (50ng/ml), a known AP-1 inducer, was used as a positive control. The mutant HPgGT at the same concentration was not able to induce AP-1 activity.

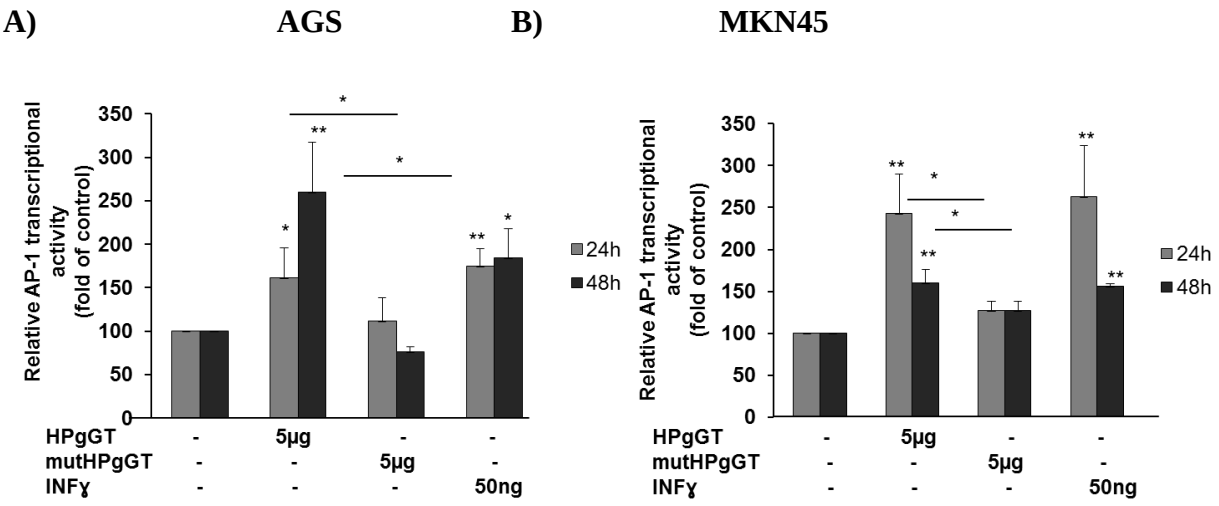


Figure 24: AP-1 transcriptional activity in HPgGT treated gastric cancer epithelial cells.

AP-1 activation in A) AGS and B) MKN45 cells transiently transfected with an AP-1 reporter plasmid and treated with HPgGT (amounts indicated were added per ml of cell culture) for 24 and 48 hours. IFNɣ (50ng/ml) was used to induce the transcriptional activity as a positive control. The bars represent mean from three independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

AP-1 is formed either as a homodimer of c-Jun or as a heterodimer of Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) proteins (Hess, Angel et al. 2004). Regulation of AP-1 activity is due to changes in transcription and mRNA stability of the individual AP-1 subunits as well as the specific interactions between AP-1 and other transcription factors or co-factors. Hence, changes in c-jun levels are central to the activation of the AP-1 pathway. c-jun expression could be confirmed by immunofluorescence. As shown in Figure 25, an enhanced nuclear signal from c-jun in HPgGT treated cells indicating increased levels of this subunit was observed after 24 hours of HPgGT treatment. Activation of the AP-1 pathway however, was not induced by the mutant HPgGT since no increases in the c-jun protein levels were detected.

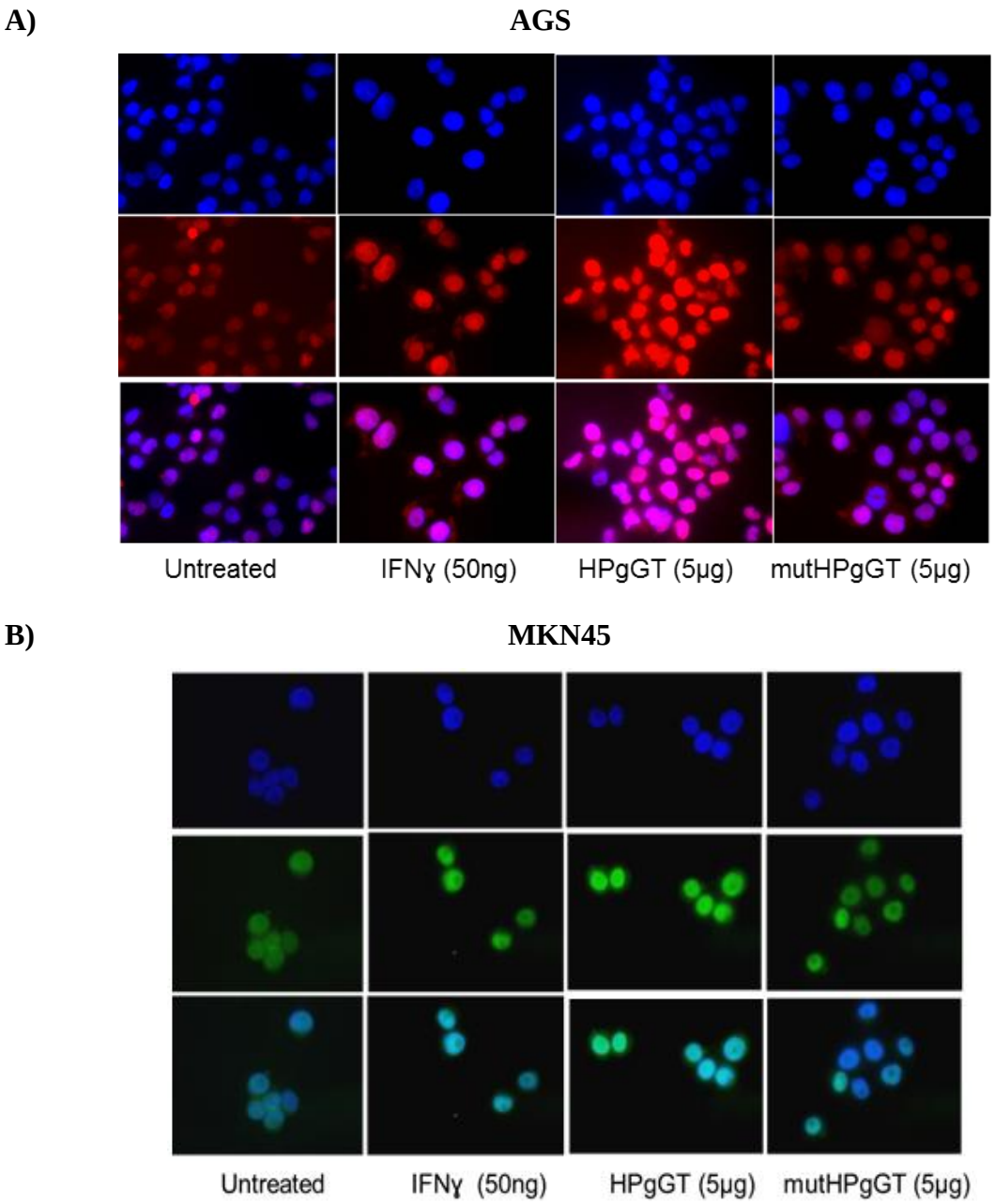


Figure 25: Increased levels of fluorescent labelled c-jun in HPgGT treated gastric cancer cells.

Increased nuclear signal of fluorescently labelled c-jun after 24 hours of HPgGT (amounts added per ml of cell culture) treatment in A) AGS cells (c-jun was labelled with Alexa 594 (Red) and nuclei stained with DAPI (blue)) and B) MKN-45 cells (c-jun was labelled with Alexa 488 (green) and nuclei stained with DAPI (blue)). IFNɣ (20ng/ml) was used as a positive control. n=2

4.3 HPgGT activates CREB

Since in addition to AP-1, CREB upregulation is also observed upon *H. pylori* infection and this is associated with increased ulceration and inflammation in the gastric mucosa (Kudo, Lu et al. 2007), it was interesting to see if this activation could be attributed to gGT.

MKN45 and AGS cells were tested for luciferase activity after a transient transfection with the CREB reporter plasmid with one CREB binding site. A significantly increased transcriptional activity upon exposure of cells to HPgGT at a concentration of 5µg/ml was observable after 24 hours and 48 hours in MKN45 cells, while the enzymatically inactive mutant was unable to activate CREB transcriptional activity, indicating that HPgGT is indeed involved in triggering the CREB signalling cascade. This significantly increased transcriptional activation was also seen in AGS cells after 24 hours of HPgGT treatment, after which there was a decrease. Forskolin, an adenylate cyclase activator able to induce the transcriptional activation of CREB was used as a positive reference control.

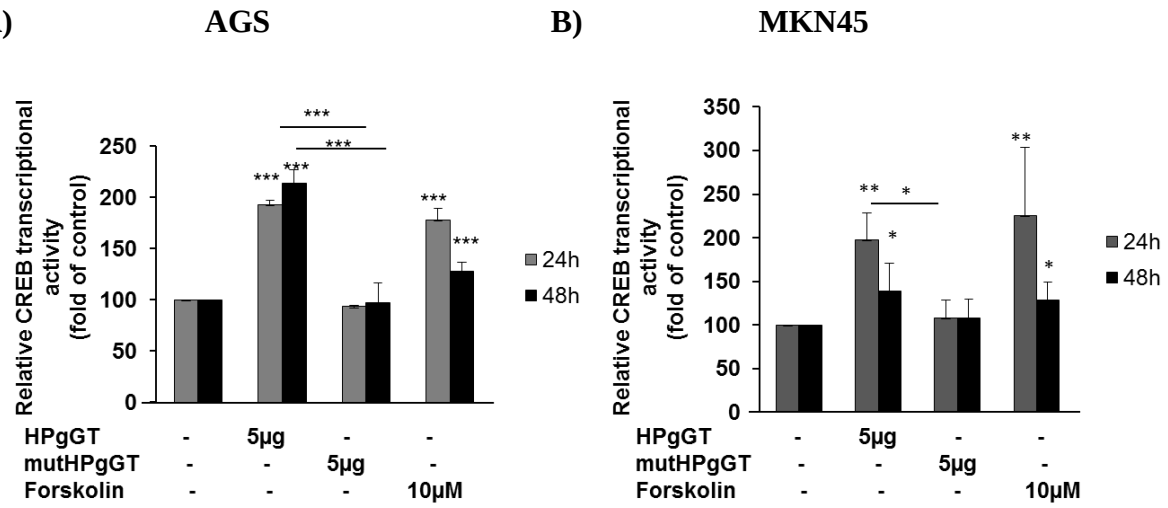


Figure 26: CREB transcriptional activity in HPgGT treated gastric epithelila cells.

CREB activation in A) AGS B) MKN45 cells transiently transfected and treated with HPgGT (amounts added per ml of cell culture) for 24 and 48 hours. Forskolin (10µM) was used to induce the transcriptional activity as a reference control. The bars represent mean from three independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

4.4 HPgGT activates NFAT

Yokoyama and colleagues showed that a functional antagonism exists between CagA and VacA in the activation of the NFAT signaling pathway by *H. pylori* (Yokoyama, Higashi et al. 2005). They further showed that NFAT activation led to regulation of certain genes involved in cell cycle regulation. To elucidate whether HPgGT could activate this signaling cascade, AGS and MKN-45 cells were transfected with a NFAT reporter plasmid containing three binding sites for NFAT. The cells were then treated with HPgGT (5µg/ml) for 24 and 48 hours. The enzymatically inactive HPgGT mutant was used as a control. Luciferase activity was measured to determine NFAT transcriptional activity.

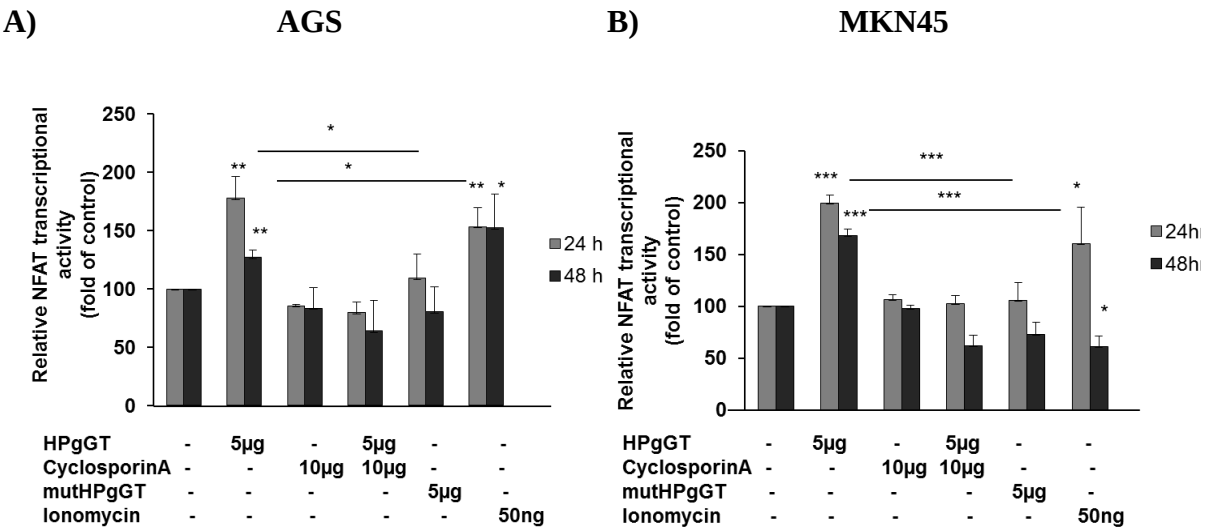


Figure 27: NFAT transcriptional activity in HPgGT treated cells.

NFAT activation in A) AGS B) MKN45 cells transiently transfected with an NFAT reporter plasmid and treated with HPgGT (amounts added per ml of cell culture) for 24 and 48 hours. Ionomycin (25ng/ml) was used to induce the transcriptional activity as a positive control. Cyclosporin A (10µg/ml), an inhibitor of calcineurin was able to block this activation. The bars represent mean from three independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

A significant increase in NFAT transcriptional activity could be detected after 24 hours of HPgGT treatment in both AGS and MKN45 cells after which there was a slight decrease (Figure 27). The mutated protein at the same concentration was unable to induce this activation. Ionomycin, a calcium ionophore, was used to activate the NFAT transcriptional activity and cyclosporine A, a calcineurin blocker, was able to inhibit HPgGT induced NFAT

transcriptional activity. However, this activation was too late to be responsible for arresting cell growth as decreased cell growth could be observed around the same time frame as the NFAT transcriptional activation.

The NFAT transcription factor family comprises of five members NFATc1, NFATc2, NFATc3 and NFATc4 (Crabtree and Olson 2002). Of these members, NFATc3 is expressed in epithelial cells and was shown to be activated upon *H. pylori* infection in gastric epithelial cells (Yokoyama, Higashi et al. 2005). Activation of NFATc3 transcriptional activity is preceded by its nuclear translocation. So activation of NFAT was further confirmed by visualization of the nuclear translocation of NFATc3. Nuclear translocation of NFAT was indeed observed after HPgGT exposure of MKN45 cells for 24 hours (Figure 28). The mutant HPgGT was, on the other hand, unable to induce this activation.

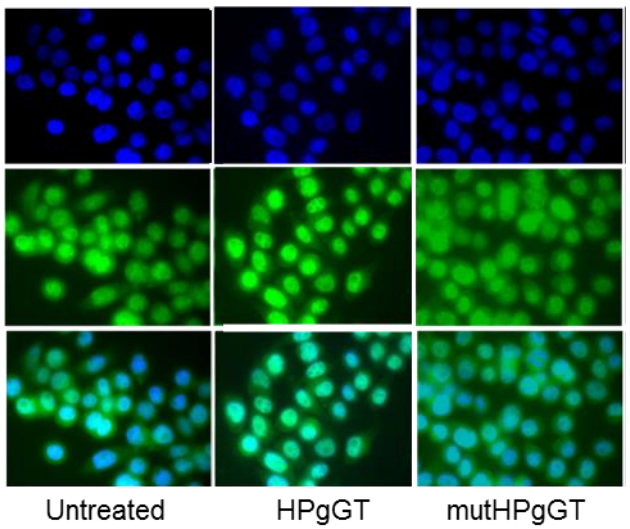


Figure 28: Nuclear translocation of fluorescently labelled NFATc3 after 24 hours of HPgGT treatment in MKN45 cells.

NFATc3 was labelled with Alexa 48 (green) and the cell nuclei stained with DAPI (blue). 5µg/ml of HPgGT or the mutant HPgGT was used. One representative of two independent experiments is shown.

5. Host transcriptional activation after *H. pylori* infection

An induction of the transcriptional activity of NF-κB, AP-1, CREB and NFAT was observed after incubating gastric cancer cells with the recombinant HPgGT. To further characterize the contribution HPgGT to the activation of these signalling cascades in the context of bacterial infection, cells were co-cultured with the *H. pylori* wildtype strain G27 and an isogenic gGT

deletion mutant. The gGT deletion mutant was contstructed by disrupting the gGT sequence of G27 wiltype bacterium by a kanamycin resistance cassette, this increased the amplified DNA length by approximately 1.5 kb (length of the resistance kanamycin cassette sequence) (Schmees, Prinz et al. 2007). To confirm the disruption of the gGT sequence in the bacterial cultures used for further experimentation, a gGT screening PCR was performed (Figure 29A). Furthermore, loss of gGT activity was also confirmed in *H. pylori* supernatants in a gGT activity assay (Figure 29B).

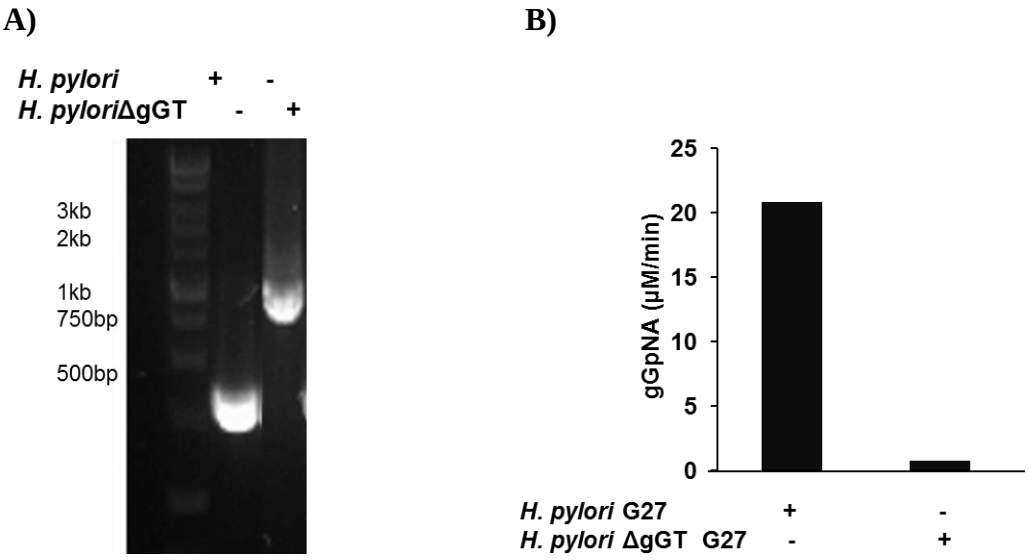


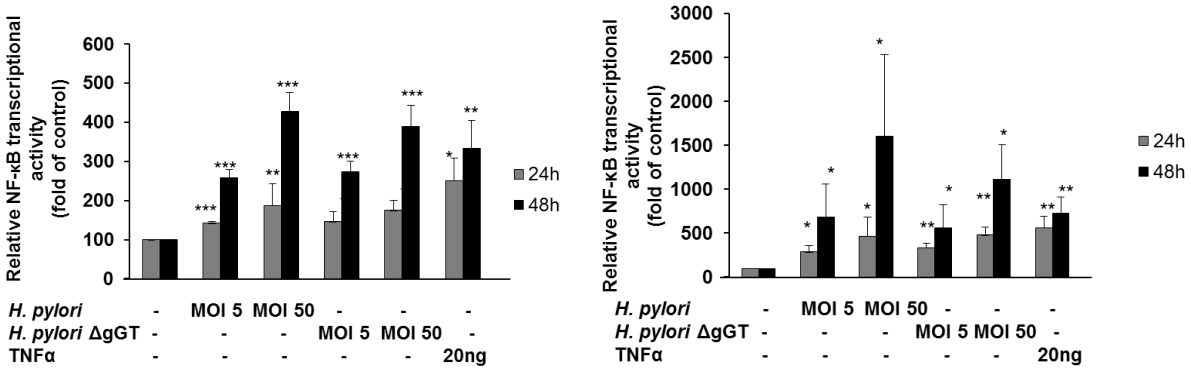
Figure 29: gGT screening PCR and activity assayfor *H. pylori* G27 strain

A) Confirmation of presence of the kanamycin resistance cassette disrupting the gGT sequence in the gGT deletion mutant. A specific screening PCR for the gGT sequence of the G27 wt and gGT knock out bacterial genomic DNA was performed. B) gGT activity test for *H. pylori* G27 and *H. pylori*ΔgGT supernatants. Kinetics for the hydrolysis of L-cglutamyl-p-nitroanilide (gGpNA) was determined at pH 8.0. Mean of duplicates from a single experiment.

MKN45 and AGS cells were then transiently transfected with NF-κB, AP-1, CREB and NFAT reporter plasmids and later infected with *H. pylori* gGT proficient and gGT deleted mutant G27 strain. The infection was carried out with two different multiplicities of infection (MOI) 5 and 50 and NF-κB (Figure 30 A), AP-1 (Figure 30 B), CREB (Figure 30 C) and NFAT (Figure 30 D)) transcriptional activity was measured after 24h and 48 h of *H. pylori* infection.

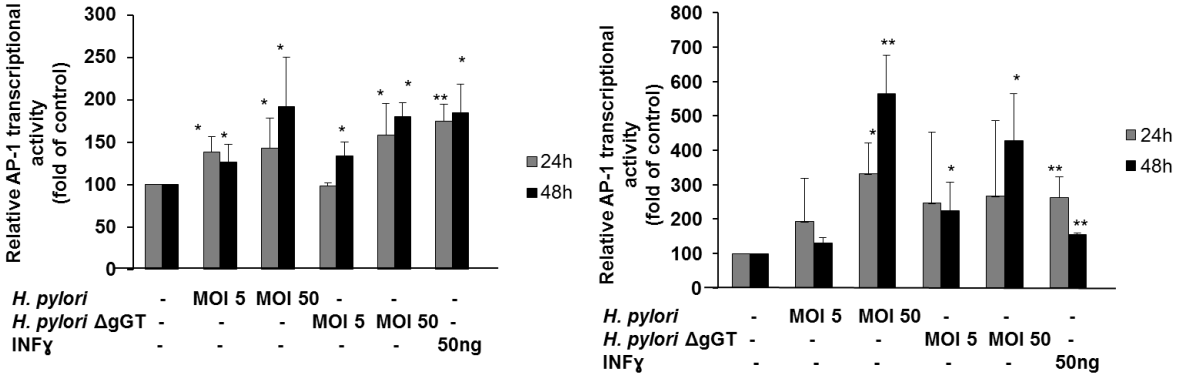
A) NF-κB

AGS **MKN45**



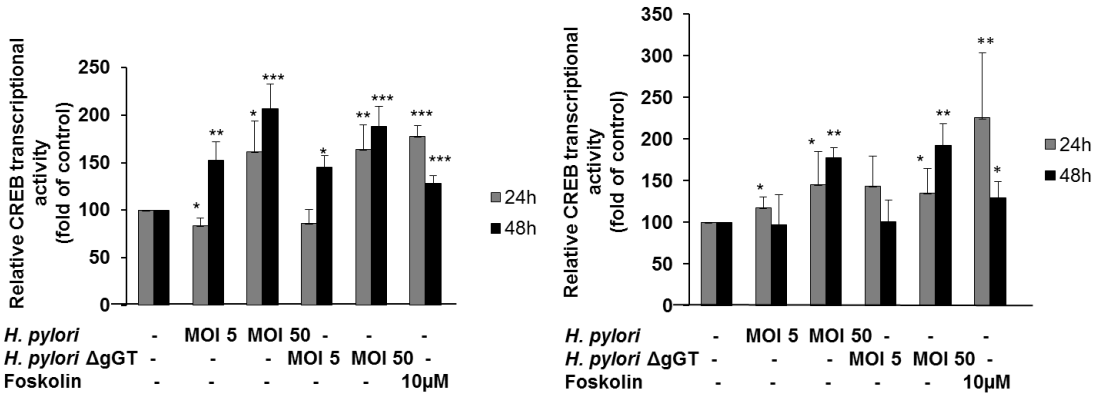
B) AP-1

AGS **MKN45**



C) CREB

AGS **MKN45**



D) NFAT

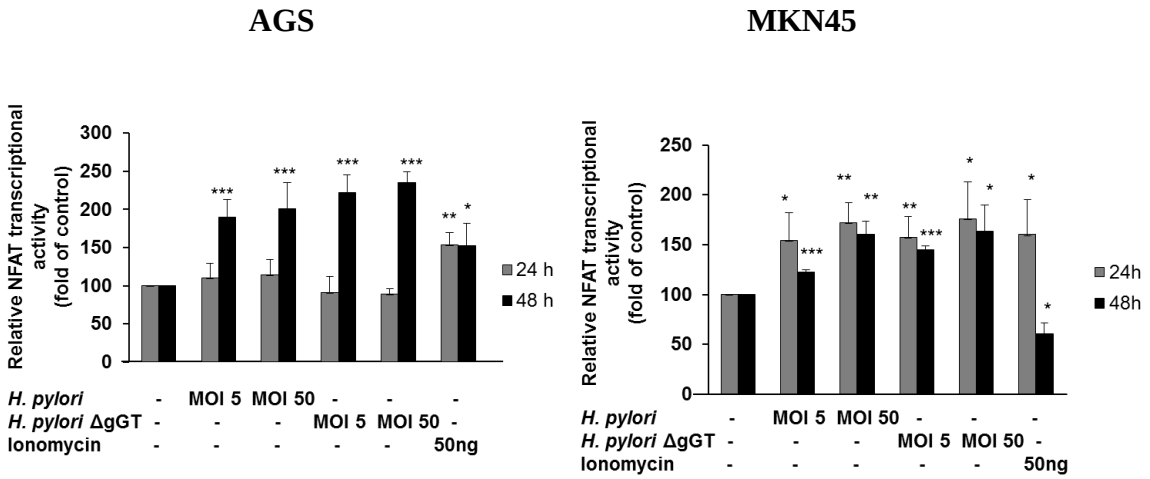


Figure 30: NF-κB, AP-1, CREB and NFAT activation in gastric epithelial cells co-cultured with *H. pylori* wt and gGT knock out strain.

NF.κB (A), AP-1 (B), CREB (C) and NFAT (D) activation in AGS and MKN45 cells transiently transfected with respective reporter plasmids and co-cultured with *H. pylori* wild type and gGT knockout G27 strain at MOI 5 and 50 for 24 and 48 hours. The bars represent mean from three independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Increased NF-κB transcriptional activity of cells treated with both the G27 wt and the gGT knock out bacterium was observed in both cell lines tested at 24 hours which was further increased after 48 hours of infection. No significant difference between the wildtype and the gGT deficient bacterium was detected.

Significant AP-1 transcriptional activation was also observed in both MKN45 and AGS cells after 48 hours of infection but again no significant differences in the transcriptional activity induced by the G27 wildtype and the gGT knock out bacterial infections were observed. NFAT and CREB transcriptional activity also followed a similar pattern in that no significant changes in the activation of the pathways by the gGT deficient and proficient bacteria could be shown. Although the results reconfirm the findings by others that *H. pylori* was able to induce the transcriptional activity of NF-κB, AP-1, CREB and NFAT, this activation however was not merely dependent on gGT, since the gGT deficient bacterium was also able to induce the transcriptional activity to a similar degree. This observation could be explained by the presence of other virulence factors such as CagA and VacA. These virulence factors are also able to induce the activation of these transcription factors in gastric epithelial cells as has been

previously described (Yokoyama, Higashi et al. 2005; Backert and Naumann 2010), and may be more potent activators of the signalling pathway than the activation induced by HPgGT.

6. Conserved *H. pylori* and *H. bilis* gGT function

HPgGT is a relatively better known example of bacterial gGT, although its effects are still not well described. As previously described, gGT is present in all GHS as well as in many EHS. To determine whether a similar effect and a possible conserved function is presented by *Helicobacter* gGTs, the effect of *H. bilis* gGT (HBgGT) on cell growth was tested. *H. bilis* as a pathogenic enterohepatic *Helicobacter* species with a wide niche makes HBgGT an ideal example to study EHS gGT associated host cell modifications that might be important in the bacterial pathogenesis. Prior to experimentation the activity and purity of the recombinant proteins were tested in a gGT activity assay and SDS-PAGE (Figure 31A, B). Furthermore, the loss of gGT activity in a *H. bilis* gGT deletion mutant was also confirmed by measurement of gGT activity in bacterial culture supernatants (Figure 31B). The gGT proficient and deficient *H. bilis* bacteria were to be employed in subsequent experiments.

The gastric cancer cell line AGS cells were used to test the conservation of function of both gGTs as this cell line is most commonly used in different HPgGT studies and therefore the effects of HPgGT are well documented in this cell line. The cells were tested for viability, proliferation and apoptosis with both HPgGT and HBgGT treatment.

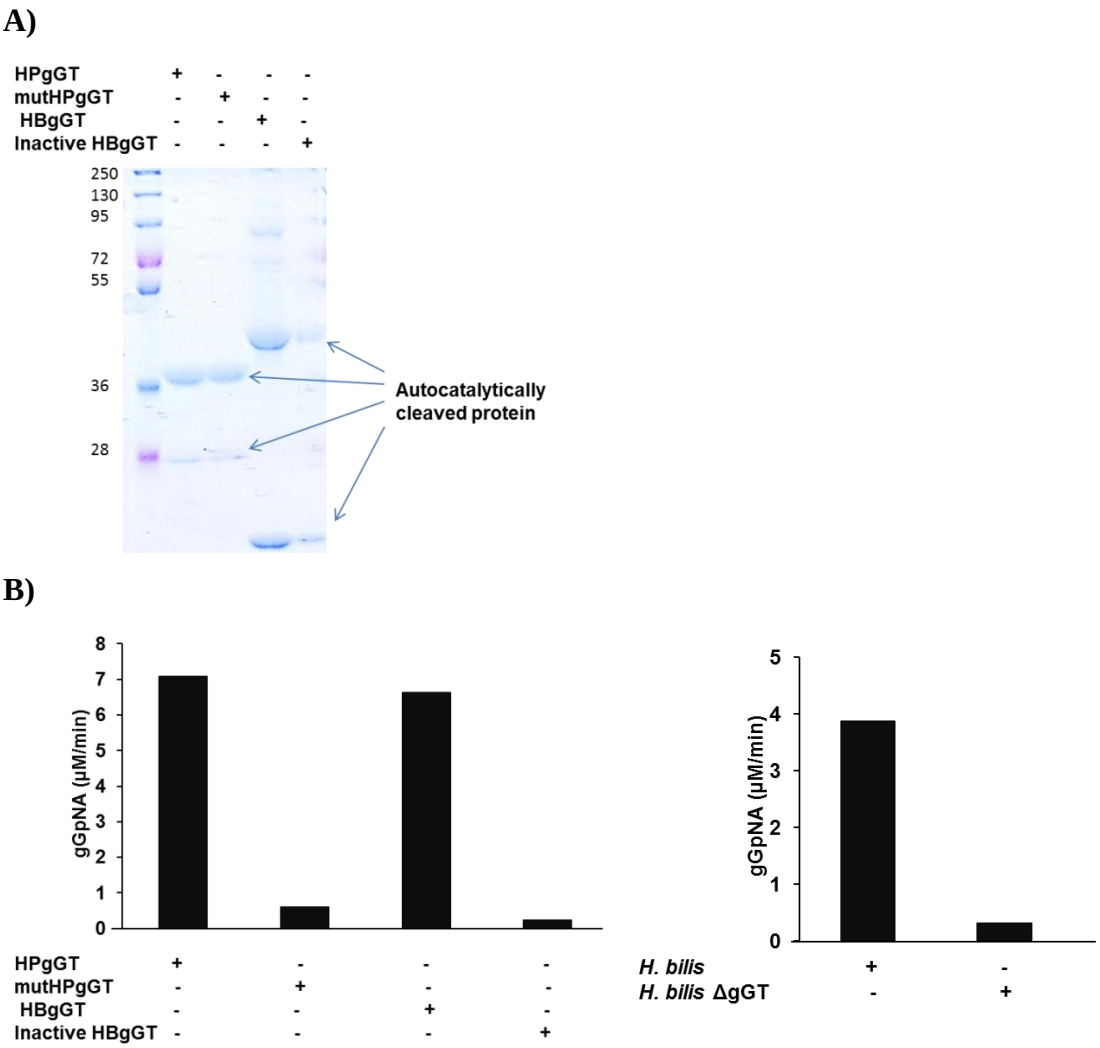


Figure 31: gGT recombinant protein quality control via SDS- PAGE and gGT activity test.

A) SDS-PAGE of purified protein fractions of HPgGT and HBgGT B) gGT activity test for HBgGT and HPgGT recombinant proteins (left) and *H. bilis* and *H. bilis*ΔgGT supernatants. Kinetics for the hydrolysis of L-glutamyl-p-nitroanilide (gGpNA) was determined at pH 8.0. Mean of duplicates from a single experiment.

6.1 HBgGT and HPgGT reduce host cell growth

Firstly, comparison of HBgGT and HPgGT effects on host cell viability were determined in AGS cells. Effect of the recombinant gGTs from the two *Helicobacter* spp., *H. pylori* and *H. bilis*, were titrated and tested on AGS cells and cell viability determined by measuring the ATP content of the cell lysates which directly correlates to the number of cells (Figure 32). Both the recombinant proteins were added at doses ranging from 0.5µg/ml to 5µg/ml to the

cells for 48 hours. The recombinant protein dose range used corresponded to the protein doses deemed to be effective for HPgGT in previous experiments (Figure 19).

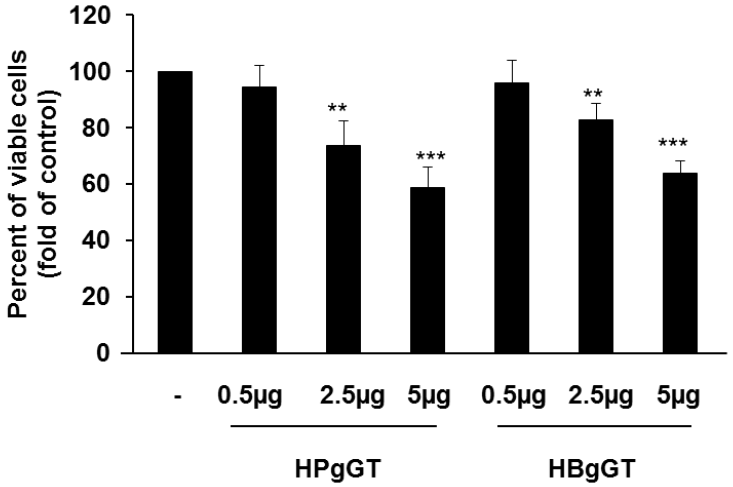


Figure 32: Cell viability after HPgGT and HBgGT treatment of AGS cells.

Exposure of AGS cells to both HPgGT and HBgGT (0.5-5µg/ml) for 48 hours led to similar reduction in viable cell numbers for both the recombinant proteins. Bars represent mean of three independent experiments as normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

HPgGT and HBgGT inhibited the viability of the cells upto 41.4%and 36.45% respectively, after 48 hours of treatment.

Since *H. bilis* is an enterohepatic bacterium, gastric lumen is not the most physiological setting for this bacterium. *H. bilis* colonizes the colon of different hosts including humans and mice. Colon cancer epithelial cell lines HCT116, DLD-1 and Caco-2 were tested in the same experimental setting to further validate this finding. Cell lines were treated with different concentrations (0.5-5µg/ml) of HPgGT and HBgGT for 48 hours and cell viability determined (Figure 33).

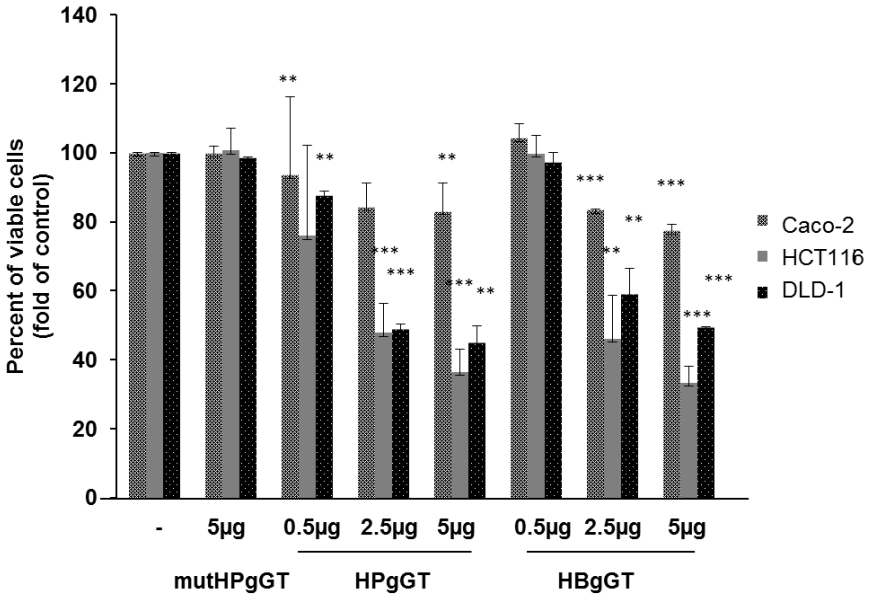


Figure 33: Cell viability after treatment of colon cancer cell lines with the recombinant HPgGT and HBgGT.
Treatment of colon cancer cell lines with the recombinant HBgGT protein (amounts added per ml of cell culture medium) resulted in reduced viability. HPgGT was taken along to compare the effects of both bacterial gGTs. Bars represent mean of three independent experiments as normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

A dose dependent decrease in cell viability could be observed for all the cell lines tested for both HPgGT and HBgGT. In Caco-2 cells, the HBgGT in comparison to HPgGT was able to induce a significant reduction in cell viability of significant reduction in cell viability of up to 20% with the 2.5µg/ml concentration of HBgGT, an even greater decrease in viability (25%) was observed when HBgGT at a concentration of 5µg/ml was used. In HCT116 cells HBgGT reduced cell viability of upto 56% at a concentration of 2.5µg/ml in comparison to 53.5% of HPgGT at the same concentration. Increased loss of cell viability was observed at higher gGT concentrations. A 78% reduction in cell viability was observed when the HCT116 cells were exposed to 5µg/ml of HBgGT as compared to 75% reduction with HPgGT. DLD-1 cells also showed significantly reduced cell viability after HBgGT treatment in doses of 2.5µg/ml (41%) and 5µg (50%).

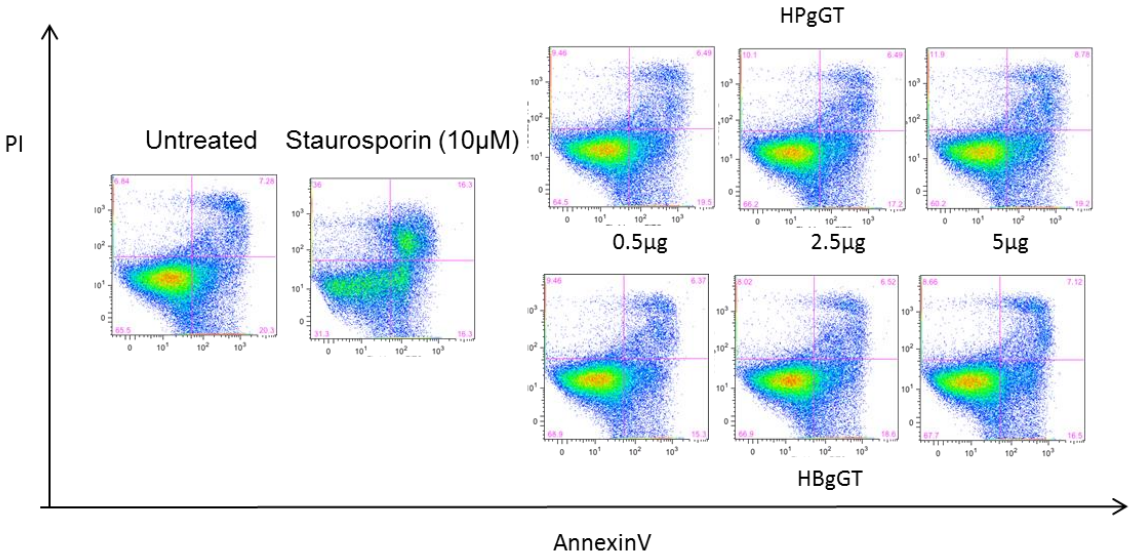
6.2 HBgGT does not induce host cell apoptosis

To rule out any apoptosis dependent cell viability effects, two approaches for an apoptosis analysis were taken. First, apoptotic cell populations were anylysed using a FACS based assay using annexinV as an early apoptosis marker and PI to stain the DNA of cells undergoing apoptosis; secondly, caspase 3/7 induction, as an early apoptotic mechanism triggered by several death signalling cascades as a prelude to apoptosis, was studied. AGS cells were again used as the model cell line and HPgGT was used as a comparison as no apoptosis in this cell line was observed in previous experiments when treated with HPgGT.

6.2.1 Annexin V PI assay

Annexin V and PI staining was used to FACS sort differentially treated cells into non-apoptotic, early apoptotic and cell populations undergoing apoptosis. AGS cells were treated for 24 hours with the recombinant HPgGT and HBgGT proteins at concentrations of 0.5, 2.5 and 5µg/ml. Staurosporine, at 10µM concentration was used as a positive control to induce apoptosis.

A)



B)

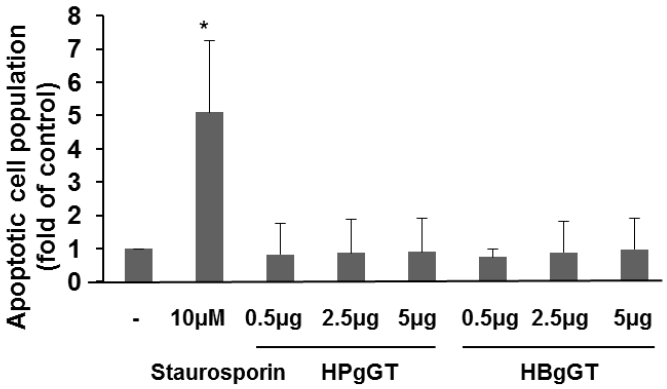


Figure 34: FACS analysis of annexin V-PI stained AGS cells after treatment with the recombinant HPgGT and HBgGT.

A) FACS analysis of annexin V-PI stained AGS cells after 24 hours of treatment with the recombinant HPgGT and HBgGT (amounts added per ml of cell culture medium). One representative experiment is shown. B) Comparison of annexinV and PI positive cell populations. Data are shown as mean of three independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Percentages of the cell populations undergoing apoptosis (annexin V+PI positive) and the ones in early stages of the apoptosis (annexin V positive) were plotted in a bar graph (Figure 34 B). No significant changes in the apoptotic cell population after 24 hours of gGT treatment could be observed for both the recombinant *Helicobacter* gGT sources compared, indicating

that a similar apoptosis independent function of reduction in cell viability exists for both gGTs.

6.2.2 Capase 3/7 induction

Kim and colleagues described a mitochondrial pathway dependent apoptosis as the main mechanism by which HPgGT is able to reduce cell viability (Kim, Lee et al. 2007). Mitochondrial pathway mediated cell death involves the activation of the caspases. The sequential activation of the caspases is central to execution of apoptosis in the cell. Several extrinsic as well as mitochondrial cell death pathways culminate in activation of caspase 3 which may also trigger other caspases (Harrington, Ho et al. 2008).

To confirm that HPgGT and HBgGT were indeed not activating such cell death pathways caspase 3/7 activity was scrutinized.

Colon cancer cell lines, HCT116 and DLD-1 were tested for specific effects of HBgGT and AGS cells for HPgGT specific effects. All cell lines used were treated with HBgGT and HPgGT at the same concentrations used for the viability assays and the apoptosis analysis via FACS i.e, 0.5-5µg/ml. Staurosporin at two different concentrations 5 and 10µM was used to induce apoptosis and therefore used as a positive control.

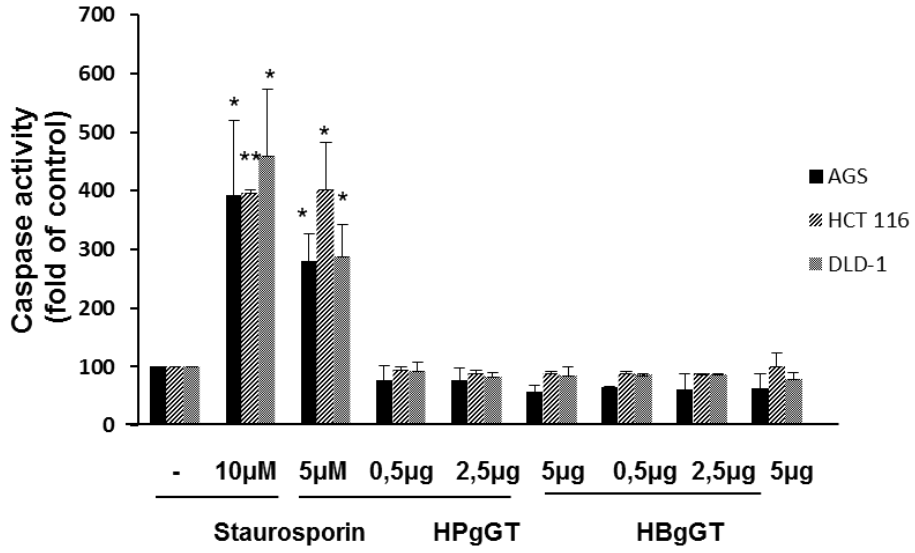


Figure 35: Caspase 3/7 assay of colon cancer cell lines and a gastric cancer cell line (AGS) after 24 hour treatment with HPgGT and HBgGT.

Caspase 3/7 assay was performed at 24 hours after treatment of AGS, HCT116 and DLD-1 cells with 0.5, 2.5 and 5µg/ml of HPgGT and HBgGT. Staurosporin at 5 and 10µM was used as a positive control. Bars represent mean of 2 independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Staurosporine treated cells showed a significant induction of caspase3/7 activity after treatment. However, in all cell lines tested, no caspase 3/7 induction after 24 hours of HPgGT and HBgGT treatment was observed. A similar apoptosis independent suppression of proliferation in gastric cancer cell line AGS and colon cancer cell lines HCT116 and DLD-1 by both *H. bilis* and *H. pylori* gGTs was observed (Figure 35).

Hence, HBgGT presented an interesting example of EHS gGT with a function that was similar to HPgGT. However it was important to analyse whether this conserved function carried out the same host cell transcriptional alterations observed for HPgGT. To test this, colon cancer cell lines HCT116 and DLD-1 were used for further experiments as *H. bilis* mainly colonizes the colon. The cells were treated with HBgGT and tested for activation of several transcription factors.

7. Host cell transcriptional dysregulation induced by HBgGT

HPgGT was able to induce the transcriptional activation of NF-κB, AP-1, CREB and NFAT. Since both the recombinant gGT proteins from *H. pylori* and *H. bilis* behaved similarly in the viability assays, it was interesting to determine whether HBgGT was also able to alter the host transcriptional activity of certain stress induced transcription factors like NF-κB, AP-1 and CREB. The concentration of HBgGT that induced the highest reduction in cell growth without inducing apoptosis (5µg/ml) was chosen for further eperiments. Since no mutant recombinant protein was available for HBgGT, a heat inactivated HBgGT at the same concentration was used as an enzymatically inactive control. Activity for both the active and the heat inactivated control was tested to confirm that no residual activity remained (Figure 31B).

7.1 HBgGT activates NF-κB

A significantly increased activation of NF-κB transcriptional activity could be observed when transiently transfected HCT116 and DLD-1 cells were exposed to HBgGT at a concentration of 5µg/ml for 24 hours. TNFα (20ng/ml) was used as a positive control to induce NF-κB transcriptional activity. No significant changes in the transcriptional activity were observed at earlier time points. This activation was abolished when HBgGT was heat inactivated, showing that the effect was dependent on the intact enzymatic activity of the protein (Figure 36).

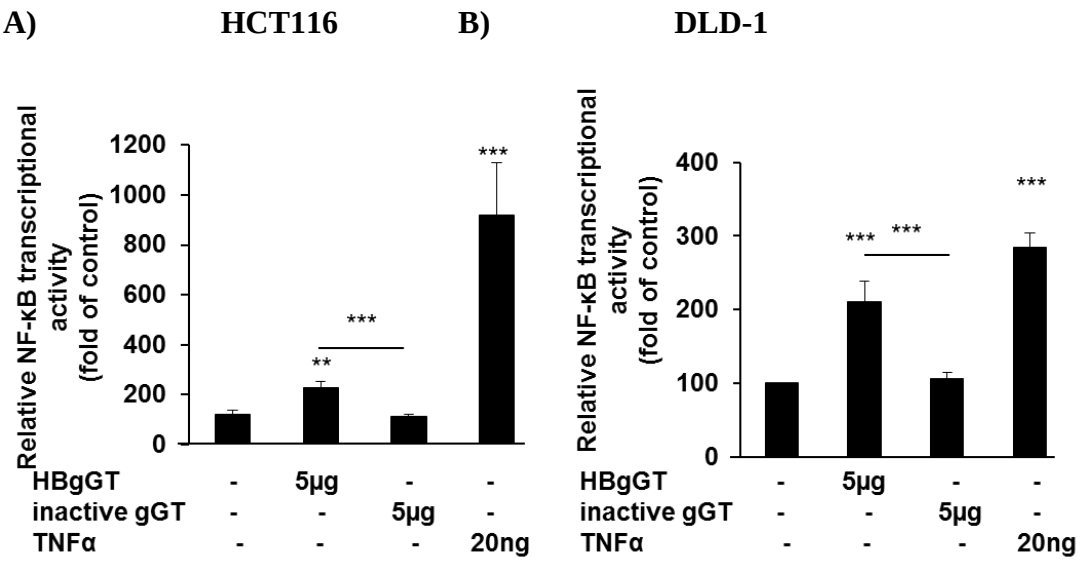


Figure 36: NF-κB transcriptional activity in HBgGT treated colon epithelial cells.

NF-κB activation in A) HCT116 B) DLD-1 cells transiently transfected and treated with HBgGT (amounts added per ml of cell culture medium) for 24 hours. TNFα (20ng/ml) was used to induce the transcriptional activity as a positive control. Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

To confirm whether this increased transcriptional activity corresponded to an activation of the pathway, immunoblot of whole cell protein lysates from similarly treated cells was performed. Blots were probed with a phospho-IκBα antibody. As mentioned before, IκBα forms an inhibitory complex with the RelA/B protein subunits of NF-κB retaining these in the cytoplasm, thereby inhibiting their activation. Activating signals promote phosphorylation of IκBα which then releases RelA/B proteins allowing their nuclear translocation. Phosphorylation of IκBα was affected when the HCT116 and DLD-1cells were treated with HBgGT for 10 hours. TNFα was used as a positive control (20ng/ml). The inactive HBgGT was however not able to induce any phosphorylation verifying that the gGT enzymatic activity is crucial in activation of the pathway (Figure 37 A, B).

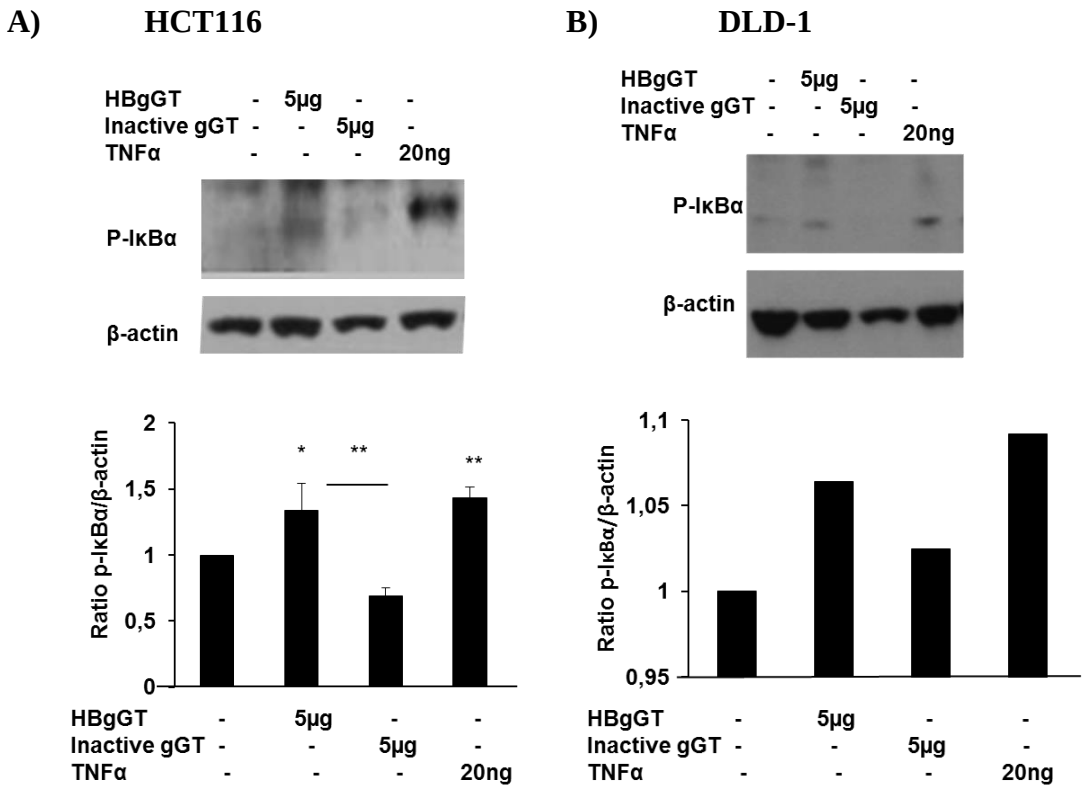


Figure 37: Immunoblot analysis of IκBα phosphorylation in HBgGT treated cells.

Phosphorylation of IκBα .Top, Immunoblot; bottom, quantification of p-IκBα and β-actin band density ratio normalized to the untreated control in A) HCT116 cells and B) DLD-1 cells. Cells were treated with 5µg/ml of active and heat inactivated HBgGT recombinant protein. TNFα (20ng/ml) was used as a positive control. Results expressed as mean three (HCT116) and of two (DLD-1) independent experiments.

Activation of the NF-κB signalling pathway could also be confirmed by nuclear translocation of p65. HCT116 cells were treated with 5µg/ml of HBgGT for 24 hours after which the cells were fixed and fluorescently labelled for p65. Physiologically p65 is located primarily in the cytoplasmic compartment of the cells. A mainly nuclear signal of p65 could be observed when the cells were treated with TNFα inducing activation of the pathway. The HBgGT active enzyme was able to induce a nuclear translocation of p65, while the inactive HBgGT on the other hand, was unable to induce any nuclear translocation confirming that indeed the pathway was triggered by HBgGT enzymatic activity (Figure 38).

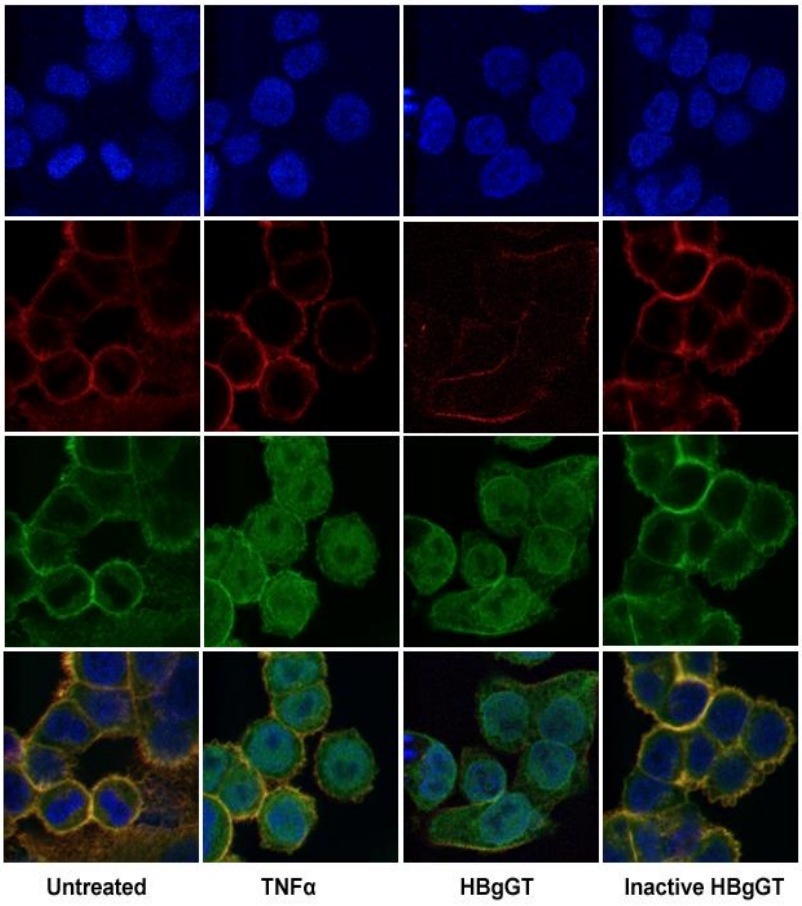


Figure 38: Nuclear translocation of p65 after HBgGT treatment of HCT116 cells.
Increased nuclear translocation of NF-κB subunit p65 after HBgGT treatment of HCT116 cells. Inactive HBgGT (amounts added per ml of cell culture medium) was used as a control. TNFα (20ng/ml) an activator of the pathway, was used as a positive control. p65 was labelled with Alexa 488 (green), the cell nuclei were stained with DAPI (blue) and phalloidin (red) was used to stain actin filaments. One representative of three independent experiments shown.

Taken together these results show that HBgGT is able to activate the NF-κB signalling cascade and that its enzymatic activity is essential for this activation.

7.2 HBgGT activates AP-1

AP-1 transcriptional activity was observed when transiently transfected HCT116 and DLD-1 cells were exposed to HBgGT. PMA (10mg/ml), a known activator of the pathway was used as a positive control. Starting 24 hours, an approximately 2 fold increase in AP-1

transcriptional activity was seen in HBgGT (5µg/ml) treated cells as compared to the untreated control. The increased transcriptional activity was dependent on intact enzymatic activity of the HBgGT as the heat inactived control enzyme was unable to induce this effect. No significant change in the transcription was observed earlier than 24 hours (Figure 39).

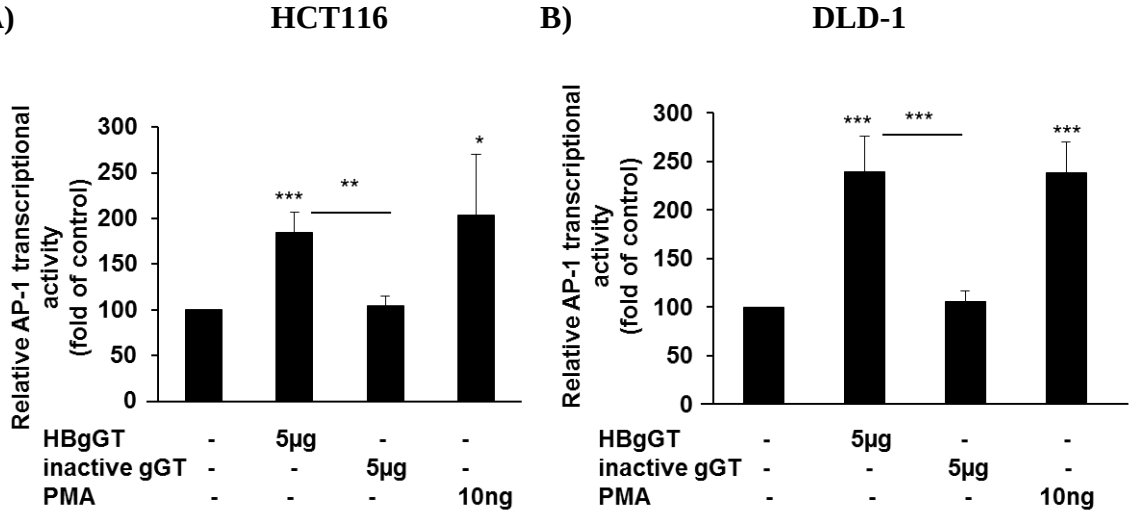


Figure 39: AP-1 transcriptional activity in HBgGT treated colon epithelial cells.

NF-κB activation in A) HCT116 and B) DLD-1 cells transiently transfected and treated with HBgGT (amounts added per ml of cell culture medium) for 24 hours. PMA (10ng/ml) was used to induce the transcriptional activity as a positive control. Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

To confirm activation of the AP-1 pathway, levels of c-jun, one of the proteins forming the AP-1 dimer, was determined, since increased c-jun levels commonly correspond with an activated AP-1 pathway. Treated cells were lysed and proteins separated via gel electrophoresis. The blots were probed for c-jun. Relative amounts of c-jun to the β-actin control were determined. Increased levels of total c-jun protein were observed in HBgGT treated cells after 6 hours (Figure 40).

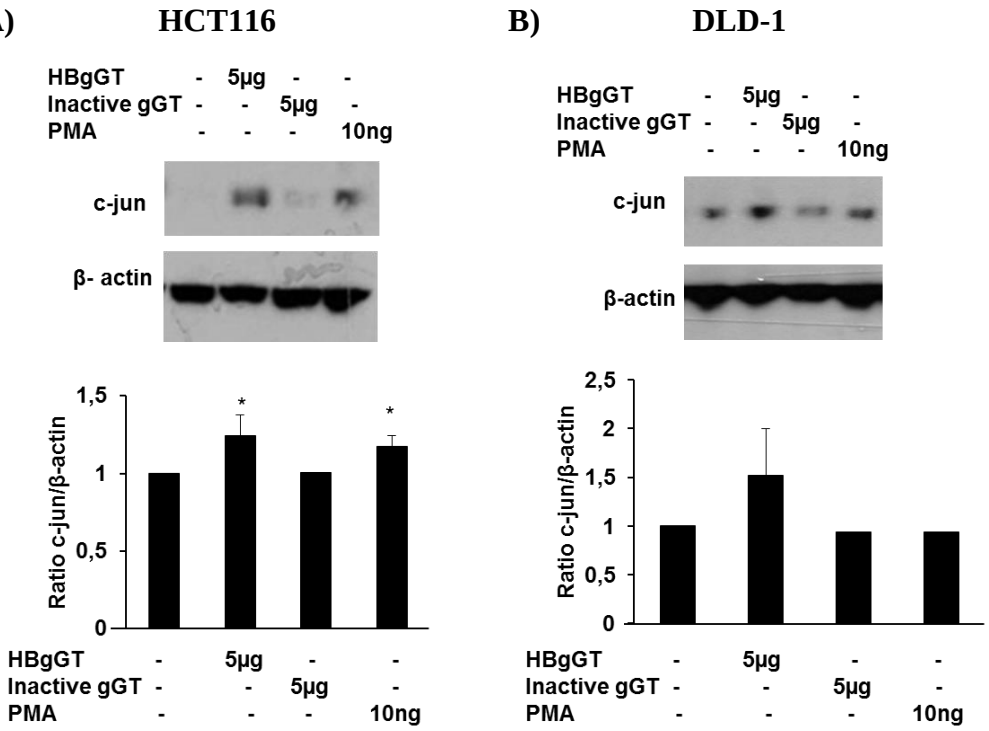


Figure 40: Immunoblot of c-jun levels in HBgGT treated cells.

Immunoblot (Top) and quantification (Bottom) of c-jun and β-actin band density ratio normalized to the untreated control in A) HCT116 cells and B) DLD-1 cells. Cells were treated with 5µg/ml of active and heat inactivated HBgGT recombinant protein. PMA (10ng/ml) was used as a positive control. Results expressed as mean of three (HCT116) and two (DLD-1) independent experiments.

7.3 HBgGT activates CREB

HBgGT also induced a significant activation of the CREB transcriptional activity in transiently transfected HCT116 cells after 24 hours of HBgGT treatment (Figure 41 A). In DLD-1 cells the basal CREB activity was very high and not easily inducible, so these cells were deemed unsuitable for study. For this purpose another colon cancer cell line, LS174T was used. HBgGT was able to induce a significant increase in CREB transcriptional activity in LS174T cells (Figure 41 B). Forskolin (10µM) was used as a positive control to induce transcriptional activation of CREB. Again, as could be seen previously, the inactivated enzyme was unable to induce the transcriptional activity of CREB. Hence HBgGT enzymatic activity was essential for CREB activation.

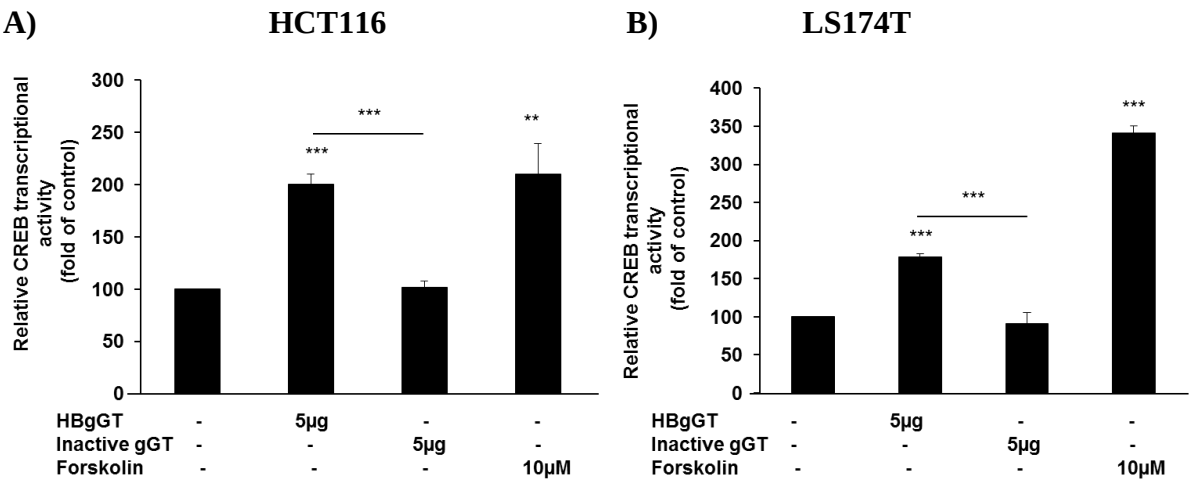


Figure 41: CREB transcriptional activity in HBgGT treated colon epihelial cells.

CREB activation in A) HCT116 and B) LS174T cells transiently transfected and treated with HBgGT (amounts added per ml of cell culture medium) for 24 hours. Forskolin (10µM) was used to induce the transcriptional activity as a positive control. Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

In order to confirm that this increased transcriptional activity corresponded to an activation of the pathway, western blot analysis was performed with a phospho-CREB antibody. Activation of the CREB signalling cascade is preceded by CREB phosphorylation which leads to its nuclear translocation and subsequent transcriptional activity. The immunoblots showed an increased phosphorylation of CREB when the cells were treated with HBgGT for 10 hours. Forskolin was used as a positive control. The inactive HBgGT was however not able to induce any phosphorylation in HCT116 cells (Figure 42).

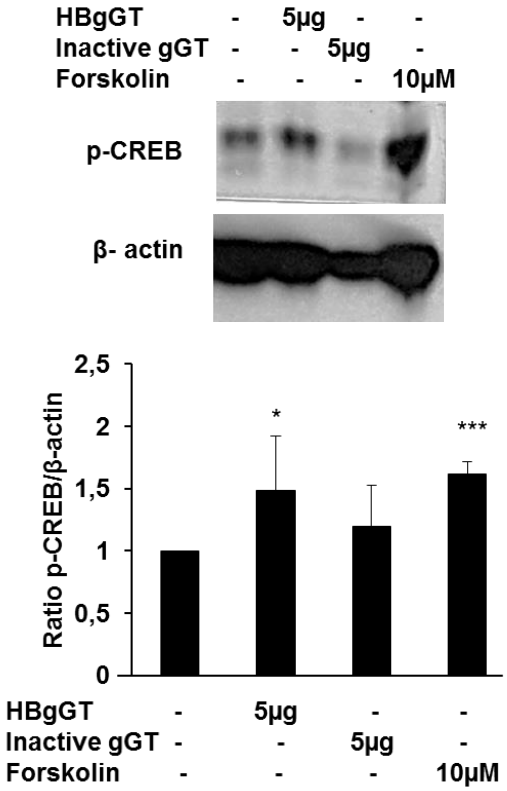


Figure 42: Immunoblot analysis of CREB phosphorylation in HBgGT treated cells.
Phosphorylation of CREB via immunoblot (Top) and quantification (Bottom) of p-CREB and β-actin band density ratio normalized to the untreated control in HCT116 cells was analyzed. Cells were treated with 5µg/ml of active and heat inactivated HBgGT recombinant protein. Forskolin (10µM) was used as a positive control. Results are expressed as mean of three independent experiments.

Although the recombinant HBgGT protein was able to activate the transcriptional activity of all these pathways however, this activation was weak. This fact made it all the more important to investigate whether this activation was strong enough to have any impact on host cells during an infection setup.

8. Role of gGT in *H. bilis* infection

The role of gGT in the context of *H. bilis* infection still remains an open question. To address this question, colon cancer epithelial cell lines HCT116 and DLD-1 were co-cultured with a bacterial suspension of *H. bilis* wild type and gGT knock out bacterium, the gGT sequence of which was replaced by a chlomphenicol resistance cassette (Rossi, Bolz et al. 2012). The bacteria were kindly provided by Rossi M. and the gGT deletion verified via PCR. The gGT

deletion mutant presented a shift in the amplified DNA band corresponding to the resistance cassette size (Figure 43).

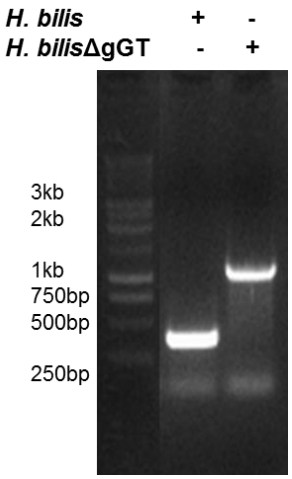


Figure 43: gGT screening PCR for *H. bilis* wiltype and gGT deletion mutant.
Confirmation of presence of the chlomphenicol resistance cassette disrupting the gGT sequence in the gGT deletion mutant. A specific screening PCR for the gGT sequence of the wildtype and gGT knock out bacterial genomic DNA was performed.

8.1 *H.bilis* infection reduces host cell viability

To determine whether any gGT dependent changes in host cell viability occurred in *H. bilis* infections, cell co-culture experiments with the bacterium were performed using wild type and a gGT knock out strain. Viability of HCT116 and DLD-1 cells was analysed by measurement of ATP content in cell lysates after a 48 hour co-culture with *H. bilis* wild type and gGT knock out bacterium at different MOIs (5 and 50).

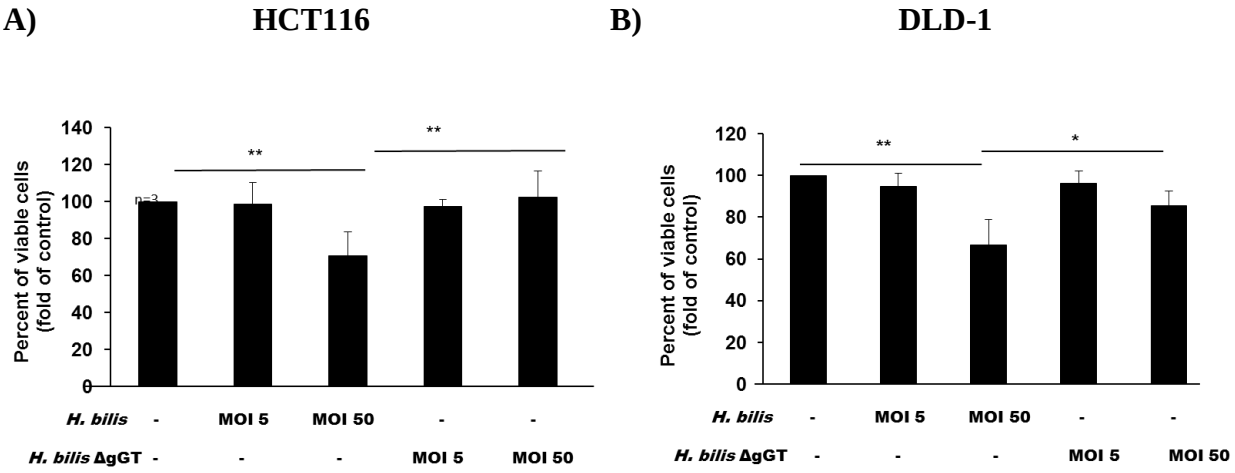


Figure 44: Co-culture of A) HCT116 and B) DLD-1 cells with *H. bilis* for 48 hours leads to a gGT dependent inhibition of proliferation.

Results are expressed as % of mean values normalized to the untreated control from three independent experiments. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Significantly reduced cell viability 29.6% for HCT116 and 33.4% for DLD-1 cells was observed in cells infected with the bacteria with a functional gGT enzyme at an MOI of 50. This effect was not present when the cells were co-cultured with the gGT deficient bacterium at the same MOI (Figure 44), indicating that the gGT enzyme is responsible for decreasing the cell viability in *H. bilis* infection.

8.2 Transcriptional dysregulation

In previous experiments with the recombinant HBgGT protein, induction of several transcription factors by the enzyme was observed. Thus, validation of HBgGTs role in bacterial pathogenesis via activation of these transcription factors in the bacterial infection model was essential. In order to achieve this, HCT116 and DLD-1 cells were co-cultured with *H. bilis* and gGT deletion mutant strain at different MOIs and measurement of changes in transcriptional activity as well as quantification of key proteins involved in the signalling cascade was performed. In the NF-κB pathway key proteins involved in the signalling cascade, IκBα and p65, were monitored. CREB phosphorylation was used as a marker for the activation of the CREB signalling cascade and levels of c-jun were detected to observe activation of the AP-1 pathway.

8.2.1 *H. bilis* activates NF-κB

It was observed that HBgGT was able to induce the activation of NF-κB transcriptional activity in colon cancer epithelial cell lines. Whether this activation by the enzyme was able to translate into a co-culture model was studied. For this purpose transiently transfected cells with a NF-κB reporter plasmid were co-cultured with *H. bilis* gGT deficient and proficient bacterium at an MOI of 5 and 50. The cells showed increased transcriptional activity which was significantly higher when the bacteria were gGT proficient. In HCT116 cells, a 4 and 10 fold increase in transcriptional activity with *H. bilis* MOI 5 and 50 respectively were observed after 24 hours of co-culture. The gGT deficient bacterium was only able to increase the transcriptional activity of NF-κB by 2 and 3 fold at MOI 5 and 50 respectively. At 48 hours post infection a similar activation pattern was observed, with the gGT proficient bacterium activating transcriptional activity by 5 and 11 folds at MOI 5 and 50 respectively as compared to the untreated control. *H.bilis* ΔgGT on the other hand, was able to only marginally increase the transcriptional activity of NF-κB by approximately 3 and 5 folds respectively with the MOIs 5 and 50 at 48 hours of co-culture in the HCT116 cells (Figure 45 A).

DLD-1 cells also behaved in a similar fashion with the wild type bacterium being able to increase the transcriptional activity of NF-κB by 1.8 at MOI 5 and 2.7 folds at MOI 50 relative to the untreated control at 24 hours and 2 and 3.2 folds respectively at 48 hours post infection. The gGT deficient bacterium was only able to activate slightly the transcriptional activity at MOI 50 by 1.8 and 1.77 folds after 24 and 48 hours, respectively (Figure 45 B).

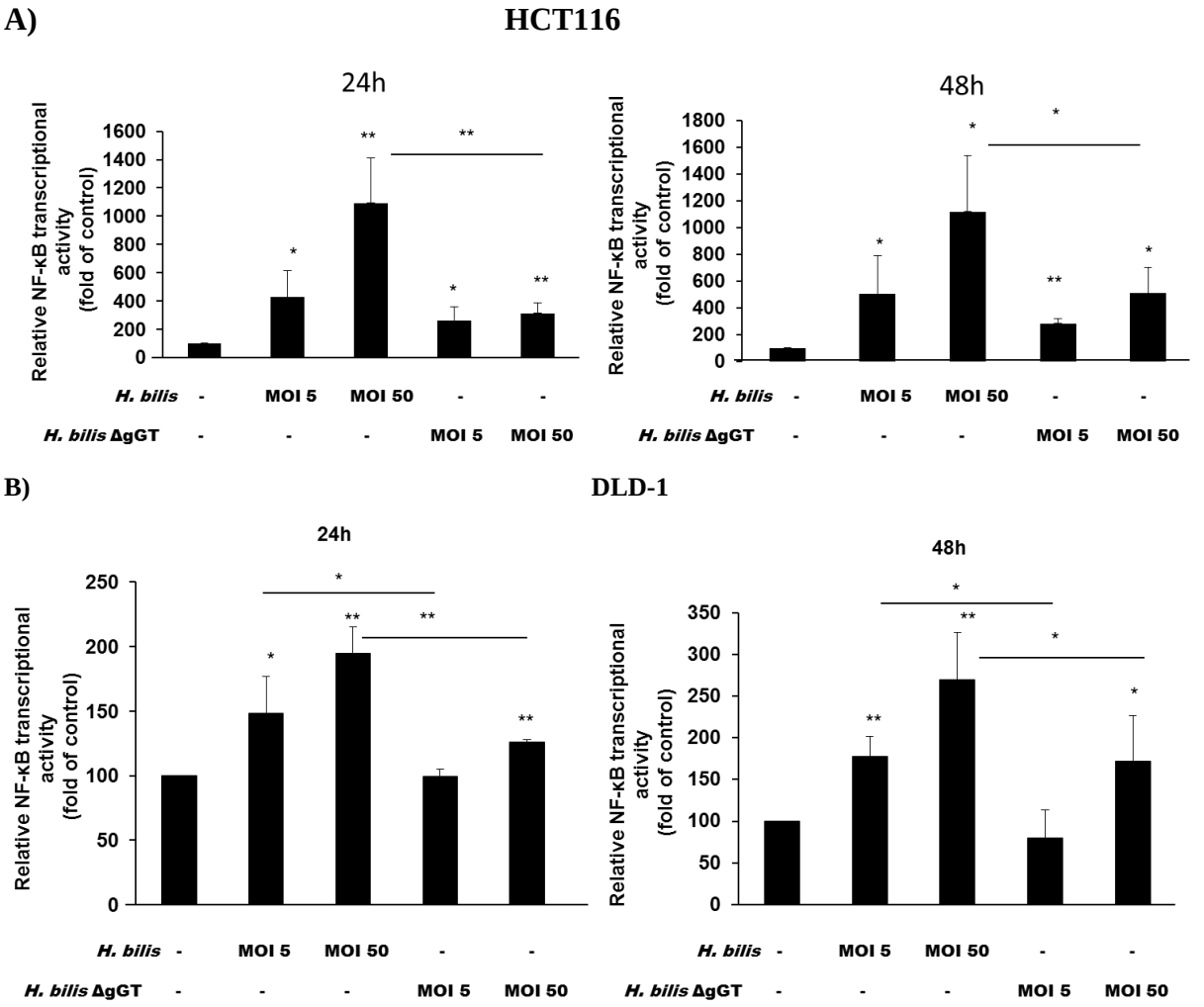


Figure 45: NF-κB transcriptional activity in *H. bilis* wt and gGT knockout infected colon cancer epithelial cells.

Increased NF-κB transcriptional activity was observed upon co-culture of A) HCT116 and B) DLD-1 cells with *H.bilis* or *H. bilis*ΔgGT at MOI 5 and 50. Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

This increase in NF-κB transcription was preceded by enhanced phosphorylation of IκBα, which was detected as early as 10 hour post infection showing activation of the NF-κB pathway by the gGT proficient bacterium. A very weak phosphorylation in case of the gGT deficient bacterium was observed. IκBα phosphorylation at 10 hours of infection is depicted in the figure for HCT116 and DLD-1 cells (Figure 46). Band density ratios between p-IκBα and β-actin loading control were calculated and normalized to the untreated control.

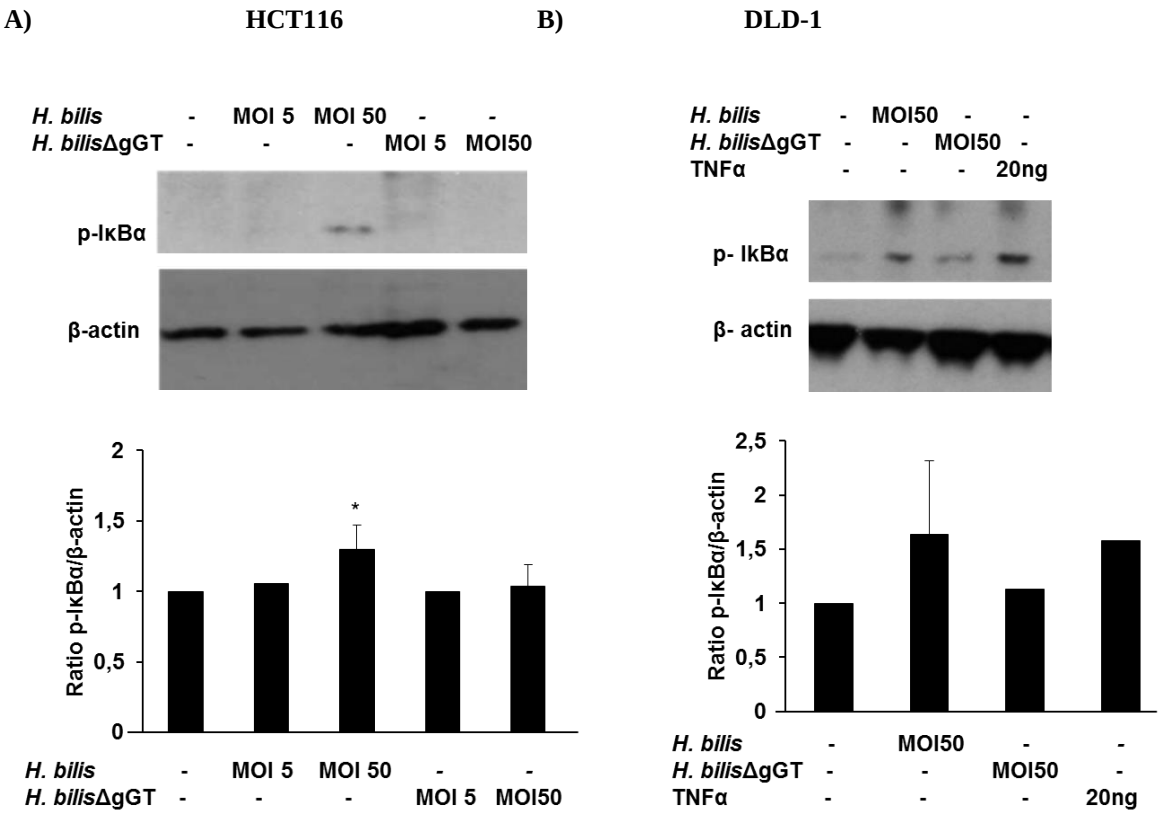


Figure 46: Immunoblot analysis of phosphorylation of IκBα in *H. bilis* infected cells.

Increased NF-κB involved increased phosphorylation of IκBα. A representative immunoblot (Top) and quantification (Bottom) of p-IκBα and β-actin band density ratio normalized to the untreated control at 10 hours of infection in A) HCT116 cells and B) DLD-1 cells. Results expressed as mean of three (HCT116) and two (DLD-1) independent experiments. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Nuclear translocation of p65 was analysed by fluorescently labelling it in infected cells after fixation. Increased nuclear translocation of the NF-κB p65 subunit was observed after *H. bilis* infection, which was enhanced in the presence of gGT, indicating a gGT dependent activation of the NF-κB pathway. TNFα was used as a positive control (Figure 47).

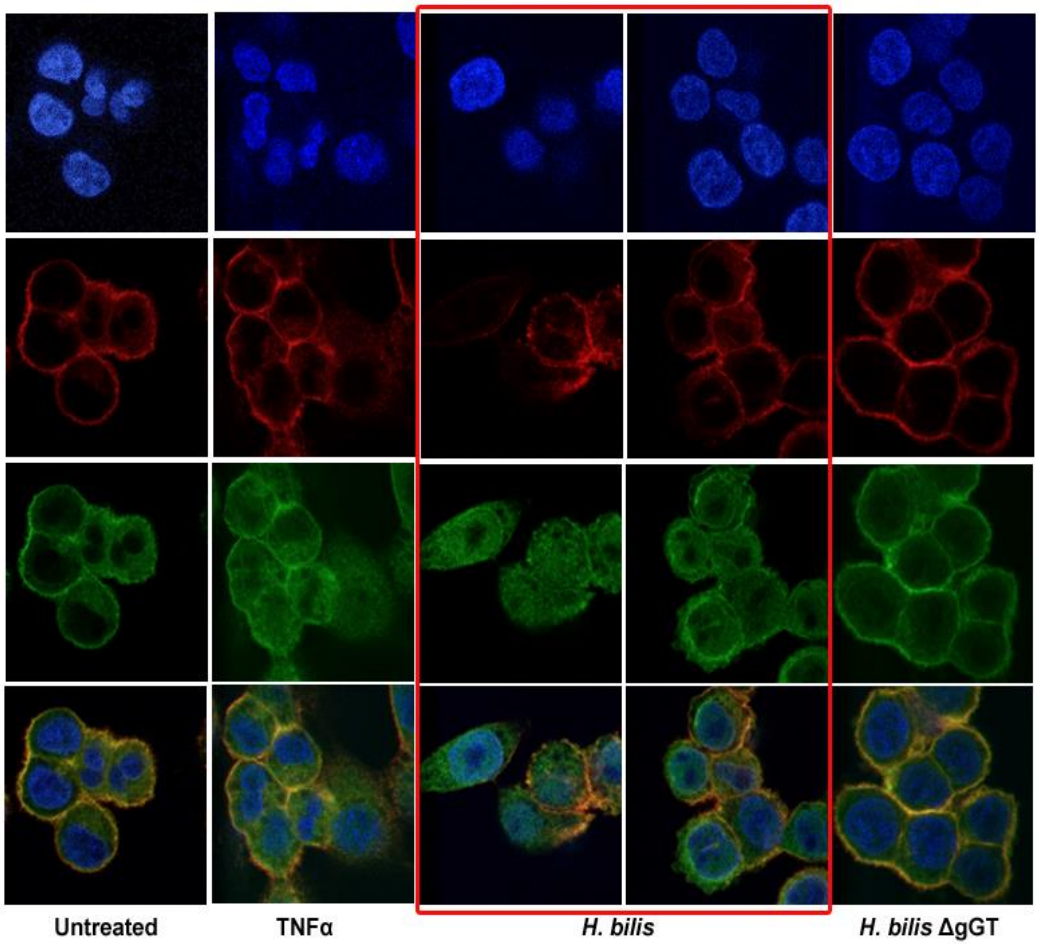


Figure 47: Increased p65 nuclear translocation in HCT116.

Increased nuclear translocation of NF-κB subunit p65 after *H. bilis* co-culture at MOI 50 in HCT116 cells. Inactive gGT deficient mutant at the same MOI was used as a control. TNFα (20ng/ml) was used to activate the pathway and used as a positive control. p65 was labelled with Alexa 488 (green), the cell nuclei were stained with DAPI (blue) and phalloidin (red) was used to stain actin filaments. One representative figure of three independent experiments is shown.

In order to confirm the role of gGT in the activation of the NF-κB signaling pathway, gain of function experiments were performed. To achieve this gGT deficient mutant *H. bilis* was supplemented with the recombinant HBgGT protein at a concentration of 5µg/ml simultaneously during cell co-culture, to compensate for the loss of function. NF-κB transcriptional activity was determined after 24 hours of co-culture. The gGT deficient *H. bilis* dependent activation of the transcription factor could be enhanced by about 140% in HCT116 and 72% in DLD-1 cells when HBgGT was added to the bacterial co-culture.

However the levels observed in case of the wildtype bacterium were not achieved. This might be due to the fact that the gGT proficient bacterium is able to continuously secrete the enzyme however in the experimental setup it was only added once. Since, it was not possible to determine how much bacterial enzyme was accumulated in the culture it was difficult to determine the physiological amounts to be added for the compensation. Despite the fact that wiltype levels of the activation of transcriptional activity were not reached by gGT compensation nevertheless increases in the activity of the gGT deficient bacterium after addition of the recombinant protein confirms that gGT is indeed an important virulence factor in *H. bilis* activation of NF-κB pathway.

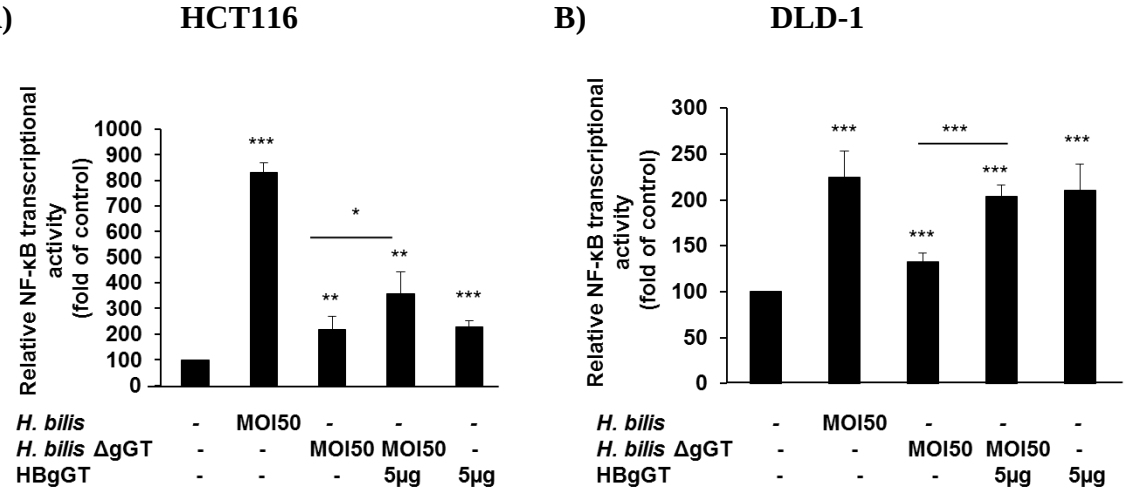


Figure 48: Gain of function in NF-κB transcriptional activity after addition of recombinant HBgGT to a co-culture with *H. bilis* ΔgGT

Gain of function in NF-κB transcriptional activity after addition of recombinant HBgGT (amounts indicated were added per ml of cell culture medium) to a co-culture with *H. bilis* wt and ΔgGT at MOI 50 for 24 hours of infection in A) HCT116 and B) DLD-1 cells. n=3. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

8.2.2 *H. bilis* activates AP-1

Since AP-1 activation was also observed when cells were treated with HBgGT, AP-1 transcriptional activity was assessed in *H. bilis* infected cells in a transient transfection system. HCT116 and DLD-1 cells were transiently transfected with the AP-1 luciferase reporter vector and infected with two different MOIs of *H. bilis* and the gGT deficient mutant. AP-1 transcriptional activity was determined after 24 and 48 hours of infection. The gGT

deficient bacterium showed decreased transcriptional activity of AP-1 as compared to the gGT proficient bacterium (Figure 49). HCT116 cells showed a significant increase in the transcriptional activity of AP-1 at 24 hours. A 10% increase in AP-1 transcriptional activity at MOI 5 was observed that increased with the MOI by 434% at MOI 50 with the *H. bilis* infected cells. At 48 hours post infection transcriptional activity of AP-1 increased by 57% with MOI of 5 and 108% with MOI 50. The gGT deficient mutant on the other hand did not show any significant changes in AP-1 activity, however a slight activation could be observed at MOI 50 (Figure 49 A). DLD-1 cells also exhibited an increase in the transcriptional activity of AP-1 at 24 hours by 46% at MOI 5 and 96% with MOI 50. A further increase in AP-1 transcriptional activity was observed at 48 hours after *H. bilis* infection with an increase of 57% with an MOI of 5 and 201% with MOI 50. The gGT deficient mutant also showed a significant activation in AP-1 transcriptional activity, however this activation was not as high as the one generated in response to the gGT proficient bacterium (Figure 49 B). This indicates that other virulence factors of the bacterium in addition to HBgGT are also involved in regulating AP-1 transcriptional activity.

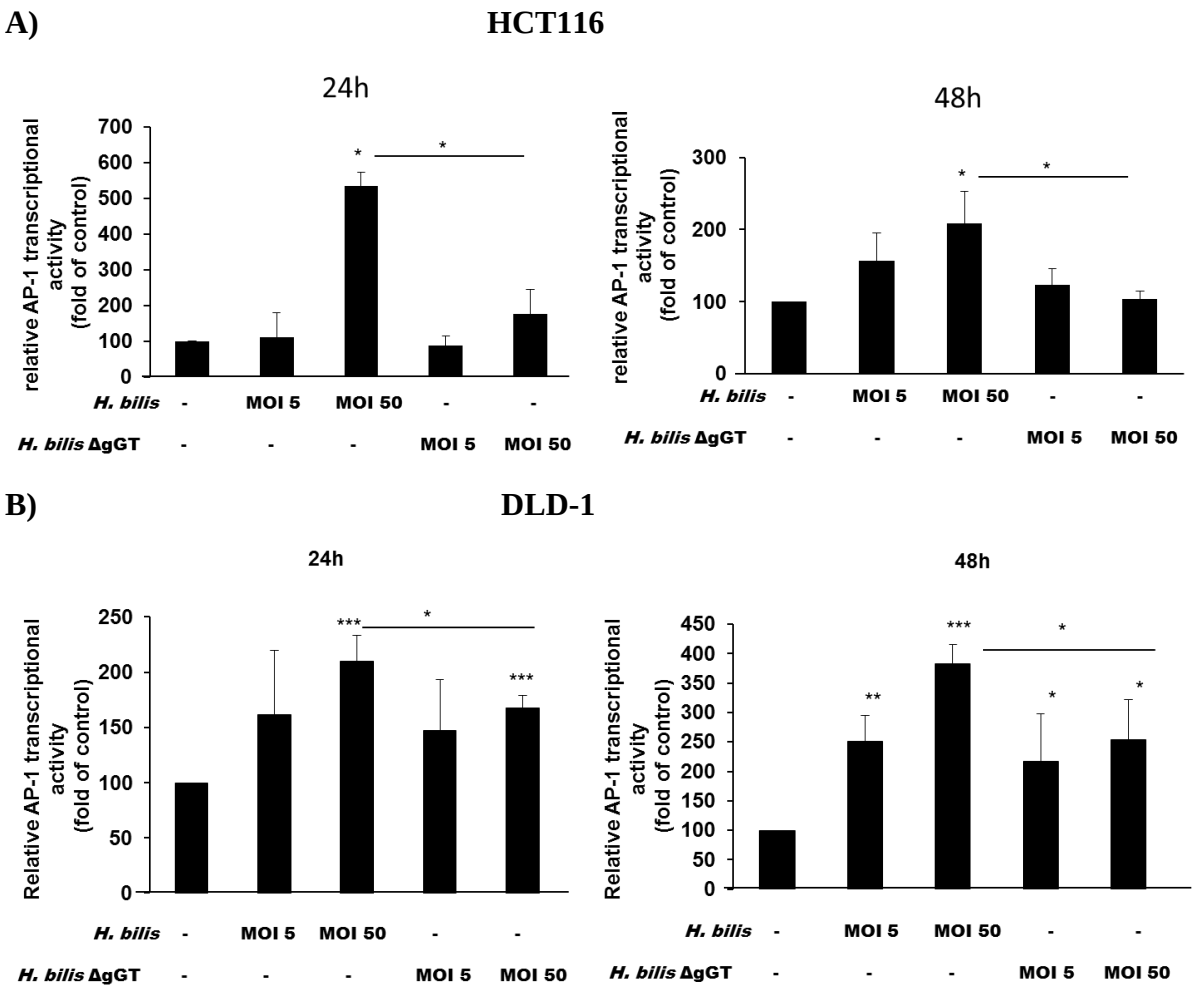


Figure 49: AP-1 transcriptional activity in *H. bilis* wt and gGT knockout infected colon cancer epithelial cells.

H. bilis co-culture induced increased AP-1transcriptional activity at 24 and 48 hours of infection in A) HCT116 and B) DLD-1 cells. Cells were infected with *H.bilis* and *H. bilis* ΔgGT at MOI 5 and 50. Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Since the transcriptional activity of AP-1 was preceded by increased c-jun levels in HBgGT treated cells as shown previously, verification of activation of the AP-1 signaling cascade was also done by analysing c-jun protein levels after *H. bilis* infection. The activation in the transcriptional activity was preceded by increased protein levels of c-jun 6 hours after infection as detected in whole cell lysates obtained after co-culture of cells with the bacterium. This activation was mainly dependent on the presence of gGT, as a very weak

activation after incubating the cells with the gGT deficient bacterium was observed, indicating that gGT is an important inducer of the AP-1 pathway in *H. bilis* infections (Figure 50).

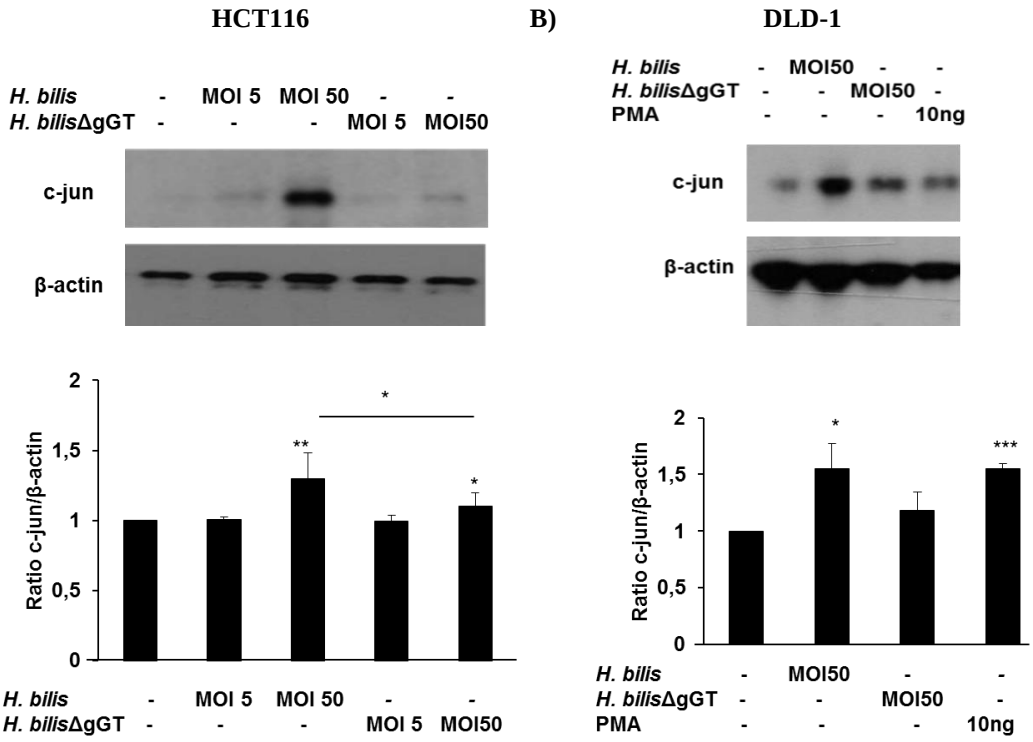


Figure 50: Immunoblot analysis of c-jun levels in *H. bilis* infected cells.

H. bilis co-culture with A) HCT116 and B) DLD-1 cells induced increased c-jun protein levels. One representative immunoblot of three independent experiments shown (Top). Mean of three separately quantified blots of ratio of band density of c-jun and β-actin normalized to the untreated at 6 hours of infection (Bottom). *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Furthermore, it was interesting to study whether the addition of the recombinant protein to the gGT deficient bacterium would compensate for the weak AP-1 activation observed. The recombinant HBgGT (5µg/ml) was added to *H. bilis* ΔgGT at the same concentration previously used. Bacterial cell co-cultures at MOI 50 were chosen for the experiments as they showed the highest AP-1 activation at 24 hours. AP-1 transcriptional activity in response to *H. bilis* ΔgGT could be significantly enhanced by 140% in HCT116 and by 72% in DLD-1 cells when the recombinant HBgGT was added to the gGT deficient bacteria in a complementation experiment (Figure 51). Hence, the addition of the recombinant protein could compensate for the loss of gGT function in the bacterium.

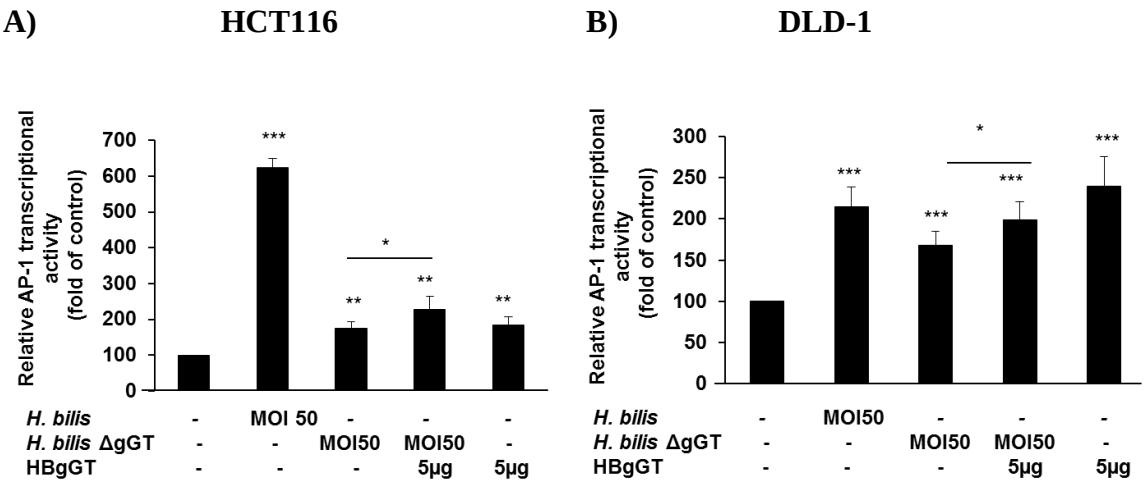


Figure 51: AP-1 transcriptional activity after addition of recombinant HBgGT to *H. bilis* ΔgGT

Partial restoration of AP-1transcriptional activity after addition of recombinant HBgGT (amounts added per ml of cell culture medium) to a co-culture with *H. bilis* ΔgGT MOI 50 at 24 hours of infection in A) HCT116 and B) DLD-1 cells. Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

8.2.3 *H. bilis* activates CREB

CREB was also among the stress response pathways triggered by the recombinant HBgGT protein. To evaluate whether this activation was present in *H.bilis* infections, transiently transfected HCT116 and LS174T cells with a CREB reporter plasmid were infected with *H. bilis* wildtype and the gGT knockout bacterium at different MOIs (5 and 50). Increased transcriptional activity could be observed for both bacteria; however the activation was only approximately 2 fold in HCT116 cells when gGT proficient bacteria were used for the infection (Figure 52 A).

A much higher level of transcriptional activity was observed in case of LS174T cells, which were used instead of DLD-1 cells because of the physiologically high CREB activation levels observed in DLD-1 cells. Significantly increased transcriptional activation was observed at MOI 5 (35%), which was even higher with MOI 50 (142%) at 24 hours. A further increase of 97% at MOI 5 and 365% at MOI 50 at 48 hours was observed. Lower levels of transcriptional activation in cells were detected when infecting cells with the gGT deficient bacterium (decrease in transcriptional activity of 67% at 24 hours and 249% at 48 hours with MOI 50)

(Figure 52 B). This differential activation levels of the gGT proficient and the deficient bacterium points to gGTs active role in CREB transcriptional activation.

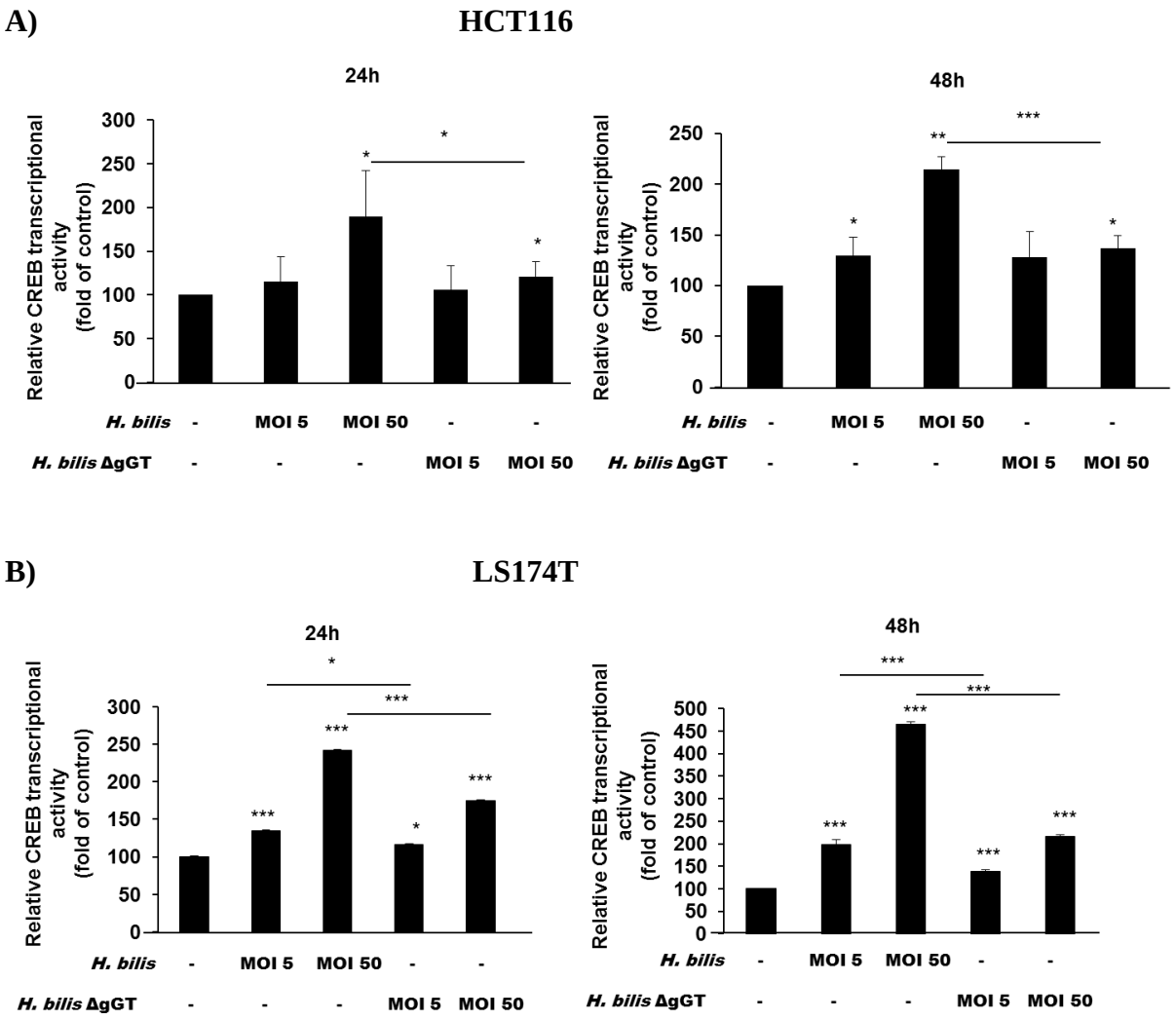


Figure 52: CREB transcriptional activity in *H. bilis* wt and gGT knockout infected colon epithelial cells.
H. bilis co-culture induced increased CREB transcriptional activity at 24 and 48 hours of infection in A) HCT116 cells and B) LS174T cells. Cells were infected with *H.bilis* and *H. bilis* ΔgGT at MOI 5 and 50. Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

As previously discussed, increased phosphorylation of CREB preceeds the nuclear translocation and subsequent activation of its transcriptional activity. Hence, levels of phosphorylated CREB were analyzed after cells were infected with the bacterium. Increased phosphorylation of CREB could be observed with the gGT proficient *H. bilis* after 6 h of

infection, which was more pronounced with a higher MOI (Figure 53) emphasizing gGTs role in CREB activation.

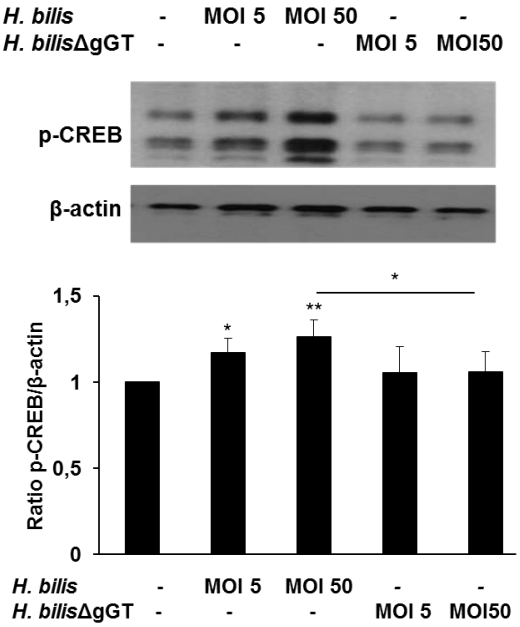


Figure 53: *H. bilis* co-culture with HCT116 cells induces phosphorylation of CREB

One representative p-CREB immunoblot is shown (Top). Mean of three independent experimental quantifications of band density ratio of p-CREB to β-actin normalized to the untreated at 10 hours of infection with *H.bilis* and *H.bilis* ΔgGT MOI 5 and 50 (Bottom). *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

A gain of function experiment was performed again to evaluate the role of HBgGT in CREB transcriptional. The recombinant HBgGT (5µg/ml) was added to *H. bilis*ΔgGT at MOI 50. It was observed that the CREB transcriptional activity in response to *H. bilis* ΔgGT could be significantly enhanced by 50% in HCT116 and at a lower level (19%) in LS174T cells when the recombinant HBgGT was added to the gGT deficient bacteria (Figure 54). Hence the addition of the recombinant protein could not completely compensate for the loss of gGT function in the bacterium but could significantly enhance the transcriptional activity induced by the gGT deficient bacterium.

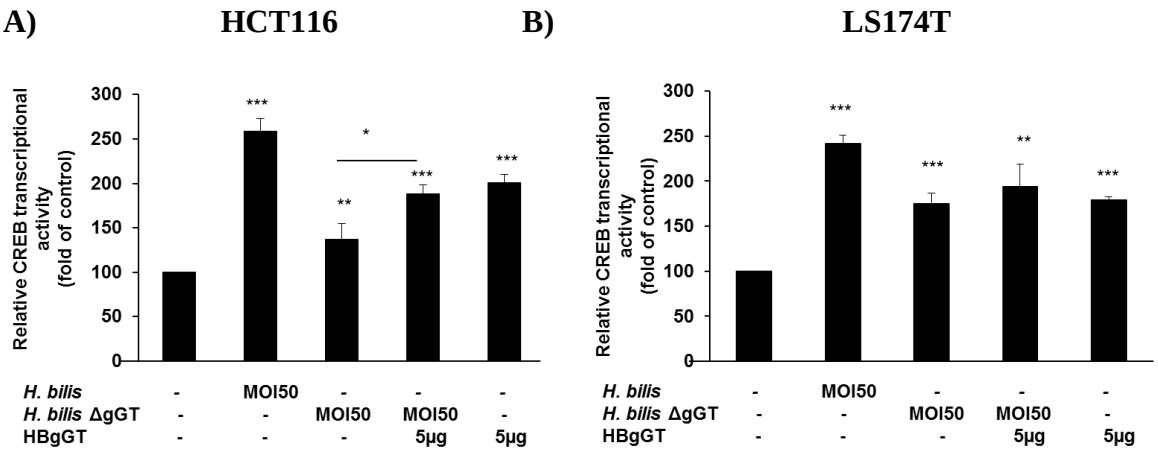


Figure 54: CREB transcriptional activity after addition of recombinant HBgGT to *H. bilis* ΔgGT

Partial restoration of CREB transcriptional activity after addition of recombinant HBgGT (amounts indicated were added per ml of cell culture medium) to a co-culture with *H. bilis* ΔgGT MOI 50 at 24 hours of infection in A) HCT116 and B) LS174T cells. Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

As HBgGT induced transcriptional activation of these stress induced pathways, it was important to see what functional advantage such activation by gGT afforded the bacterium. In order to elucidate which functional cellular responses are involved in gGT-modulated activation of these pathways, downstream target genes regulated by these transcription factors and involved in induction of inflammatory stress responses in the host were studied.

9. Target genes involved in gGT-induced oxidative stress signalling

NF-κB, AP-1 and CREB play important roles in determining the severity and outcome of several diseases including ulceration, carcinoma and IBD. Consistent with this role transcriptional activation of these factors trigger the expression of several cellular stress response regulating genes which are their downstream targets. Some of these target genes like COX-2, IL-6, IL-8 are important inducers of inflammation. HPgGT has been shown to induce activation of IL-6, COX-2 and IL-8 (Busiello, Acquaviva et al. 2004; Gong, Ling et al. 2010), however, the role of HBgGT in the activation of these target genes has not been explored yet.

9.1 IL-8

H. pylori infection is able to induce IL-8 production from gastric epithelial cells (AGS). This activation was dependent on CagA, VacA and gGT virulence factors (reviewed in Backert and Naumann 2010). Since it had already been shown that HPgGT is able to induce IL-8 production, it remained to be explored if HBgGT also behaved in a similar manner.

HCT116 and DLD-1 cells were co-cultured with *H.bilis* and the gGT knock out strain. Cell supernatants were collected and IL-8 chemokine measured by IL-8 enzyme linked immunosorbent assay. An infection ratio of 50 was used for these assays as this was the MOI that was most effective in the activation of the transcriptional regulators of IL-8 studied previously (NF-κB, AP-1 and CREB).

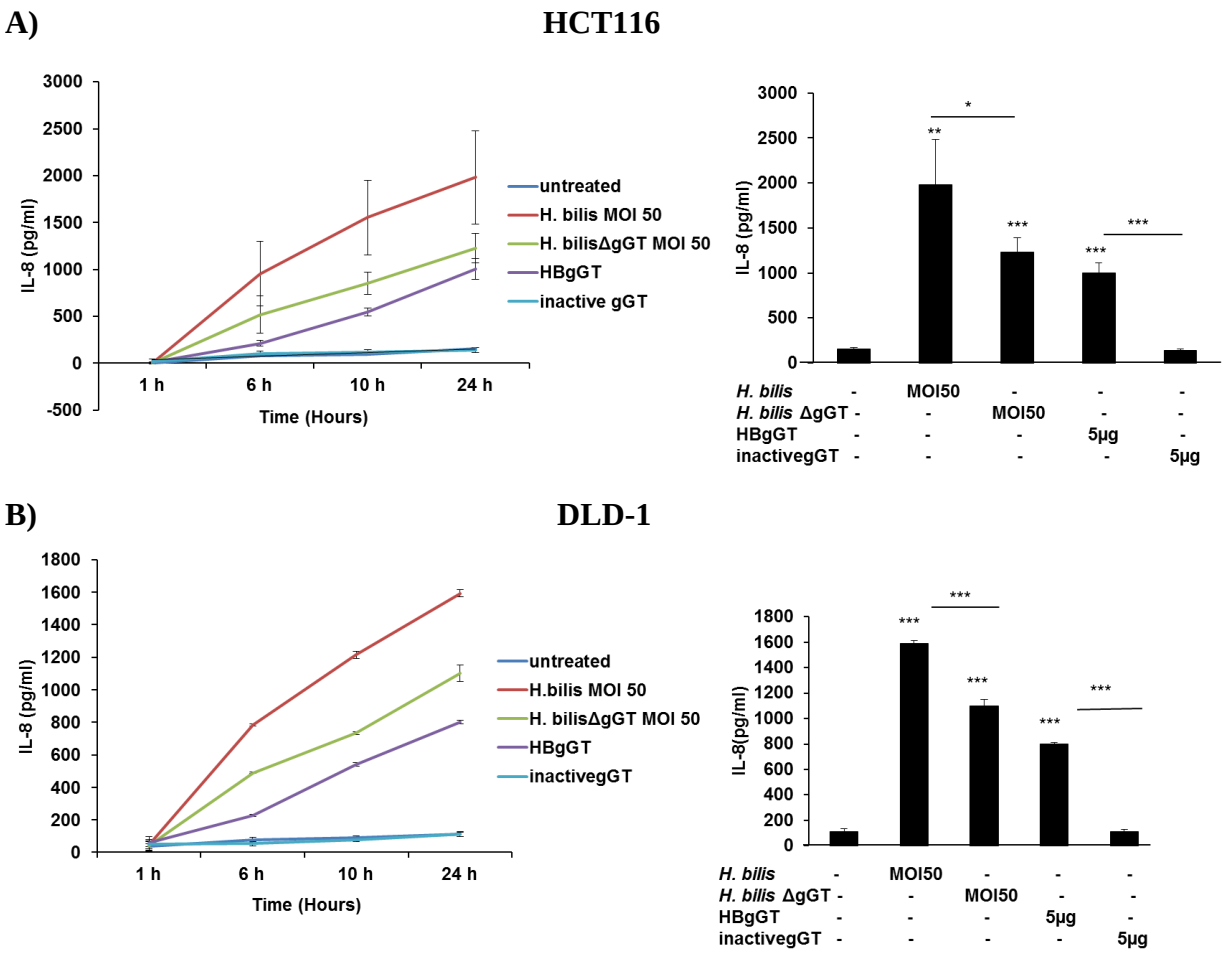


Figure 55: Secretion of IL-8 after gGT treatment and *H. bilis* co-culture with colon epithelial cells.
Differential production of IL-8 involved in presence/absence of gGT after *H. bilis* co-culture or stimulation with the recombinant HBgGT (amounts indicated were added per ml of cell culture medium) in A) HCT116 cells, time course of IL-8 production in presence/absence of gGT (Left) and IL-8 levels after 24 hour of treatment (Right). B) DLD-1 cells, time course of IL-8 production in presence/absence of gGT (Left) and IL-8 levels after 24 hour of treatment (Right). Values expressed as mean of three independent experiments. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Increased IL-8 levels could be detected in the supernatants from both cell lines after *H. bilis* infection starting 6 hours post infection (Figure 55). The IL-8 production was significantly decreased by 752 pg/ml for HCT116 cells and for 493 pg/ml DLD-1 cells at 24 hours when the gGT was knocked out from the bacterium (Figure 55). The recombinant protein was also able to induce IL-8 production, but at a lower level (1003 pg/ml for HCT116 cells and 799 pg/ml for DLD-1 cells). These results indicate that IL-8 production is significantly influenced by gGT in *H. bilis* infections, although this might not be the only virulence factor responsible

for induction of this pro-inflammatory cytokine by the bacterium in colon epithelial cells, since the gGT deficient mutant was also able to induce IL-8 secretion from cells.

9.2 IL-6

Lu H. et al reported IL-6 production from primary epithelial cell cultures in *H. pylori* infected cells and linked this to activation of the NF-κB, AP-1 and CREB pathway by the bacterium (Lu, Wu et al. 2005). To investigate whether *H. bilis* could also influence the levels of this pro-inflamatory cytokine, IL-6 production was tested in culture supernatants after culture of HCT116 and DLD-1 cells with *H. bilis* and *H. bilis* ΔgGT at different time points ranging from 1hour post infection to 24 hours. No increase in IL-6 secretion was detected for both cell lines since the cytokine levels were below the detection limit of the ELISA (data not shown).

9.3 COX-2

Elevated COX-2 mRNA and protein levels have been reported for *H. pylori* infections. Since COX-2 is also a downstream transcriptional target of the NF-κB, AP-1 and CREB transcription factors, it was interesting to see if any changes in COX-2 protein levels could be detected in case of *H. bilis* infections and whether such activation depended on gGT. Cell lysates from gGT proficient and deficient *H. bilis* infected HCT116 and DLD-1 cell cultures were obtained after different time of infection. The cell lysates were probed for the COX-2 protein levels by western blot. No changes in the COX-2 protein levels could be detected with the infection, indicating that *H. bilis* does not influence COX-2 levels (Figure 56).

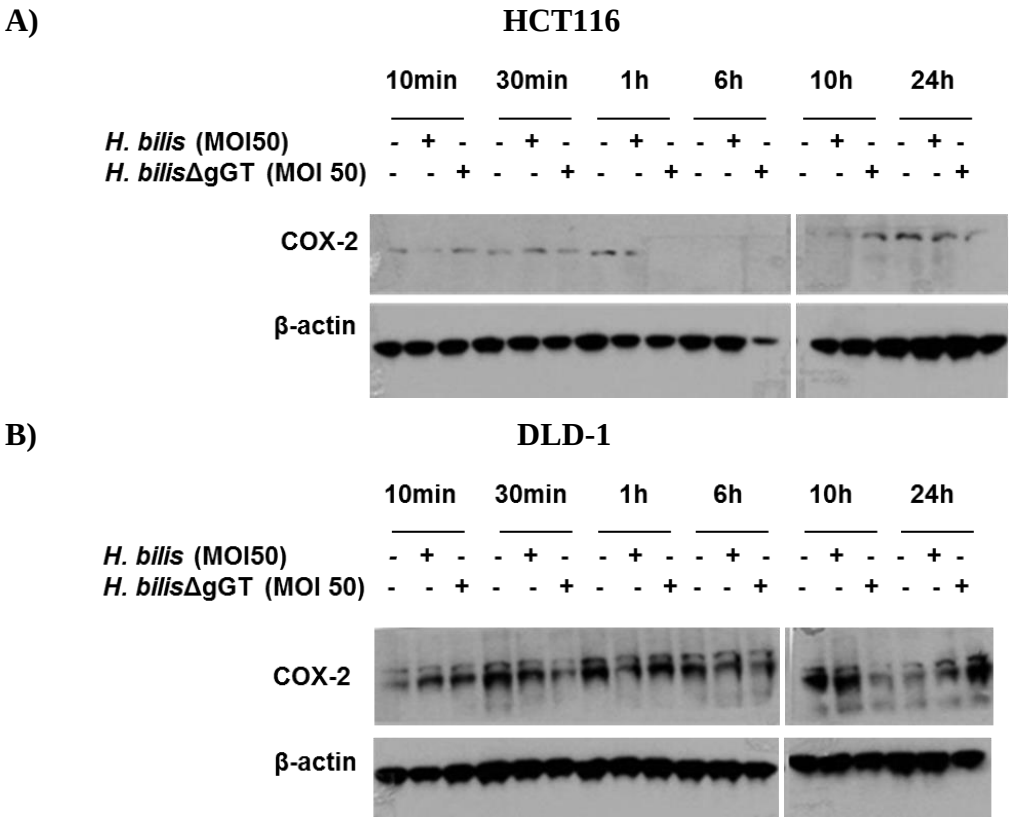


Figure 56: COX-2 levels in *H. bilis* gGT proficient and deficient infected cells.

A) HCT116 and B) DLD-1 cells were infected with *H. bilis* and the gGT deficient bacterium at an MOI of 50 and COX-2 protein levels from lysates at different time points evaluated. One representative of two independent experiments is shown.

10. Mechanism of gGT mediated transcriptional activation

It is confounding how a bacterial enzyme is able to modify host cellular response. Additionaly upstream signalling events leading to the activation of the transcriptional regulators may give an indication as to which activation signals are involved.

10.1 gGT induced cellular stress is dependent on glutamine deprivation

As previously described, gGT helps in the glutamate uptake by the bacterium by hydrolysing glutamine and glutathione. The net uptake of these substances however results in a net decrease in extracellular glutamine and glutathione which is essential in maintaining the redox balance in the host cell.

Glutamine deprivation has been shown to induce activation of the NF-κB and AP-1 pathways and involved in IL-8 production in Caco-2 cells (Marjon, Bobrovnikova-Marjon et al. 2004; Liboni, Li et al. 2005; Xue, Sufit et al. 2011; Lesueur, Bole-Feysot et al. 2012).

To see whether HBgGT was indeed able to influence the host stress responses via glutamine deprivation, HCT116 and DLD-1 cells were treated with HBgGT pre-incubated medium supplemented with L-glutamine after the incubation to replenish depleted glutamine. The gGT was inactivated after 24 hours of preincubation to stop the depletion of the supplemented glutamine. As a control cells were incubated in glutamine free medium while the rest of the cells treatments had a regular concentration of glutamine (2mM) in the medium. Culture of cells without glutamine and subsequent infection led to loss of cell cultures at earlier time points than at which the analysis was performed. This indicated that glutamine starvation makes the cells much more susceptible to bacterial infection induced cell death and since no cell survived the analysis, this condition was excluded from further experiments.

Glutamine starved cells showed a significant increase in transcriptional activity of NF-κB, in HCT116 and DLD-1 cells after 24 hours. Addition of the HBgGT preincubated medium activated the transcriptional activity of NF-κB to a similar extent as that observed by the recombinant protein at 24 hours with a 2 fold activation in HCT116 and DLD-1 cells. Supplementation of the medium with glutamine abolished the activation of NF-κB by HBgGT (Figure 57A).

Secondly, to determine whether this held true in an infection setup with the live bacterium, cells co-cultured with *H. bilis* were also simultaneously supplemented with external L-glutamine. Cells co-cultured with the live bacterium were supplemented with different amounts of L-glutamine. A concentration of 5mM was found to be the most effective in relieving the activation of the pathways. The supplementation however was not able to completely relieve the activation of the pathway, although a significant decrease in NF-κB transcriptional activity in *H. bilis* infected and glutamine supplemented HCT116 cells and DLD-1 cells was observed (Figure 57B).

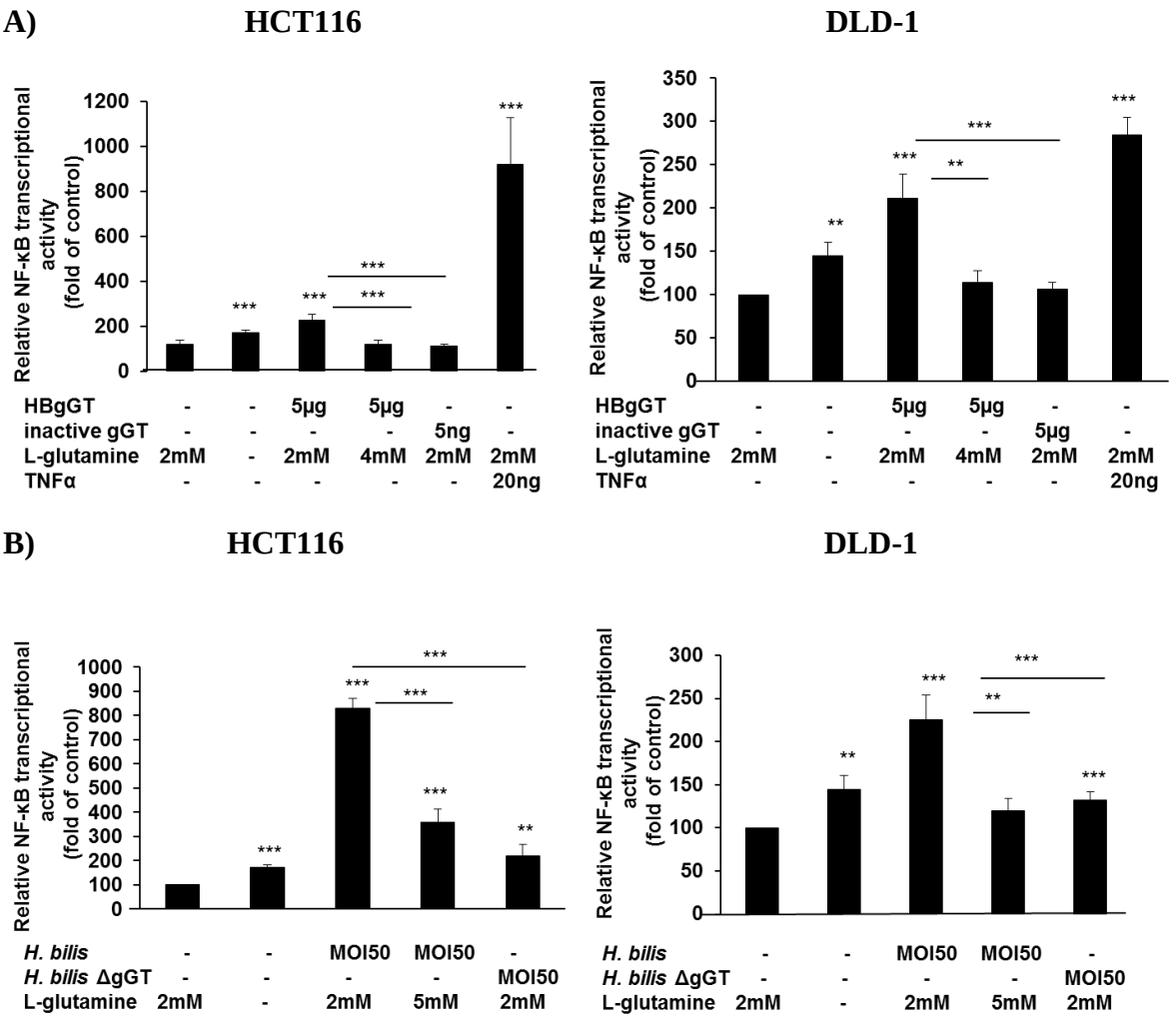


Figure 57: Transcriptional activity of NF-κB after glutamine supplementation of HBgGT treated epithelial cells and *H. bilis* co-cultures.

Transcriptional activation of NF-κB after glutamine supplementation at 24 hours HCT116 and DLD-1 cells were used in a transient transfection with A) cells treated with a HBgGT pre-incubated medium (5µg of protein added per ml of cell culture medium). L-glutamine was added to the cells (final amount in the medium indicated). TNFα was used to induce the transcriptional activation of NF-κB. B) Co-culture with *H. bilis* MOI 50. L-glutamine free medium was used to starve the cells of glutamine. L-glutamine was added to the cells (final amount in the medium indicated). Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control.

To confirm whether this decrease in transcriptional activity corresponded with activation of the pathway, phosphorylation of IκBα was also evaluated in cells. A decrease in the phosphorylation of IκBα could also be observed after supplementation of the HBgGT pre-

incubated medium with glutamine (4mM) in HCT116 cells (Figure 58A). So glutamine supplementation relieved the activation of the NF-κB pathway by HBgGT. *H. bilis* infected and glutamine supplemented cells after 10 hours of co-culture were also evaluated for IκBα phosphorylation. A decrease in the phosphorylation of IκBα could be observed after supplementation of the cells with glutamine (5mM) at the time of infection in both cell lines (Figure 58B). Thus, an overall decrease in the NF-κB signalling activation after glutamine supplementation could be observed for both the recombinant HBgGT treated cells and the ones infected by *H. bilis*. These results indicate that glutamine depletion is an essential part of activation of the NF-κB pathway by gGT with the recombinant protein as well as in a co-culture setup.

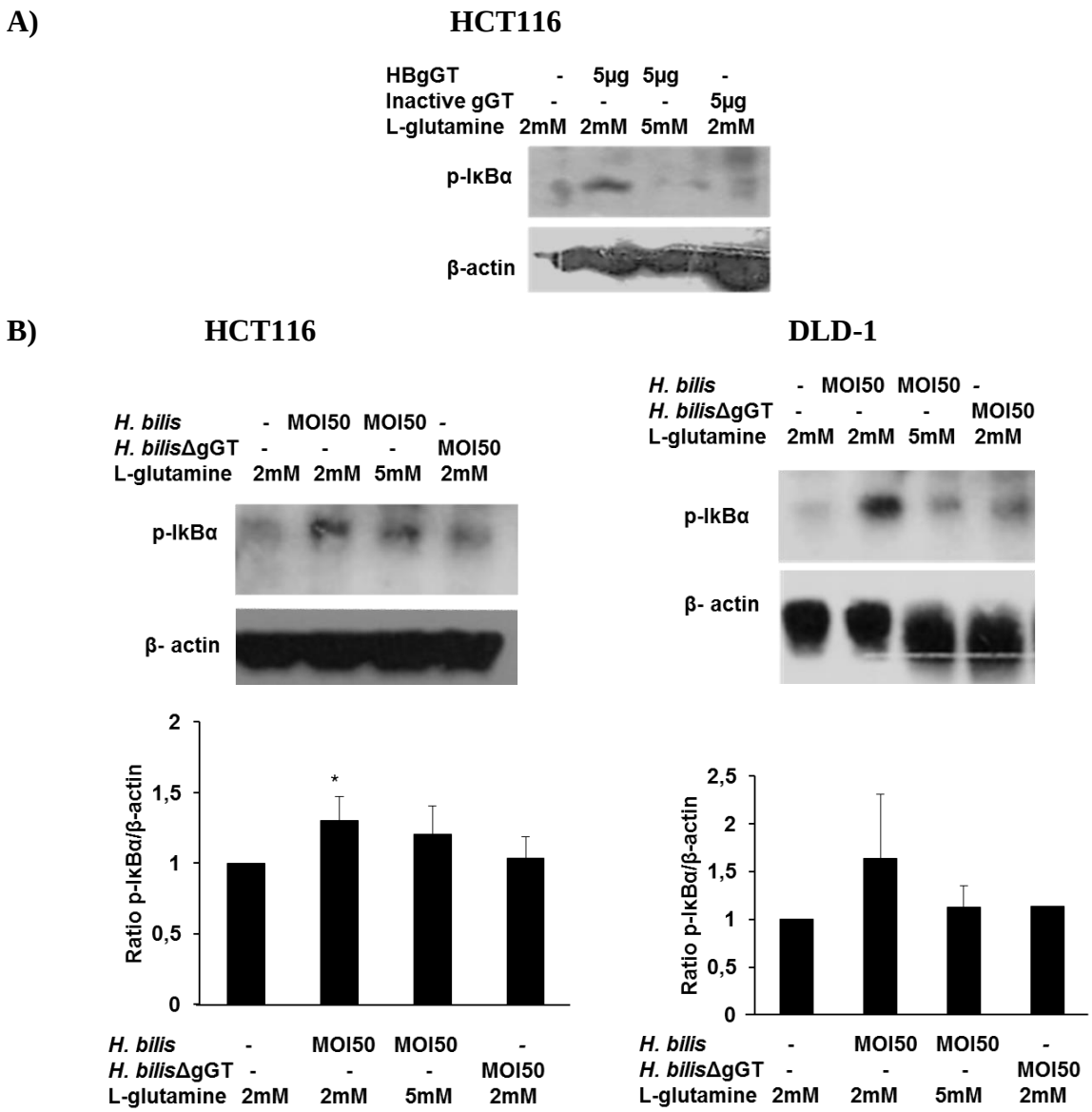


Figure 58: IκBα phosphorylation after glutamine supplementation of HBgGT treated epithelial cells and *H. bilis* co-cultures.

A) p-IκBα blot of HCT116 cell lysates treated with HBgGT and glutamine supplemented for 10 hours B) p-IκBα blots (Top) and quantification (Bottom) of band density ratio of p-IκBα to β-actin normalized to the untreated of *H. bilis* co-cultured HCT116 and DLD-1 cells after glutamine supplementation of the medium at 10 hours after infection. Results expressed as mean three (HCT116) and of two (DLD-1) independent experiments. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Changes in the transcriptional activity and activation of the signalling cascades of the other transcription factors (AP-1 and CREB) activated by HBgGT were next scrutinized after glutamine supplementation.

Glutamine starved cells also showed a significantly increased transcriptional activity of AP-1. A significant increase in AP-1 transcriptional activity in HCT116 and DLD-1 cells was observed upon glutamine starvation. HBgGT preincubated medium also activated the transcriptional activity of AP-1 as that previously shown to be induced by the recombinant protein at 24 hours with a significant activation of AP-1 transcriptional activity in HCT116 and DLD-1 cells. Moreover, supplementation of the medium with glutamine abolished this activation by the enzyme (Figure 59A). Again, in order to determine whether this applied in a co-culture setup, cells infected with *H. bilis* were also simultaneously supplemented with external L-glutamine. The supplementation was not able to completely relieve the activation of AP-1 transcriptional activity, although a significant decrease in *H. bilis* infected and glutamine supplemented HCT116 and DLD-1 cells was observed (Figure 59B).

Lastly, to confirm whether this decrease in transcriptional activity corresponded with activation of the pathway, c-jun levels were evaluated by western blot in cells. Increased c-jun levels were also observed in cells treated with the HBgGT preincubated medium. The increase in c-jun levels could be suppressed when glutamine was added to the preincubated medium exposed cells (Figure 60 A). Cells infected with *H. bilis* for 10 hours were also tested for changes in c-jun levels. A decrease in total c-jun levels could be observed after supplementation of the cells with glutamine (5mM) at the time of infection in both cell lines (Figure 60B).

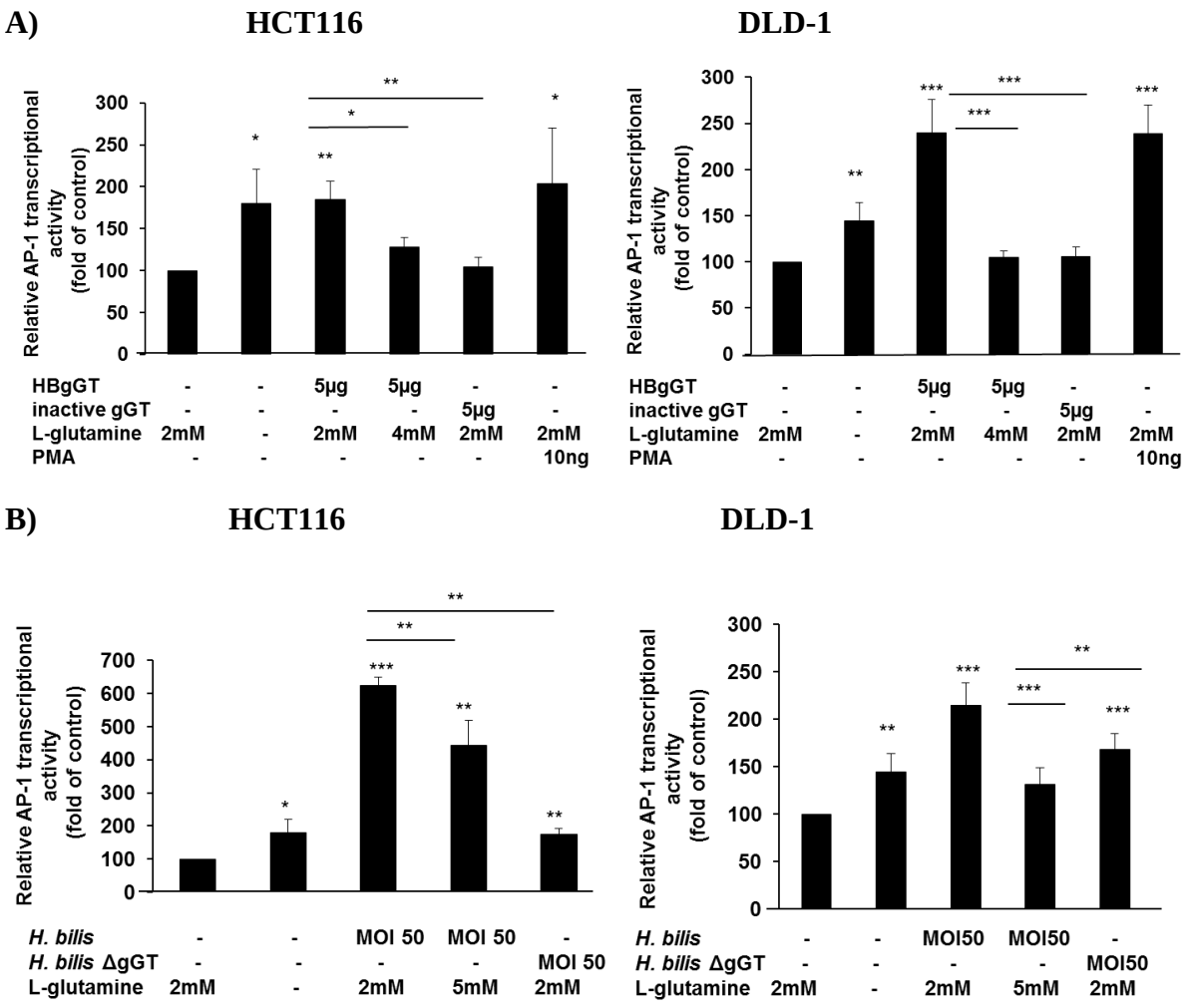


Figure 59: Transcriptional activity of AP-1 after glutamine supplementation of HBgGT treated epithelial cells and *H. bilis* co-cultures.

Transcriptional activation of AP-1 after glutamine supplementation at 24 hours A) HCT116 and DLD-1 cells were used in a transient transfection and treated with HBgGT pre-incubated medium (5µg of protein added per ml of cell culture medium). L-glutamine was added to the cells (final amount in the medium indicated). PMA was used to induce the transcriptional activation of AP-1. B) HCT116 and DLD-1 cells were used in a transient transfection and co-cultured with *H. bilis* MOI 50. L-glutamine was added to the cells (final amount in the medium indicated) L-Glutamine free medium was used to starve the cells of glutamine. Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control.

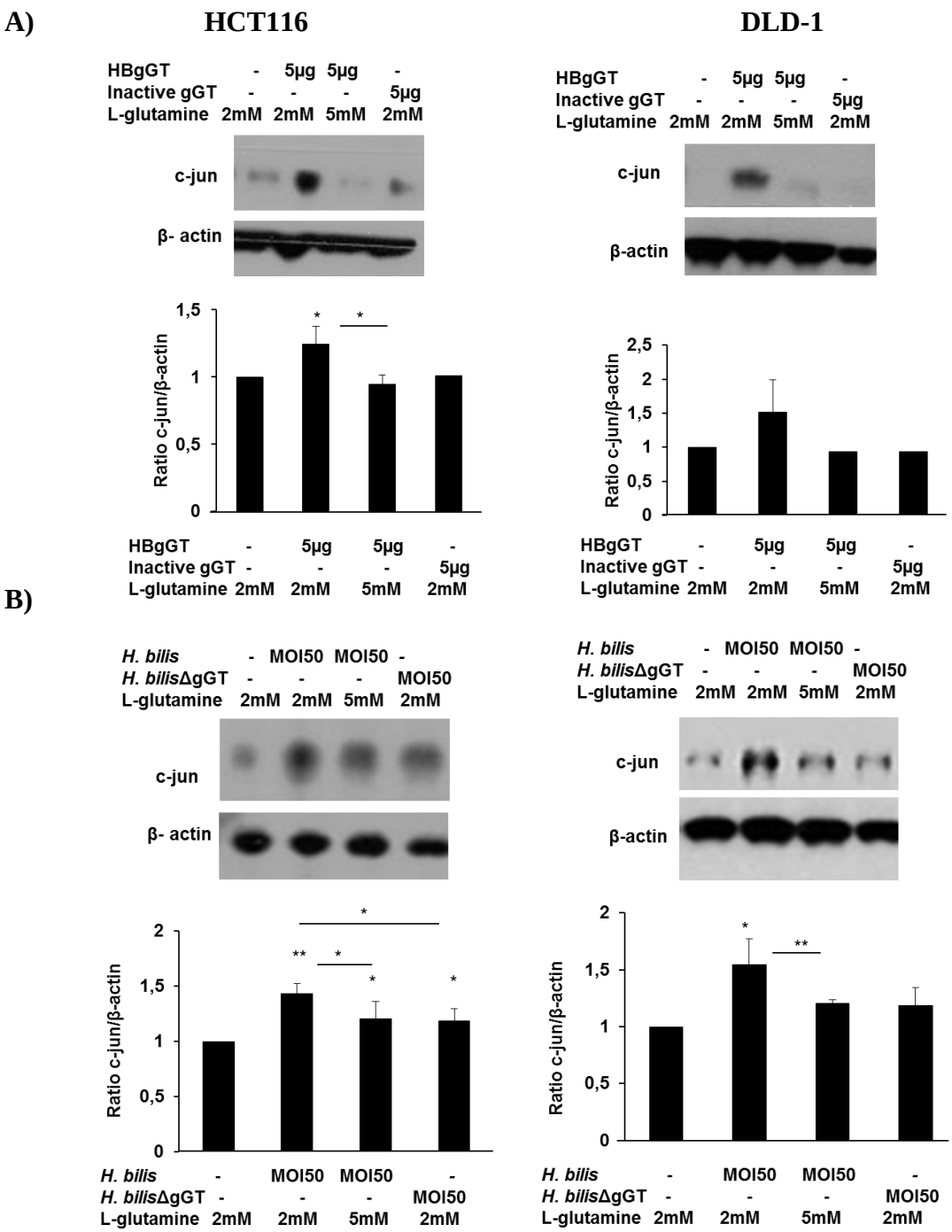


Figure 60: c-jun levels after glutamine supplementation of HBgGT treated epithelial cells and *H. bilis* co-cultures.

A) c-jun blots (Top) and quantification (bottom) of band density ratio of c-jun to β-actin normalized to the untreated of HCT116 and DLD-1 cells after glutamine supplementation of the HBgGT pre-incubated medium (10 hour treatment). Results expressed as mean of three (HCT116) and two (DLD-1) independent experiments. B) c-jun blots (Top) and quantification (bottom) of band density ratio of c-jun to β-actin normalized to the untreated of *H. bilis* co-cultured HCT116 and DLD-1 cells after glutamine supplementation of the medium (10 hour treatment). Results expressed as mean of three independent experiments. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Altogether, this data suggests that glutamine depletion by gGT is an important part of its AP-1 activation mechanism.

CREB transcriptional activity could also be stimulated upon glutamine starvation in HCT116 and LS174T cells which showed a significantly increased transcriptional activity in HCT116 and LS174T cells. HBgGT preincubated medium also significantly activated the transcriptional activity of CREB as that induced by the recombinant protein at 24 hours in HCT116 and LS174T cells. Again supplementation of the medium with glutamine abolished this activation by the enzyme (Figure 61A). Secondly, to determine whether this held true in an infection setup with the live bacterium, cells co-cultured with *H. bilis* were also simultaneously supplemented with external L- glutamine. The supplementation again was not able to completely relieve the activation of CREB transcriptional activity, although a significant decrease in *H. bilis* infected and glutamine supplemented HCT116 cells and LS174T cells was observed (Figure 61B).

For confirmation of activation of the CREB pathway, CREB phosphorylation was evaluated by western blot with protein lysates from cells treated for 10 hours. A decrease in phospho-CREB to the basal levels could be observed after supplementation of the cells with glutamine (4mM) in HBgGT preincubated medium treated cells (Figure 62A). A decrease in phosphorylation of CREB could also be observed in glutamine (5mM) supplemented HCT116 cells at the time of infection (Figure 62B).

Decreased activation of the CREB pathway by gGT, in both recombinant protein treated and *H. bilis* infected cells after glutamine supplementation emphasize the role of glutamine deprivation as a mechanism of gGT mediated activation of this pathway.

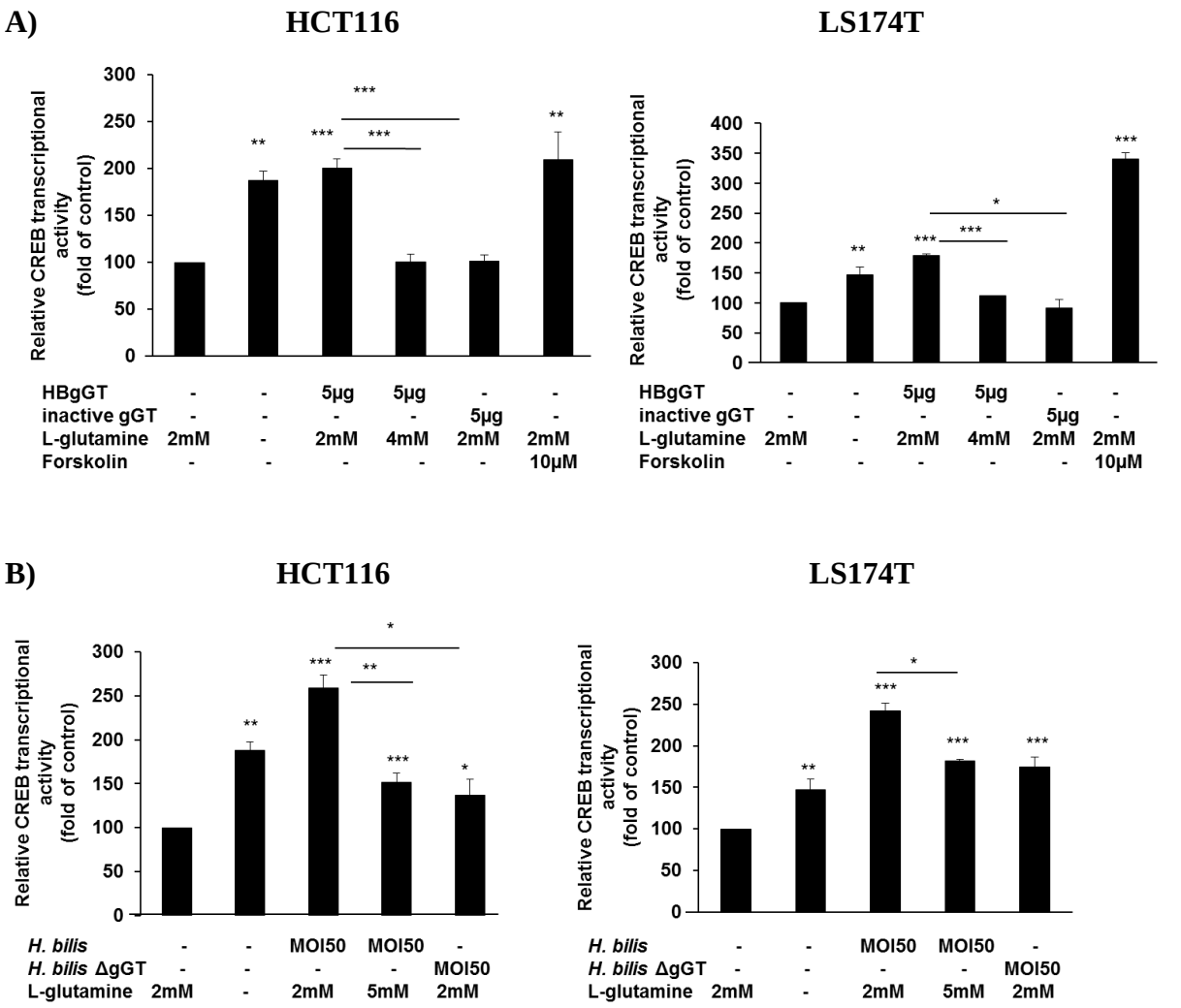


Figure 61: Transcriptional activity of CREB after Glutamine supplementation of HBgGT treated epithelial cells and *H. bilis* co-cultures.

Transcriptional activation of CREB after Glutamine supplementation at 24 hours A) HCT116 and LS174T cells were used in a transient transfection and treated with a HBgGT pre-incubated medium (5µg of protein added per ml of cell culture medium). L-glutamine was added to the cells (final amount in the medium indicated). Forskolin (10µM) was used to induce the transcriptional activation of CREB. B) HCT116 and LS174T cells were used in a transient transfection and co-cultured with *H. bilis* MOI 50. L-Glutamine free medium was used to starve the cells of glutamine. Bars represent mean of relative luciferase values to renilla of 3 independent experiments normalized to the untreated control.

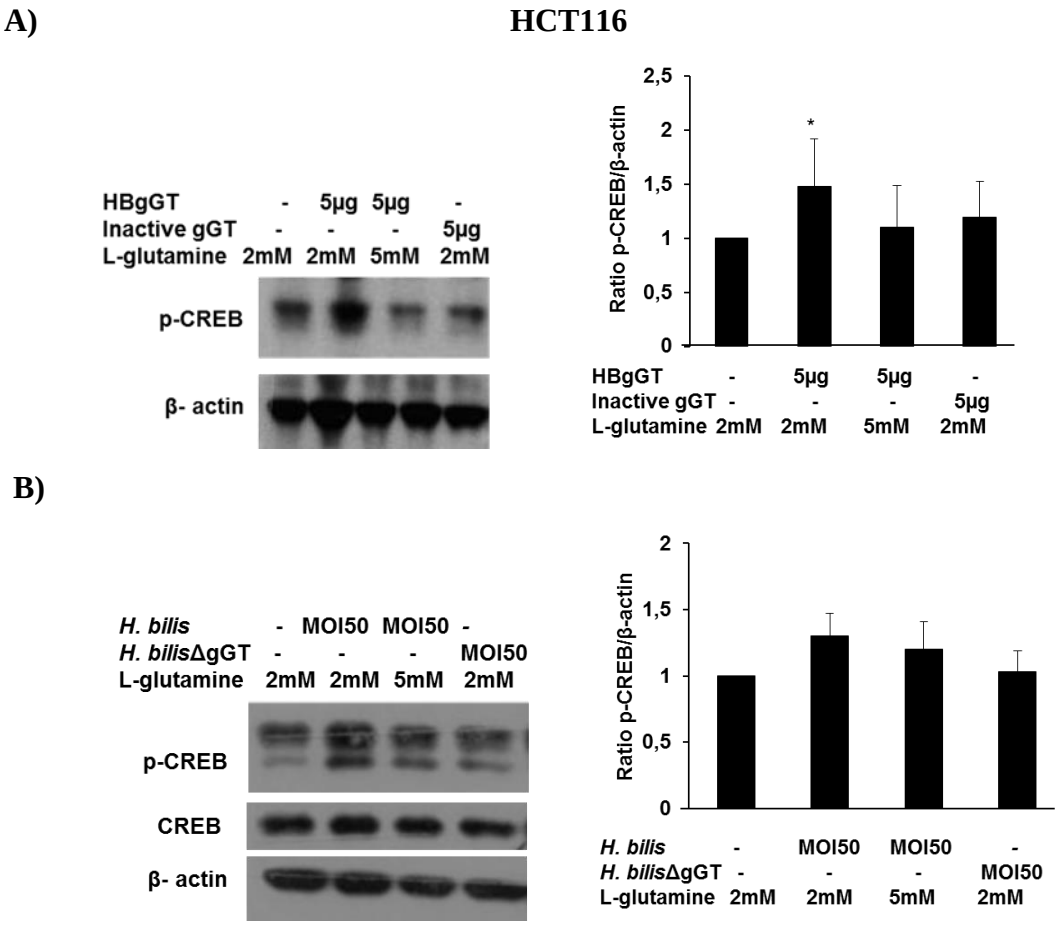


Figure 62: CREB phosphorylation after glutamine supplementation of HBgGT treated epithelial cells and *H. bilis* co-cultures.

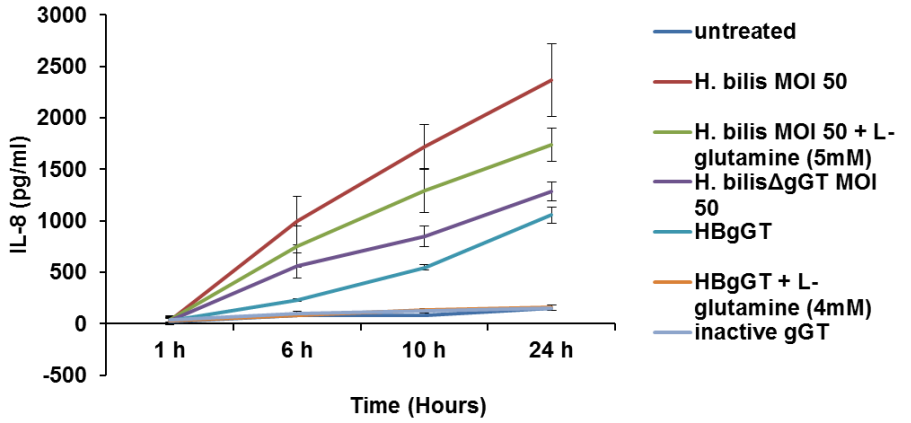
A) p-CREB blots (left panel) and quantification (right panel) band density ratio of p-CREB to β-actin normalized to the untreated of HCT116 cells exposed to HBgGT preincubated for 10 hours B) p-CREB blots (left panel) and quantification (right panel) band density ratio of p-CREB to β-actin normalized to the untreated of *H. bilis* co-cultured HCT116 cells after glutamine supplementation of the medium (10 hours of treatment). Results expressed as mean of three (HBgGT) and two (*H. bilis* co-culture) independent experiments. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Hence replenishment of the exogenous glutamine sources of the cells was able to rescue the HBgGT modulated induction of the stress regulated transcription factors. The incomplete rescue by glutamine supplementation in the co-culture with live bacterial might be due to continuous exhaustion of the glutamine resources by the active enzyme produced by *H. bilis*.

Whether glutamine supplementation was also able to relieve the downstream production of the pro-inflammatory cytokine IL-8 was next investigated. The IL-8 production after HBgGT exposure could be completely reversed with glutamine supplementation of the pre-incubated medium (Figure 63). In an *in vitro* infection setup, glutamine supplementation of the cells was also able to decrease IL-8 production after *H. bilis* co-culture significantly with a decrease from 2365 pg/ml to 1737 pg/ml of IL-8 release in HCT116 cells (Figure 63). The gGT deficient mutant *H. bilis* was also able to induce IL-8 production (1287 pg/ml), but these values were much lower than those observed in case of the gGT proficient bacterium.

This data suggests that Il-8 secretion induced by gGT is partly due to a glutamine depletion mechanism in cells treated with the recombinant protein or infected by *H. bilis*, thereby promoting inflammation.

A)



B)

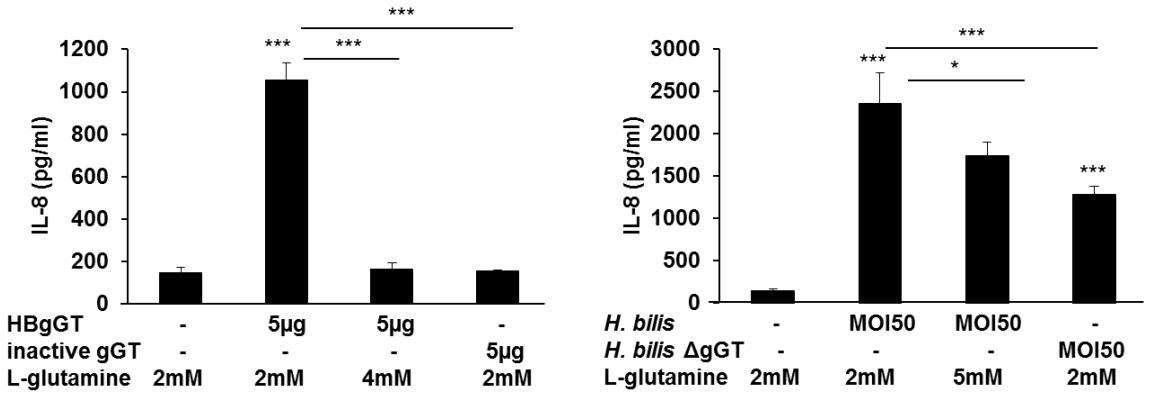


Figure 63: IL-8 production after glutamine supplementation of HBgGT treated and *H. bilis* infected cells.
A) Time course of IL-8 production in presence of HBgGT treatment and *H. bilis* co-culture in HCT116 cells. B) IL-8 levels after 24 hour of treatment with HBgGT pre incubated medium (5µg of protein added per ml of cell culture medium) and glutamine supplemented HBgGT pre incubated medium (left). Glutamine supplementation reduces IL-8 production in response to *H. bilis* for 24 hours (right). L-glutamine was added to the cells (final amount in the medium indicated). Bars represent mean values of duplicates from three independent experiments. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

10.2 gGT upregulates ROS production from epithelial cells

Glutamine and glutathione depletion from the cells exposes the cells to cell damage by reactive oxygen species by impairing the redox system of the host cell. ROS are potent inducers of many host stress signalling cascades. Host cell damage by bacterial gGT in *H. pylori* and *H. suis* infected epithelial cells, induced by ROS in presence of glutathione has been observed (Flahou, Haesebrouck et al. 2011).

Hence ROS production could be an important aspect of HBgGT modulated host cellular responses. To determine superoxide production from cells, HCT116 and DLD-1 were treated with HBgGT as well as co-cultured with *H. bilis* wild type and gGT knock out bacterium. After 10 hours the cells were washed with DMEM and cultured for an hour with a NBT (Nitroblue tetrazolium chloride). Superoxide in the cells reacted with NBT to form dark blue formazan crytals which could be visualized and quantified.

Enhanced formazan crytal formation could be seen in presence of gGT with bacterial co-culture as well as when the cells were treated with the recombinant protein (Figure 64). Quantification of formazan crystal formation in the lysates of treated cells in presence of HBgGT showed a significant increase as compared to the untreated control or when the gGT was heat inactivated (Figure 65). These results show increased ROS production from cells exposed to HBgGT.

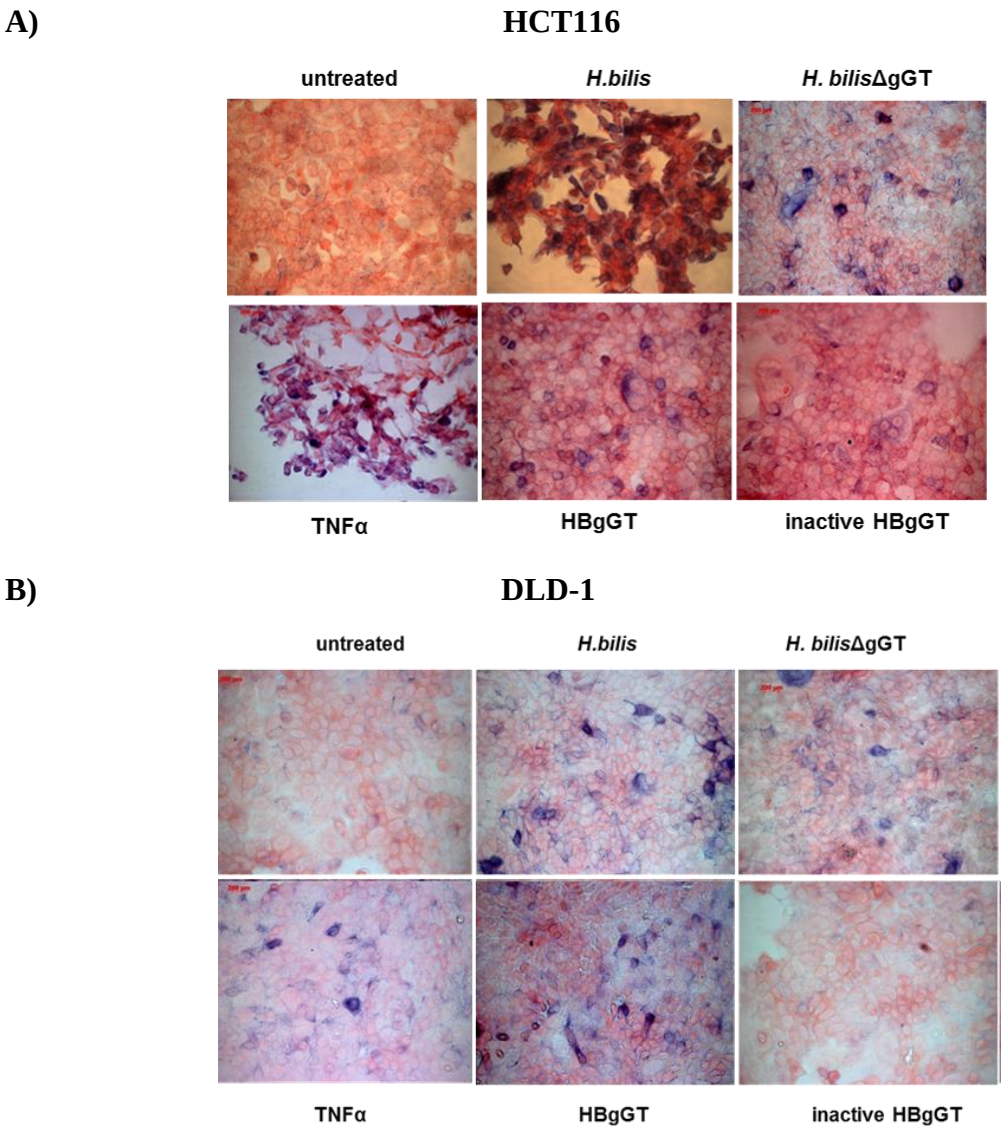


Figure 64: Formazan crystal formation measure by visualization in HBgGT treated and *H. bilis* infected cells.

Superoxide production from cells was measured by visualization of formazan crystal formation in an NBT assay. A) HCT116 and B) DLD-1 cells after incubation with NBT in response to *H. bilis*, gGT deficient and proficient at MOI 50, HBgGT (5µg/ml) and the inactive gGT for 10 hours. Formazan crystals stained dark blue and cells counterstained with safranin (orange). TNFα was used to induce superoxide production as a positive control. Representative figure of three independent experiments shown.

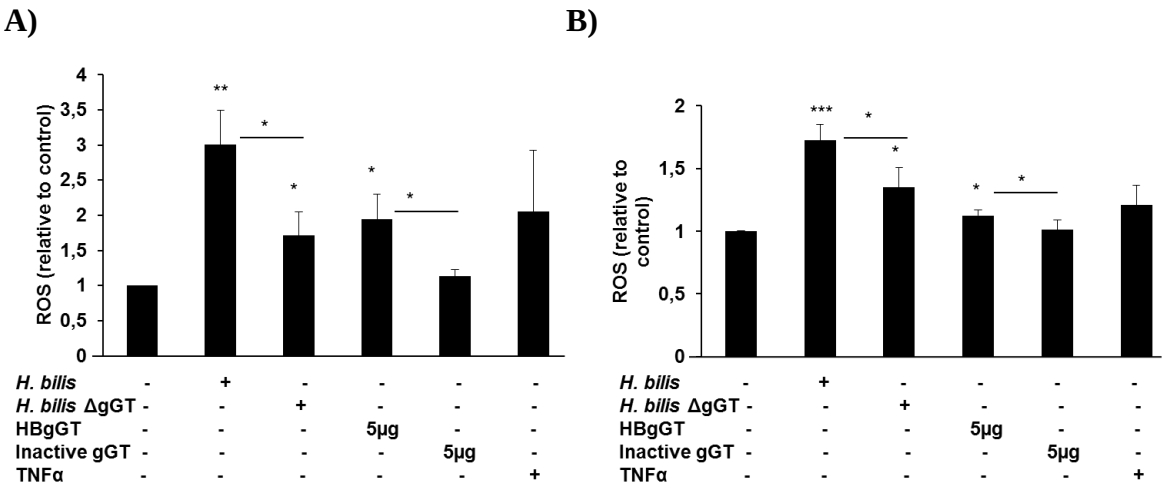


Figure 65: NBT assay quantification of formazan crysal formation in HBgGT treated and *H. bilis* infected cells.

Formation of formazan crystals after incubation with NBT in response to *H. bilis* gGT deficient and proficient co-culture at MOI 50 in A) HCT116 B) DLD-1 cells. Cells were also treated with HBgGT (5µg/ml) and the inactive gGT for 10 hours. Quantification of crystal formation in treated cell lysates by measuring OD at 650 nm. TNFα was used to induce superoxide production. Bars represent mean values of three independent experiments normalized to the untreated control. *=p value of <0.05, **=p value <0.005, ***=p value <0.0005.

Chapter 4: Discussion

Helicobacter pylori infects about half of the world population. All of the infected individuals develop an asymptomatic gastritis but approximately 20% develop complications like gastritis, ulcer and about 1-2% of this progress to gastric carcinoma due to the chronic infection. Other non-*pylori Helicobacter species*, like *H. bilis* have been associated with similar chronic illnesses. In addition, *H. bilis* infection has been associated with incidences of chronic diarrhea and cholangiocarcinoma in humans (Boonyanugomol, Chomvarin et al. 2012); as well as IBD and colitis in mice (Shomer, Dangler et al. 1997; Maggio-Price, Treuting et al. 2006; Liu, Ramer-Tait et al. 2011). Since all of these diseases result from chronic inflammation and proliferative changes in the epithelia it is important to decipher the bacterial factors responsible for inducing such changes in the host cells. This can also form the basis for future approaches of targeted therapy and vaccine development. Many virulence factors of *H. pylori* have been described. CagPAI, CagA, urease and VacA are some of the most investigated virulence factors described in this species. On the other hand, only a few virulence factors have been described for *H. bilis*. Cytolethal distending cytotoxin and urease are among the few virulence factors commonly found in the *Helicobacter* genus.

4.1 gamma-Glutamyl transpeptidase as an important virulence determinant in *Helicobacter* infections

In 2003 gamma-glutamyl transpeptidase enzyme was first described to be a new apoptosis inducing protein from *H. pylori* supernatants (Shibayama, Kamachi et al. 2003). Since then it had been described to be a proliferation limiting factor for T lymphocytes (Gerhard, Schmees et al. 2005; Schmees, Prinz et al. 2007). Furthermore, HPgGT was described to be essential for colonization of the gastric mucosa of mice by *H. pylori* (Chevalier, Thiberge et al. 1999). Both these observations point to a significant immunomodulatory role for the bacterial enzyme. Clinical importance for gGT function was recently demonstrated in a study where a significant correlation between gGT activity and peptic ulcer disease was reported (Gong, Ling et al. 2010). The importance of gGT can also be deduced from the fact that this enzyme is not only important for *H. pylori* but seems to be conserved in all GHS and is present in many EHS. For instance *H. bilis*, an EHS, possesses two putative gGT sequences, one of which was found to respresent the functional active enzyme (Rossi, Bolz et al. 2012). gGT

from *H. bilis,* which had not been previously characterized was not only analysed for the first time in relation to cell growth but also host transcriptional alterations and its mechanism of action were also explored in this study.

4.2 *Helicobacter* gGT reduces host cell growth

The mucosal epithelial surface presents a first line of defence against invading bacteria. *H. pylori* colonizes the stomach where it eventually attaches to and in some cases invades the epithelial cells. *H. bilis* on the other hand has the ability to colonize the bile duct, liver tissue, colon and caecum. Although many bacterial factors contribute to the establishment of infection in both bacteria, gGT is a common bacterial factor presented by both. Hence, it was interesting to analyze how this bacterial enzyme might influence the epithelial cells. Gastric cancer epithelial cells as well as other cell lines were tested for viability after exposure to HPgGT. Colon cancer epithelial cell lines on the other hand were tested for proliferative changes after HBgGT treatment. Gastric epithelial cells exhibited the most prominent decrease in cell viability after exposure to HPgGT and the colon epithelial cells reacted in a similar manner to HBgGT. The keratinocytes on the other hand were almost completely insensitive to the gGT treatment. Both gGTs were able to inhibit cell growth to a similar extent in the epithelial cell lines, supporting the hypothesis that the *Helicobacter* gGT is not only physically but also functionally conserved among the genus. This is the first report describing such a conserved function of *Helicobacter* gGTs. The decrease in cell growth observed in the gastric epithelial cells is also in accordance with several reports which have described gGT from *H. pylori* and *H. suis* to be responsible for reduced cell viability in epithelial cells (Kim, Lee et al. 2007; Fehri, Koch et al. 2010; Flahou, Haesebrouck et al. 2011; Boonyanugomol, Chomvarin et al. 2012). This reduction in cell viability may result in the corrosion of the epithelial layer by the bacterium resulting in the development of peptic ulcer disease. Recently, a significant correlation between the development of peptic ulcer disease and gGT activity in infected patients was recently described. Thus, clinical importance of HPgGT was highlighted by the fact that a significantly higher gGT activity was observed in *H pylori* isolates obtained from patients with peptic ulcer disease than isolates from patients with nonulcer dyspepsia (Gong, Ling et al. 2010). Although a majority of the current literature available on gGT effects on epithelial cells describe a loss of cell viability upon gGT exposure, most of these reports fail to describe a comprehensive mechanism of

gGT action. In order to investigate whether this reduction in cellular growth of gGT exposed cells was due to apotosis, cells were tested for apoptosis via FACS sorting of annexinV and PI staining. However, no significant induction of apoptosis for both HPgGT and HBgGT could be observed in this assay. Furthermore, no changes were detected in caspase 3/7 levels in gGT treated cells. The fact that both HPgGT and HBgGT were not able to induce caspase 3/7, which is central to induction of apoptosis, indicates that the cellular growth reduction induced by gGT is independent of apoptosis. Both the FACS analysis of apoptotic cell populations and caspase 3/7 assay rules out apoptosis as a likely mechanism for gGT modulated reduction in cell growth.These findings were in contrast to those observed by Kim et al. who pointed to a mitochondrial pathway induced cellular death after exposure of cells to HPgGT (Kim, Lee et al. 2007). These differences in observations could be due to the differences in the experimental setup used in this study. The serum starvation of cells employed in the afore mentioned study might increase cellular stress making the cells exceedingly sensitive to gGT, which in turn coerce the cells to cell death rather than inducing a cell cycle arrest as was observed in the experiments presented here. The G1 cycle cell arrest observed in epithelial cells exposed to HPgGT was later published by Kim et al. where they indicated that HPgGT was able to arrest the cells by down-regulation of cyclin E, cyclin A, Cdk 4, and Cdk 6, and the up-regulation of the cyclin-dependent kinase (Cdk) inhibitors p27 and p21. However, the signalling cascades leading to activation of these cell cycle mediators were not described (Kim, Lee et al. 2007).

4.3 gGT alters host transcriptional activity

The second aim of this study was to observe which sort of host cellular responses the *Helicobacter* gGT triggers in epithelial cells. For this purpose, the analysis of several central transcription factors often found upregulated in *Helicobacter* infections was performed. Results showed an increased transcriptional activity of NF-κB, AP-1, and CREB by HPgGT as well as HBgGT. This indicates that the conservation of function of both the gGTs is not only limited to reduction of cell growth but they also behave in a similar manner regarding the alteration of host epithelial cell signalling. In addition, HPgGT was also able to induce increased transcriptional activity of NFAT. All these transcription factors are also involved in regulating the cell turnover. However the late onset of activation of the transcriptional activities of these transcription factors in response to gGT makes their involvement unlikely

in induction of cell cycle arrest observed in these cells. Next, to observe whether these cellular changes depended on gGT during infection, it was important to study these effects in relation to the whole bacterium, where other factors are also involved. The role of gGT in a co-culture model was analysed, whereby gastric epithelial cells and colon epithelial cells were cultured with *H. pylori* and *H. bilis* gGT proficient and deletion mutants respectively. Unfortunately, role of HPgGT in the activation of these transcription factors was not clear as co-culture of gastric epithelial cells with *H. pylori* gGT proficient and a deletion mutant activated the transcriptional activity to a similar extent This indicates that other virulence factors are more potent in induction of these transcription factors in *H. pylori* infection. Indeed, several virulence factors are involved in *H. pylori* mediated activation of these pathways. CagA, peptidoglycan, VacA as well as urease are potent inducers of NF-κB (Lu, Wu et al. 2005; Choi, Park et al. 2007; Hisatsune, Nakayama et al. 2008; Backert and Naumann 2010). AP-1 activators in the *H. pylori* arsenal include peptidoglycan injected by the cagPAI into the cells, since the activation is dependent on an intact injection pillus (Meyer-ter-Vehn, Covacci et al. 2000; Ding, Olekhnovich et al. 2008; Allison, Kufer et al. 2009; Backert and Naumann 2010; Cho, Lim et al. 2010). VacA is so far the only virulence factor that has been described to induce CREB activity (Juttner, Cramer et al. 2003; Chang, Wu et al. 2005; Kudo, Lu et al. 2007; Hisatsune, Nakayama et al. 2008). A functional antagonism also exists between cagA and VacA in NFAT activation in gastric epithelial cells, where cagA was described to be the agonist and VacA antagonized NFAT activation induced by cagA (Yokoyama, Higashi et al. 2005). Other functionally antagonistic relationships between the virulence factors may exist. These virulence factors may override the gGT mediated activation in *H. pylori* infection and may compensate for its loss in the gGT deletion mutant. However, gGTs' role may not be disregarded completely as it still may be an important inducer of these pathways in concert with other virulence factors at a certain phase of the infection. Further studies with multiple deletion mutants may shed some light on this interaction.

In *H. bilis* co-cultures with colon cancer cell lines the effect of gGT was much more clearer. The bacterium was able to significantly increase the transcriptional activity of NF-κB, AP-1 and CREB in these cells. Furthermore, the activation of these pathways was confirmed by observation of increased nuclear translocation of p65 and phosphorylation of IκBα in case of NF-κB, increased levels of c-jun for AP-1 and phosphorylation of CREB for the CREB pathway activation. This activation was significantly enhanced when the bacterium was gGT proficient. HBgGT hence plays a major role in the activation of these pathways in *H. bilis*

infection and the resultant pathological effects that might be observed in response to this. Residual activation of the pathways, in host cells when infected with the gGT knockout bacterium also shows that other virulence factors like the cytolethal distending toxin, peptidoglycan and LPS may be additionally responsible for some of these effects. However, the specific involvement of gGT in the activation of these signalling cascades was confirmed by complementation of the gGT knockout bacterium by addition of the recombinant HBgGT protein that was able to enhance the transcriptional activity and activation of all these pathways. However, the levels still did not correspond to those observed in the wild type bacterium. The fact that the recombinant HBgGT could not completely compensate for loss of gGT production in *H. bilis* ΔgGT strain could be due to the continuous replenishment of gGT sources in the gGT proficient bacterium. HBgGT was only added once at commence of the co-culture in contrast to the wildtype bacterium where the continuous secretion of the enzyme by the bacterium may lead to an overall higher cumulative gGT activity. The results described in the present study are in accordance with earlier reports where NF-κB and CRE activation after *H. bilis* infection have also been observed in human bile duct cells (Takayama, Takahashi et al. 2010). However, this is the first report of activation of these pathways by *H. bilis* in colon epithelial cells. Since *H. bilis* is able to infect both bile duct and colon of the host and cause subsequent inflammation in both tissues, the activation of these pathways by gGT may be central to this effect in both tissues.

Activation of the NF-κB, AP-1 and CREB pathways is critical in the induction of the pro-inflammatory chemokine IL-8 in epithelial cells during *H. pylori* infection (Reviewed in Backert and Naumann 2010). IL-8 is the single most upregulated chemokine in *H. pylori* infection in epithelial cells (Eftang, Esbensen et al. 2012). IL-8 is often upregulated in oxidant stress and recruits inflammatory cells leading to a further increase in oxidant stress mediators making it an important parameter in localized inflammation. IL-8 was also found to be highly expressed in pateints with ucerative colitis (Gologan, Iacob et al. 2012), but so far there are no reports on IL-8 induction by *H. bilis*. Increased inflammation as a result of the activation of these stress induced pathways may be a consequence in gGT proficient *H. bilis* infections. Indeed, co-culture of cells with *H. bilis* as well as the recombinant HBgGT protein was able to induce IL-8 secretion from HCT116 and DLD-1 cells. A significant reduction in IL-8 secretion was observed when the gGT deficient bacterium was used for the co-culture. Thus, gGT seems to be an important factor in determining the intensity of the pro-inflammatory response generated in intestinal epithelial cells via IL-8 secretion in *H. bilis* infection.

Subsequently such an enhanced inflammatory response by the host cells in response to gGT proficient *H. bilis* bacterium may be responsible for increased virulence of the bacterium.This increased virulence could be potentially decreased by blocking gGT activity and could be potentially employed for targeted therapy.

4.4 Mechanism of gGT mediated alterations in host cell signaling

Since gGT has been established as an important virulence factor in the *Helicobacter* arsenal, the next question arises as to how a bacterial enzyme may trigger such a variety of host cell responses. The answer may lie in its intrinsic enzymatic activity. Mechanisms behind gGT mediated effects have not been explored but some reports have discussed the possible mechanisms behind the gGT induced reduction in cell viability (Shibayama, Wachino et al. 2007; Flahou, Haesebrouck et al. 2011). According to some of these reports the mechanism of action of gGT may on the one hand be due to the continuos hydrolysis of beneficial glutamine and glutathione from the host cells and on the other hand may be due to the reactive oxygen species produced in the process.

4.4.1 gGT-modulated transcriptional dysregulation in host cells is partly due to glutamine deprivation

As previously discussed, glutamine and glutathione serve as sources of glutamate for *Helicobacter sp. (H. pylori, H. bilis, H. suis* and *H. felis)* however, the bacterium is unable to absorb them in this form. In mammalian cells glutathione is an ubiquitous substance present in the cytosol in mM quantities (Kaplowitz, Aw et al. 1985). Glutamine is also essential for maintaining homeostasis and normal integrity of the intestinal mucosa (Plauth, Raible et al. 1999; Boelens, Nijveldt et al. 2001)). Glutamine and glutathione function as an anti-oxidant by detoxification of oxidizing substances (Kaplowitz, Aw et al. 1985; Dominici, Paolicchi et al. 2003; Njalsson and Norgren 2005). Depletion of host cellular glutamine and glutathione resources leads to an impaired redox balance in the host cells, triggering a cascade of oxidative stress response elements. HPgGT is able to hydrolyse glutamine and glutathione. Glutamine and glutathione depletion leaves the cells exposed to free radicals. It has been reported that *H. pylori* infected gastric epithelial cells have significantly lower glutathione levels than uninfected cells (Jung, Lee et al. 2001). The need for additional glutathione synthesis by the cell and consumption of extracellular glutamine would also impose excessive energy requirements for the host cell, reducing the growth and viability of the cell (Lord

1999). Moreover, glutamine depletion has also been reported to influence the NF-κB and AP-1 pathways in intestinal epithelial cells. It was shown that extracellular glutamine supplementation resulted in nuclear degradation of p65 (Lesueur, Bole-Feysot et al. 2012). While, glutamine deprivation of cells was also shown to activate AP-1 (Marjon, Bobrovnikova-Marjon et al. 2004). Moreover, glutamine supplementation markedly improved the outcome of experimentally induced colitis in rats by attenuating cytokine induced inducible nitric oxide production and nuclear translocation of nuclear factor-κB p65 subunit (Xue, Sufit et al. 2011). These evidences point to a crucial protective role of glutamine against activation of the NF-κB and AP-1 pathways in intestinal epithelial cells. Since gGT is able to activate both these pathways; its role in glutamine hydrolysis makes glutamine depletion by this enzyme a highly likely mechanism of activation of these pathways. In this study glutamine supplementation experiments were performed for HBgGT and the live bacterium. The focus in this case was HBgGT, the reasons were two fold; First, HPgGT had been previously studied in this context and secondly, because HPgGT effects in the bacterial model could not be dissected from those of the other *H. pylori* virulence factors. Results of this study demonstrated that glutamine supplementation showed a compensatory effect by decreasing the gGT induced transcriptional activation of NF-κB and AP-1 as well as CREB. Glutamine supplementation in case of the recombinant protein could compensate for the depletion. In the case of co-culture of cells with the live bacterium this compensation was not complete, although a significant decrease in the transcriptional activities of these transcription factors was observed. This could be due to the continuous depletion of glutamine by the still active enzyme secreted by the live bacterium as opposed to the glutamine depleted pre-incubated medium where the gGT is heat inactivated after a 24 hour incubation step. These findings support the theory that gGT triggers host transcriptional activation via a glutamine depletion mechanism.

Some evidence pointing to glutamine as an important preemting factor in determining the intensity of host inflammatory responses induced via activation of the mentioned pathways exists. Bobrovnikova-Marjon and colleagues found increased NF-κB (p65/p50) and AP-1 (Fra-1/c-Jun, JunD) DNA-binding activities in response to glutamine deprivation, leading to an increased IL-8 expression. It was also observed that increased inflammation in intestinal epithelium could be caused by glutamine depletion *in vivo* (Becker, Wu et al. 2000; Coeffier, Miralles-Barrachina et al. 2001; Coeffier, Marion et al. 2002) and glutamine supplementation reduced this intestinal inflammation (Li, Liboni et al. 2004). *In vitro* deprivation of Caco-2

cells from glutamine resulted in increased nuclear p65 as well as increased ubiquitination of I-κB in H4 cells, resulting in increased IL-8 production in response to LPS (Plauth, Raible et al. 1999). In accordance with these observations, glutamine supplementation in *H. bilis* infected cells was also able to decrease the IL-8 production in HCT116 cells in response to the infection. Replenishing the depleted glutamine, in a pre incubated medium with the HBgGT, by supplementation of the medium with additional glutamine, rescued the cells from gGT-induced IL-8 production. Clinical studies have proven the efficacy of glutamine administration in patients with trauma, sepsis, and major surgery (Boelens, Nijveldt et al. 2001). Glutamine administration also conferred a protective effect in bacterial toxin-induced apoptosis in both intestinal cell lines and rabbit ileal loops (Carneiro, Fujii et al. 2006), as well as ammonia induced gastric epithelial cell death (Nakamura and Hagen 2002). Taking these into account, glutamine therapy may indeed relieve some of the *H. bilis* induced IBD and colitis symptoms. Established murine models for *H. bilis* infection related IBD and colitis could be employed for such an investigation. Since, glutamine depletion plays a central role in gGT modulated inflammation, glutamine administered via diet to diseased mice could be monitored for relief of symptoms, differential pathology and inflammatory response.

As mentioned previously, glutamine is important anti-oxidant and its depletion leads to a redox imbalance in the host cells. It can therefore be speculated that this impaired redox balance may lead to an increase in the accumulated reactive oxygen species in affected host cells. Therefore, ROS production may be another mechanism by which gGT modulates host cell signaling.

4.4.2 gGT induced pathogenesis via generation of free radicals

Reactive oxygen species (ROS) and reactive nitrogen species are mediators of cell signaling in epithelial cells. In the presence of molecular oxygen and iron or copper ions, a number of antioxidants paradoxically generate reactive oxygen species (ROS) leading to free radical damage of nucleic acids and oxidative modification of lipids and proteins. Human γ-glutamyltransferase (HsgGT) in the presence of glutathione (GSH) and transferrin, as an iron source was able to generate ROS (Drozdz, Parmentier et al. 1998). Bacterial gGT (*H. pylori* and *H. suis*) also induced ROS in epithelial cells in presence of glutathione leading to host epithelial cell death (Flahou, Haesebrouck et al. 2011).

Increased levels of superoxide production from *H. bilis* infected cells were observed in this study, which depended on the presence of gGT. This increased superoxide production by the

cells in response to gGT may be another mechanism by which HBgGT is able to affect cell growth and induce NF-κB, AP-1 and CREB, all of which are also known to be triggered in response to oxidative stress.

It was shown that polyamines e.g polyamine spermine oxidation by the enzyme spermine oxidase (SMO) leads to the generation of H_2O_2 from epithelial cells in response to *H. pylori* infection. Free radical metabolites of H_2O_2 like OH^- can be highly toxic to the cells by damaging macromolecular structures like DNA, lipids and proteins leading to cell death (Rahman and MacNee 1999; Rahman and MacNee 2000; Xue, Sufit et al. 2011). Increased H_2O_2 production by gastric epithelial cells in response to HPgGT was also reported by Gong et al (Gong, Ling et al. 2010). H_2O_2 in turn leads to generation of free oxidative radicals like the superoxide. Increased superoxide production may in turn result in oxidative DNA damage in the host cells, decreasing host cell viability.

Molecular mechanisms of ROS action have only partly been deciphered yet. It is hypothesized that oxidation of disulfide groups in redox sensitive proteins with highly conserved cysteine residues may cause structural changes leading to the exposure of active sites and subsequent activation. Such molecular targets include transcription factors (NF-κB and AP-1), signaling molecules such as Ras/Rac or JNK, protein tyrosine phosphatases and p21 (Rahman and MacNee 2000). Felty and Roy observed that stimulation of redox sensor kinase A-Raf, AKT or PKC, activates transcription factors NF-kB, CREB, or AP-1 via the MEK/ERK pathway (Felty and Roy 2005). ROS induced activation of NF-kB, AP-1 and CREB may in turn play a major role in inflammation. It has been shown that oxidative stress induced by H_2O_2 and TNF-α increases the activation of AP-1 and NF-κB, which regulate chromatin remodeling leading to IL-8 expression (MacNee and Rahman 2001). HBgGT-induced superoxide production and increased IL-8 production in HCT116 and DLD-1 cells points to the fact that HBgGT might be an important factor in the development of inflammatory disorders in *H. bilis* infections. Having said that, it will be important to extend these observations to a *H. bilis* infection animal model to validate these findings.

Deprivation of beneficial glutamine and glutathione, and possibly production of toxic ammonia, accounts for one of the mechanisms whereby HPgGT damages the gastric cells (Shibayama, Kamachi et al. 2003): The results presented here suggest a similar reduction in cell growth by HBgGT via increased ROS production from the cells affecting cell growth, eventually leading to loss of viability. Model of gGT induced host cell response modulation is summarized in figure 66.

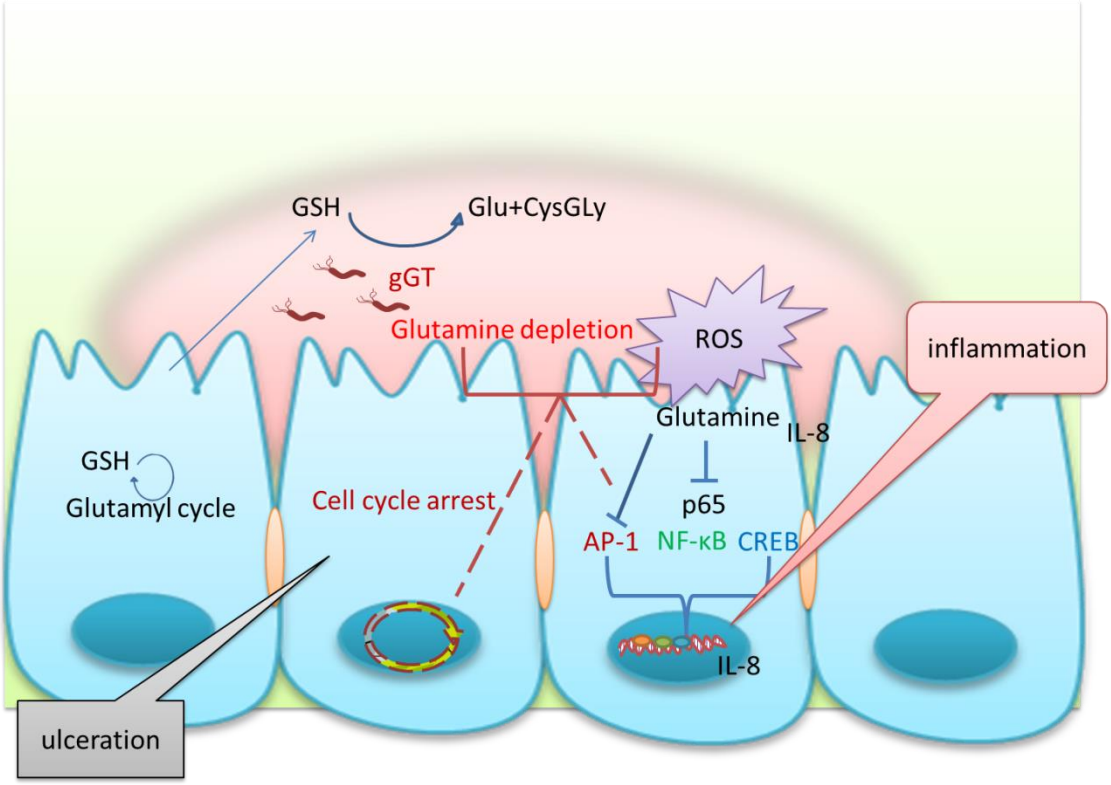


Figure 66: Summary of gGT modulated host cell responses.
Helicobacter gGT modulated host cell response is glutamine and glutathione degradation dependent with subsequent generation of free radicals inducing several oxidative stress response cascades in host cells and changes in cell growth.

4.1 Conclusions

An apoptosis independent suppression of cell proliferation by both *H. bilis* and *H. pylori* gGTs was observed indicating a conserved gGT function in these bacteria.

The recombinant HPgGT and HBgGT in epithelial cells activated the CREB, AP-1 and NFkB pathways. *H. bilis* co-culture with colon epithelial cells also led to activation of these pathways. The activation of NF-κB, AP-1and CREB by the bacterium was largely dependent on gGT as the gGT deficient bacterium exhibited decreased ability to activate the pathways.

HBgGT mediated host transcriptonal changes are mainly imposed due to oxidative stress caused by utilization of the extracellular glutamine sources by gGT and enhanced ROS

production as either by products of the glutamine metabolism or depletion of the antioxidant glutathione from the cells.

The concomitant activation of host oxidative stress response elements, production of IL-8 pro-inflammatory chemokine as well as reduction in host cell viability by bacterial gGT may be the contributing factors in *H. pylori* and *H. bilis* infection induced ulceration and inflammation.

4.2 Limitations of the study and Future prospects

The results of this study point to a significant role of *Helicobacter* gGT in induction of inflammation in epithelial cells. However, the conclusions that can be drawn from the data are limited in the fact that no *in vivo* experimental approaches were used. *In vivo* validation is an essential subsequent step that is required to prove the role of gGT in *H. bilis* induced inflammation and pathogenesis. Murine experimental models are now available for *H. bilis* induced IBD and colitis. Thus role of gGT in induction of inflammation and determining the pathological outcome of *H. bilis* infected intestinal mucosa could be investigated *in vivo*. Moreover, the protective effect of glutamine therapy in infected mice could be studied.

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Appendix

Publications from thesis

1. Rossi, M., C. Bolz, Revez J, Javed S, El-Najjar N, et al. (2012). "Evidence for conserved function of gamma-glutamyltranspeptidase in Helicobacter genus." PLoS One 7(2): e30543.
2. Javed S, Mejias-luque R, Behnam Kalali, Christian Bolz, Gerhard M. “Helicobacter bilis gamma glutamyl transpeptidase enhances inflammatory stress response in infected cells via oxidative stress.” Submitted for publication.

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