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DISSERTATION

Human beta defensin 3 induction in gastric epithelial cells under *Helicobacter pylori* infection and cytokine-dependent modulation

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Abbrevations

AMP	antimicrobial peptide
AP-1	activator protein 1
APC	antigen presenting cells
ATP	adenosine triphosphate
BHI	brain heart infusion
BSA	bovine serum albumin
CagA	cytotoxin associated gene product A
CD	cluster of differentiation
CFU	colony forming units
DC	dendritic cell
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
FoxP3	forkhead box P 3
GGT	gamma glutamyltransferase
GM-CSF	granulocyte macrophage colony stimulating factor
h p.i.	hours post infection
hBD	human beta defensin
hbEGF	heparin binding EGF-like growth factor
HGFR	hepatocyte growth factor receptor
hi	heat-inactivated
Hop	Helicobacter outer membrane porins
HP-NAP	neutrophil-activating protein from *H. pylori*
HRP	horseradish peroxidase
HtrA	high-temperature requirement A protein
IFN	interferon
IL	interleukin
kDa	kilodalton
LPS	lipopolysaccharide
MACS	magnetic-assisted cell sorting
MALT	mucosa-associated lymphatic tissue

MAPK	mitogen-activated protein kinases
MHC	mayor histocompatibility complex
MOI	multiplicity of infection
MWCO	molecular weight cut off
NF-κB	nuclear factor-kappa B
NI	not infected
NK	natural killer cells
NO	nitric oxide
NOD	nuclear oligomerization domain
OD	optical density
OipA	outer inflammatory protein A
OMV	outer membrane vesicle
PAI	pathogenicity island
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood monocytes
PRR	pattern recognition receptors
PVDF	polyvinylidene difluoride
RPM	rounds per minute
SD	standard deviation
SDS	sodium dodecyl sulfate
SN	supernatant
T4SS	type IV secretion system
TBS	tris-buffered saline
TGF	transforming growth factor
T_H	T-helper cells
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumor necrosis factor
T_{REG}	regulatory T-cells
VacA	vacuolating cytotoxin A
wt	wild type

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Abstract

Helicobacter pylori is a human gastric pathogen, chronically infecting about half of world's population causing the development of peptic ulcer disease and gastric cancer. Many facets of the initiated immune response following *H. pylori* infection remain elusive. In recent years it has been shown that epithelial cells are capable of actively counteracting *H. pylori* by the secretion of human beta defensin 3 (hBD-3). Yet, knowledge of inducing mechanisms and the regulation of hBD-3 in gastric epithelial cells remains fragmentary. Aims of the present study were to expand knowledge about the induction of hBD-3 in gastric epithelial cell lines following infection with *H. pylori*, to test other stimulants leading to hBD-3 expression and to examine the modulation of hBD-3 induction by the adaptive immune response. For this purpose various gastric cancer cell lines were tested for hBD-3 induction in combination with different bacterial preparations and/or addition of several recombinant cytokines. Furthermore, the influence of the adaptive immunity on hBD-3 expression of epithelial cells was examined by the stimulation of epithelial cells with supernatants of a co-cultivation system of activated dendritic cells (DC) with T-cells. Induction was monitored by western blot and ELISA. Here, it could be demonstrated that an effective induction of hBD-3 in gastric epithelial cells is dependent on bacterial viability and the level of induction is regulated according to the number of infecting bacteria. Stimulation of gastric epithelial cells with pro-inflammatory cytokines alone was shown to be insufficient in inducing hBD-3. Yet, when infection with viable bacteria was combined with Interferon-γ (IFN-γ), production of hBD-3 was strongly reinforced. In addition, this combinatorial treatment enhanced cell death. When testing the ability of the adaptive immunity to modulate hBD-3 induction in epithelial cells, it could be shown that supernatants of DC/T-cells activated via *E. coli* LPS induced hBD-3. In contrast to that *H. pylori*-activated DC/T-cells rather showed an inhibiting effect on hBD-3 induction. Alltogether, the present study provides an insight into the immune reaction of gastric epithelial cells and demonstrates that hBD-3 induction is mediated by viable bacteria and implicates a modulation by the adaptive immune response. Simultaneously, this modulation seems to be inhibited by *H. pylori* which could represent a novel immune evasion mechanism of *H. pylori* by suppressing an effective innate immune response. The enhanced cell death dependent on IFN-γ treatment further implies that this cytokine is an important factor for pathogenesis of *H. pylori*-related gastric injury.

Zusammenfassung

Helicobacter pylori ist ein im Magen des Menschen vorkommender Krankheitserreger, mit dem 50% der Weltbevölkerung chronisch infiziert sind und der mit der Entwicklung peptischer Ulcera und von Magenkrebs assoziiert ist. Bisher sind die Abläufe der Immunantwort auf eine Infektion mit *H. pylori* nicht vollständig verstanden. In den letzten Jahren konnte gezeigt werden, dass gastrische Epithelzellen über die Sekretion von humanem beta Defensin 3 (hBD-3) aktiv an der Bekämpfung der Infektion mit *H. pylori* beteiligt sind. Die Induktionsmechanismen dieses Defensins in gastrischen Epithelzellen sind jedoch nicht vollständig geklärt. Die vorliegende Studie hatte zum Ziel diese Mechanismen zu analysieren. Hierfür wurden neben *H. pylori* auch weitere bekannte Stimuli eingesetzt, um ihre Kapazität hBD-3 zu induzieren bzw. die hBD-3 Expression zu modulieren genauer betrachten zu können. Dafür wurde ein Modell gewählt, bei dem gastrische Krebszelllinien infiziert wurden sowie verschiedene bakterielle Stimuli und rekombinante Zytokine appliziert wurden um die Auswirkung auf die Induktion von hBD-3 zu messen. Der Einfluss der adaptiven Immunität auf die hBD-3 Produktion in Epithelzellen wurde anhand der Behandlung gastrischer Epithelzellen mit den Überständen eines Co-Kultur Modelles von aktivierten dendritischen Zellen (DC) mit autologen T-Zellen getestet. hBD-3 wurde mittels Western blot und ELISA detektiert. Es konnte gezeigt werden, dass die Induktion von hBD-3 in gastrischen Epithelzellen von der Viabilität der Bakterien abhängig ist und nach der Menge der Bakterien reguliert wird. Eine Behandlung mit rekombinanten Zytokinen alleine führte zu keiner Induktion. Die Stimulation der Zellen mit Interferon-γ (IFN-γ) während einer Infektion führte jedoch zu einer deutlich verstärkten Produktion von hBD-3 und gleichzeitig zu vermehrtem Zelltod. Die Untersuchung des Einflusses der adaptiven auf die angeborene Immunität zeigte, dass *E. coli* LPS aktivierte DC/T-Zellen hBD-3 Expression induzieren während die Aktivierung mit *H. pylori* zu einem inhibierenden Effekt führte. Die vorliegende Arbeit erlaubt einen Einblick in die angeborene Immunität gastrischer Epithelzellen und konnte zeigen, dass die Induktion von hBD-3 durch die adaptive Immunantwort moduliert werden kann, wobei IFN-γ ein wichtiges Signalmolekül darstellt. Gleichzeitig scheint *H. pylori* diese Modulation zu inhibieren, was auf eine neue Strategie der pathogen-vermittelten Unterdrückung einer effektiven Immunantwort hindeutet. Der durch IFN-γ hervorgerufene vermehrte Zelltod in infizierten Zellen impliziert eine wichtige Rolle dieses Zytokins bei der Pathogenese gastrischer Läsionen unter Infektion mit *H. pylori.*

1. Introduction

1.1 *H. pylori* - a human gastric pathogen

Helicobacter pylori is a gastric pathogen chronically infecting 50% of world's population. Infected individuals develop a chronic gastritis that mostly remains asymptomatic, but infection can also lead to the formation of peptic ulcers, and a link between long term infections with *H. pylori* and the formation of gastric cancer could clearly be established (Parsonnet et al., 1991). Hence, *H. pylori* was ranked as a class one carcinogen by the World Health Organization (Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). First reports of spiral shaped bacteria colonizing the stomach of dogs were already provided by Bizzozero in 1893 but it took almost a century to establish the connection between infection and gastric disease. In 1984, *H. pylori* was identified as an infective cause of gastritis and gastric and duodenal ulcer disease by Marshall and Warren (1984). Since then, research has focused on the understanding of mechanisms that lead to the establishment of infection and its related diseases. *H. pylori* is capable of colonizing an adverse biological niche in the gastric mucosa and this pathogen has perfectly adapted to the human host. Once an infection is acquired it can persist for decades or even for an entire life (Kuipers et al., 1993). *H. pylori* has evolved different virulence factors that on the one hand enable it to withstand the conditions found in the stomach and on the other are capable of actively modulating and suppressing the host's immune response (Wilson and Crabtree, 2007). Yet, many facets of the interplay of *H. pylori* and its host still remain elusive. So gathering further insight into the biology and the interaction mechanisms of *H. pylori* with the immune system, enabling it to establish and subsequently maintain an infection, is crucial for the development of an effective treatment and the understanding of *H. pylori`s* impact on its human host.

H. pylori is a Gram-negative bacterium belonging to class of ε-proteobacteria, order of Campylobacterales. It measures about 3 µm in length and 0.5 - 1 µm in width and is helix shaped and flagellated. The bacterium is microaerophilic, which means that it requires at least 2% O_2 but does not tolerate high oxygen concentrations. It is able to survive low pH levels but prefers a pH at around 6 (Stingl et al., 2002). The core genome comprises around 1,150 genes (Dorer et al., 2011). *H. pylori* lacks distinct DNA mismatch repair systems leading to a high mutation rate and wide genetic variability and allelic diversity (Dorer et al., 2011). Furthermore, variability is fostered as *H. pylori* is competent for DNA uptake (Haas et al., 1993) and shows frequent inter- and

intragenomic recombination (Dorer et al., 2011; Suerbaum et al., 1998). Horizontal gene transfer between different strains via a ComB type IV secretion system has also been described (Hofreuter et al., 2001).

1.2 Epidemiology and transmission of *H. pylori*

About half of the world's population is infected with *H. pylori,* but infection rates vary vastly between developed and developing countries. Whereas in the latter around 80% of the adults are infected due to reduced hygienic standards, in developed countries the prevalence of infection is at less than 40% (Goh et al., 2011; Perez-Perez et al., 2004; Tonkic et al., 2012). Within developed and developing countries, prevalence of infection within different populations is also dependent on factors such as the socioeconomic status, origin or age (Perez-Perez et al., 2004). It has been shown that in populations with a low socioeconomic status, the infection rate is higher and infection is more common in immigrants originating from developing countries (Perez-Perez et al., 2005). Additionally, the elderly generation is more commonly infected with *H. pylori.* This effect is a result of the hygienic conditions these generations had to face in the past when infection rates were higher (Kuipers et al., 1993), underlining the notion that an infection once it is acquired persists for decades. In industrialized countries infection rates are declining (Tonkic et al., 2012), which can be a result of improving living standards and the successful treatment of infection with antibiotics.

The transduction of *H. pylori* is mainly thought to take place by an oral - oral or fecal - oral path and it is acquired in early childhood (Goh et al., 2011). Transmission routes differ between developed and developing countries. In developed countries infection does not spread epidemically but mainly within families, and infection during childhood is more common (Raymond et al., 2004; Schwarz et al., 2008). In populations with a high prevalence of *H. pylori* and with low hygienic standards, as it is found in developing countries, the transmission routes are more likely to be horizontal and interfamilial transmission plays a minor role (Goh et al., 2011). Here, additionally multi-colonization with different *H. pylori* strains is observed (Raymond et al., 2004).

1.3 Disease caused by *H. pylori*

Infection with *H. pylori* leads to the development of a chronic active gastritis (Marshall and Warren, 1984) which, in infected individuals, shows a wide variety of clinical

symptoms. Some develop severe dyspepsia (Kandulski et al., 2008) others are completely asymptomatic (Kandulski et al., 2008). 10 - 20% of infected develop peptic ulcer disease (Crabtree et al., 1991). Soon after its discovery, *H. pylori* was linked to the development of gastric cancer. In cohort studies it could be shown that there was an increased risk of developing gastric adenocarcinoma of the intestinal type in individuals infected with *H. pylori* (Parsonnet et al., 1991). This relation between *H. pylori* infection and development of gastric cancer has been further analyzed and a cascade for the development of gastric cancer was proposed by Correa (1988). It implies that a chronic active gastritis caused by a long persisting infection can lead to the development of atrophic gastritis which can proceed to an intestinal metaplasia. At this stage, infection is lost and the metaplastic areas are free from colonization with *H. pylori*. These metaplastic lesions tend to further dedifferentiate into a dysplastic stage. Finally 1 - 2% of all infected individuals develop gastric adenocarcinoma of the intestinal type, mainly in the antrum and pylorus region. This stepwise model for the development of gastric cancer could be confirmed by other studies (Uemura et al., 2001). It is estimated that around 60% of all gastric cancer cases and 5 - 6% of all cancers every year can be attributed to *H. pylori* infection (Parkin, 2006). *H. pylori* has also been linked to the development of gastric mucosa-associated lymphatic tissue (MALT) lymphoma, a none-Hodgkin B-cell lymphoma of the marginal zone (Fischbach, 2004). Out of approximately 15,000 new cases a year, 79% in developing countries and 74% in developed countries are caused by *H. pylori* infection (Parkin, 2006). At an early stage of this disease, eradication of *H. pylori* leads to complete remission in almost 2/3 of the patients (Fischbach, 2004).

1.4 Virulence factors

1.4.1 *H. pylori* escapes low pH

H. pylori can survive in the acidic pH of the stomach and even though the pH in the gastric lumen can go down to 1 - 2, this pathogen is capable of maintaining a neutral intracellular pH (Stingl et al., 2002). The bacterium shows a special mechanism to cope with this hostile environment, which is dependent on the cell surface-associated and intracellularly found enzyme urease and an inner-membrane-bound, pH-sensitive urea channel (HPurel). This channel allows urea to enter the cytoplasm under acidic conditions (Weeks et al., 2000). Urea is metabolized to NH_3 and CO_2 via the enzyme urease (Stingl et al., 2002), the most abundant protein of *H. pylori* and an important

virulence factor. NH_3 is then used to neutralize H^+ leading to the formation of NH_4 and that way a pH of 6 in the bacterial periplasma is maintained. Yet in an *in vivo* model it could be shown, that this effect is only limited to a short period of few minutes, depending on the pH and pepsins in the gastric juice (Schreiber et al., 2005). So, as resistance against low pH is limited, *H. pylori* needs to be capable of moving quickly from the gastric lumen towards the epithelial cells, penetrating the mucus layer. Here, *H. pylori* makes use of its flagellae and it has been shown that flagellation is crucial for establishing an infection but does not affect bacterial growth in vitro (Eaton et al., 1996). In the mucus layer, *H. pylori* follows a rising pH gradient and colonizes a layer in the mucus in the vicinity of the gastric epithelium (the so-called juxtamucosa) which shows a pH close to 6 (Schreiber et al., 2004). This whole process is a first and very important immune evasion mechanism, as the gastric acid is usually capable of killing most ingested bacteria. This mechanism therefore forms the basis for *H. pylori* to successfully colonize in the human stomach.

1.4.2 Adherence and colonization

H. pylori mainly colonizes the mucus layer close to the epithelial surface. However, a small amount of bacteria get into direct contact with epithelial cells. There is a homeostasis of non-adherent and adherent bacteria, the latter seem to be a reservoir of bacterial renewal (Tan et al., 2009). The adherent bacteria have been shown to disrupt the epithelial barrier, penetrate the lamina propria and can also be found in intracellular spaces (Necchi et al., 2007). Adherence is mediated via several different adhesins of the helicobacter outer membrane porins (Hop) protein family like HopS (BabA), HopZ and HopP (SabA) (Bauer and Meyer, 2011; Petersson et al., 2006). In addition, *H. pylori* upon infection disrupts cellular polarity of epithelial cells and gains access to nutrients, thereby securing its survival (Tan et al., 2009). The complex interplay of adherence, inflammation and uptake of nutrients is important for the maintenance of an infection (Kirschner and Blaser, 1995).

1.4.3 The *cag* pathogenicity island

The cytotoxin-associated gene pathogenicity island (*cag*PAI) is a 40 kilobase gene region encoding major virulence factors of *H. pylori*. Strains harboring the PAI gene locus are called type 1 strains, strains without type 2 strains. Infection with *cag*PAI positive strains leads to activation of the transcription factors activator protein 1 (AP-1)

and nuclear factor-kappa B (NF-κB) (Naumann et al., 1999), secretion of interleukin-8 (IL-8) and cellular elongation in AGS cells (Backert et al., 2004). Type 1 strains cause stronger inflammatory responses and are more likely to cause gastric ulcers and cancer in infected hosts (Huang et al., 2003; Kuipers et al., 1995). In the *cag*PAI, different Cag proteins are encoded forming a type IV secretion system (T4SS) (Pham et al., 2012) that interacts with integrins expressed on host cells (Kwok et al., 2007; Pham et al., 2012). The T4SS is seen as the most important trigger for NF-κB activation in epithelial cells (Schweitzer et al., 2010). This T4SS encoded in the *cag*PAI is one of four T4SS found in *H. pylori* and delivers bacterial factors such as peptidoglycans (Viala et al., 2004a) and the cytotoxin-associated gene product A (CagA), which is also part of the *cag*PAI, to host epithelial cells (Backert et al., 2000; Bauer et al., 2012; Odenbreit et al., 2000). CagA is an important factor leading to many pathologies related to type 1 strains. After translocation to host cells, CagA is phosphorylated at tyrosine residues by tyrosinkinases Src and Abl (Selbach et al., 2002). The phosphorylation takes place at a specific amino acid sequence, the EPYA motif (Stein et al., 2002). After phosphorylation, CagA forms a phosphotyrosine-dependent complex with SHP-2, a tyrosine phosphatase (Bauer et al., 2012; Higashi et al., 2002). The CagA/SHP-2 complex modulates and suppresses many different intracellular signaling pathways. It dephosphorylates and blocks the EGFR which is activated in infection with *H. pylori* (Bauer et al., 2012), or leads to disruption of the cytoskeleton, which in vitro can be seen as the ‘hummingbird phenotype’ in AGS cells (Moese et al., 2004).

1.4.4 Vacuolating cytotoxin A

The protein VacA, a cytotoxin produced by *H. pylori,* is important for its capability to infect humans (Cover and Blanke, 2005). This toxin is multifunctional and released via type V secretion into the extracellular space (Schmitt and Haas, 1994). It is seen as an important factor for the development of peptic ulcer disease and cancer and causes a stronger lymphocytic reaction (Wada et al., 2004). It interacts with a multitude of different components of the host’s cells. It can be inserted directly into the cellular membrane and form channels leading to a leakage of ions and small molecules from infected cells (Szabo et al., 1999). When VacA is taken up by cells via pinocytosis, it disrupts the function of the endolysosmal system by forming anion channels leading to vacuolization within cells (Leunk et al., 1988). This cytotoxin leads to an alteration of the permeability of the mitochondrial membrane and can cause the release of cytochrome c

(Kimura et al., 1999) which can lead to the induction of apoptosis (Kuck et al., 2001). Furthermore, cellular signaling pathways are affected as seen in a VacA-dependent activation of mitogen-activated protein kinase (MAPK) pathways (Nakayama et al., 2004). One central role of VacA, making it an important factor for the long term colonization of a host, is its immunomodulatory function: It inhibits T-cell activation and expansion, as production of interleukins like IL-2 is hampered and the activation of nuclear factor of activated T-cells (NF-AT), a transcription factor important in T-cell activation, is disrupted possibly via calcineurin inhibition (Boncristiano et al., 2003; Gebert et al., 2003). Besides hampering activation, VacA also inhibits proliferation of activated $CD4^+$ T-cells (Beigier-Bompadre et al., 2011).

1.4.5 Outer membrane structure

Another factor critically determining virulence of *H. pylori* is the structure of its outer membrane. Lipopolysaccharide (LPS) is an integral part of the outer membrane of Gram-negative bacteria and it is a potent trigger of the immune response. The general structure of LPS consists of a hydrophobic part anchoring it in the outer membrane, the lipid A component, which is made of fatty acids bound to phosphorylated glucosamine disaccharides. The lipid A is directly attached to the core oligosaccharide, which is further decorated with a polysaccharide chain, the O-antigen. LPS containing this O-antigen is called smooth, whereas LPS missing it is called rough. The most important component of LPS for activation of the innate immune response is the lipid A (Cullen et al., 2011) which in *H. pylori* has been shown to be under-phosphorylated and under-acylated (Muotiala et al., 1992). Upon infection, bacterial molecules like the LPS are sensed by toll-like receptors (TLR), but *H. pylori`s* altered LPS structure tremendously weakens its endotoxic activity (Muotiala et al., 1992). Additionally, there is evidence that the innate immune response is even suppressed instead of activated by *H. pylori* LPS (Lepper et al., 2005). Moreover, the under-phosphorylation of the lipid A component leads to a higher resistance of *H. pylori* towards cationic antimicrobial peptides (AMP) (Cullen et al., 2011). On top of the modification of the lipid A component, the polysaccharide chains of the O-antigen can be fucosylated at the terminal unit (Moran, 2008). These fucosylated polysaccharides mimic human Lewis antigens are also found on human gastric epithelial cells (Appelmelk et al., 2000). Different effects of this mimicry are discussed and this is seen as an immune evasion mechanism and an adaptation to the human host, leading eventually to an autoimmunity and development

of gastric atrophy (Appelmelk et al., 1997; Moran, 2008). Other findings suggest a role of bacterial adhesion to the host cell for Lewis antigens (Appelmelk et al., 2000). Furthermore, the outer membrane has been shown to contain cholesterol α-glucosides which is rare in bacteria and animals (Haque et al., 1996). *H. pylori* extracts cholesterol from its host and glycosylates it, forming cholesterol α-glucosides that protect the bacteria from phagocytosis by macrophages and inhibit T-cell proliferation (Wunder et al., 2006).

1.4.6 Gamma-glutamyltransferase

Gamma-glutamyltransferase (GGT) is another virulence factor *of H. pylori*. It is an enzyme present in many mammalian and bacterial cells. It transfers gamma glutamyl groups from gluthathione to acceptors and is important for the detoxification of drugs and xenophobics. The GGT of *H. pylori* has been shown to inhibit growth of AGS cells and induce apoptosis via the induction of cell cycle arrest in G1 phase (Kim et al., 2010). Further, it fosters production of H_2O_2 in gastric epithelial cells and leads to activation of NF-κB signaling and release of IL-8 (Backert and Clyne, 2011; Gong et al., 2010). *H. pylori* GGT has also been linked to the release of EGFR-related peptides heparin binding EGF-like growth factor (hbEGF) and amphiregulin and the release of prostaglandin E2 (Busiello et al., 2004). In addition to that, it could be shown that patients suffering from ulcer disease are infected with *H. pylori* strains that show higher GGT activity (Gong et al., 2010). Recently, GGT in combination with VacA, was reported to alter DC maturation leading to the induction of forkhead box P 3 $(FoxP3)^+$ regulatory T-cells (T_{REG}), being a critical factor for the development of tolerance of *H. pylori* infection in mice (Oertli et al., 2013).

1.4.7 Neutrophil-activating protein

Neutrophil-activating protein from *H. pylori* (HP-NAP) is another highly conserved virulence factor critically influencing the adaptive and innate immune response. It is chemotactic to monocytes and neutrophils and increases adhesion of neutrophils to endothelial cells (Dundon et al., 2002). Furthermore, it leads to activation of monocytes and induces production of reactive oxygen species in neutrophils (Dundon et al., 2002). These effects are a result of the activation of the ERK/p38 MAPK pathways in neutrophils by HP-NAP (Nishioka et al., 2003). On top of this, HP-NAP fosters differentiation of monocytes towards dendritic cells (DC) and leads to secretion of IL-12

and IL-23 by neutrophils, promoting a T_H1 polarization of T-cells and enhanced production of interferon-γ (IFN-γ) (Amedei et al., 2006).

1.4.8 Outer inflammatory protein and high temperature requirement protein A

Outer inflammatory protein (OipA) is an outer membrane protein and a member of the Hop protein family (HopH). The *oipA* gene is present in all *H. pylori* strains but its expression is regulated by phase variation based on a slipped strand repair mechanism (Yamaoka et al., 2000). It is involved in attachment to host cells and leads to induction of IL-8 *in vitro* (Yamaoka et al., 2000). Expression of OipA further has been demonstrated to be related to the development of duodenal ulcer and the expression of IL-8 *in vivo* (Yamaoka, 2002). Another *H. pylori* virulence factor recently described is high temperature requirement A (HtrA) protein. This secreted protease is capable of cleaving E-cadherin of epithelial cells leading to disruption of the epithelial barrier, enabling *H. pylori* to invade the intercellular space (Hoy et al., 2010). So *H. pylori* gets into contact with the basolateral side of epithelial cells, where integrins are expressed, allowing the T4SS to interact with the host cell (Bauer and Meyer, 2011).

1.5 The host immune response and the course of infection with *H. pylori*

1.5.1 The innate immunity

The innate immunity is the first line of defense any pathogen has to overcome in order to infect its host. This part of the immune reaction is induced rapidly or is constitutively active. Basic physiologic features of the host, such as the acid pH of the stomach and a protective mucus layer can be seen as a first part of the immunity, as they prevent most pathogens from colonizing. When a pathogen has overcome the physiological barriers it is confronted by the innate immunity of the host's epithelial cells and other cells of the innate immune system like macrophages or granulocytes. Here, pathogens are sensed via specific pattern recognition receptors (PRR). PRR are a heterogeneous group of receptors which can be found on the cell surface or in intracellular space and include TLRs, C-type lectin receptors (CLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Ishii et al., 2008). PRRs are capable of recognizing pathogen associated molecular patterns (PAMPs) which are bacterial or viral elements such as LPS, bacterial or viral RNA and DNA or flagellin. For example, TLR2 and TLR4 can sense bacterial LPS and peptidoglycans, TLR3 and TLR9 sense RNA and DNA, TLR5 senses flagellin,

and NOD1/2 are intracellular receptors of the NLR family that can sense peptidoglycans (Ishii et al., 2008). Upon activation, TLRs form homo- or heterodimers leading to the recruitment of the adapter molecule MyD88 and the formation of a signaling complex resulting in MAPK pathway-dependent AP-1 activation or the induction of NF-κB (Banerjee and Gerondakis, 2007). This gives rise to the expression of genes encoding a pro-inflammatory response (Banerjee and Gerondakis, 2007).

1.5.2 The role of epithelial cells in *H. pylori* infection

Upon infection, *H. pylori* is sensed by the innate immunity of gastric epithelial cells. NOD1 has been shown to play an important role in the innate immunity of gastric epithelial cells against *H. pylori, as* it is activated by peptidoglycans delivered to the cells by *H. pylori* either via the T4SS (Viala et al., 2004b), or via outer membrane vesicles (OMV) shed by *H. pylori* (Kaparakis et al., 2010; Viala et al., 2004a) . The interaction between *H. pylori* peptidoglycan and NOD1 leads to AP-1 and NF-κB activation and subsequent production of IL-8 (Allison et al., 2009; Bhattacharyya et al., 2002). IL-8 is an important mediator in the early immune response, leading to neutrophil recruitment. The role of TLRs in *H. pylori* infection is controversial. Gastric epithelial cells have been shown to express TLRs (Schmausser et al., 2004). However, *H. pylori* is capable of preventing an effective activation of the innate immune response due to modification of its PAMPs. Its LPS for example shows low endotoxic activity (Muotiala et al., 1992) as it inhibits TLR4 signaling and is only a weak activator of TLR2 (Lepper et al., 2005). *H. pylori* flagellin furthermore has been shown to be a poor agonist of TLR5 (Gewirtz et al., 2004). So *H. pylori* is being recognized by gastric epithelial cells via TLRs but it evades effective activation due to a modified PAMP structure, indicating that these signaling pathways only take a back seat in immune activation. Furthermore, it could be shown that gastric epithelial cells upon infection shed EGFR-related peptides like hbEGF leading to a paracrine stimulation of the epithelial layer affecting its homeostasis (Romano et al., 1998). Another facet of the innate immunity of epithelial cells is their recently discovered capability to actively take countermeasures against *H. pylori*. Upon sensing the pathogen, epithelial cells start producing AMPs, such as human beta defensin (hBD)-2, and hBD-3 (Bauer et al., 2012; Grubman et al., 2010). The latter has been shown to have a strong bactericidal effect against *H. pylori* (Bauer et al., 2012; Kawauchi et al., 2006). Here, *H. pylori* has evolved a mechanism for escaping the bactericidal effect of hBD-3. Upon infection, CagA is translocated via the

T4SS to epithelial cells where it is phosphorylated and binds to SHP-2. The CagA/SHP-2 complex dephosphorylates and blocks the EGFR. As the induction of hBD-3 is dependent on EGFR signaling, inhibition of this receptor leads to a cut down of hBD-3 production (Bauer et al., 2012).

1.5.3 Other cells of the innate immune system and their role in *H. pylori* infection

A second line of the innate immune reaction is formed by phagocytic cells like neutrophils, monocytes and macrophages that could be shown to infiltrate the site of inflammation (Necchi et al., 2009). All of these cells can coordinate the immune response and upon contact with pathogens produce bactericidal substances such as NO, O_2^-, H_2O_2 or OH^- (Gobert et al., 2001). *H. pylori*-activated macrophages additionally produce chemo-attractants and cytokines, such as tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-23 and IL-12 (Fehlings et al., 2012; Wilson and Crabtree, 2007). However, *H. pylori* blocks the bactericidal activity of macrophages by inhibiting NO production by macrophages via depletion of arginine, the precursor of the synthesis of NO (Gobert et al., 2001). What is more, a VacA-dependent apoptosis of macrophages and an interruption of the phagosome maturation has been described (Bauer and Meyer, 2011).

1.5.4 Activation of adaptive immunity

Simultaneously with the above-mentioned innate defense against infection, activation of the adaptive immune response is initiated. Antigen presenting cells (APCs) like DCs are recruited to the site of infection. Immature DCs show a high endocytic activity and are characterized by the expression of HLA-DR, CD11c, DC-SIGN, and CD206 (Bimczok et al., 2010). Invading pathogens are phagocytosed and DCs are activated. Activated DCs prime the adaptive immune system towards the pathogen by presenting specific pathogenic antigens loaded onto major histocompatibility complex (MHC) class II molecules. This is recognized by naïve $CD4^+$ T-cells via their specific T-cell receptor/CD3 complex. They bind to the MHC-II-bound antigen with the aid of the CD4 co-receptor. This is followed by binding of CD28 expressed on T-cells to the co-stimulatory molecules CD80 and CD86 expressed on DCs following activation. This is a prerequisite for T-cell activation and leads to secretion of IL-2 which triggers clonal expansion of T-cells. Furthermore, upon activation, DCs, macrophages and other cells start producing cytokines. The composition of the cytokine mixture found in a tissue produced by cells like DCs, macrophages or neutrophils leads to a specific activation

and differentiation of the T_H-cells towards different subsets such as T_H1, T_H2, T_H17 or inducible regulatory cells T-cells (iT_{REG}). IL-12 and IFN-γ promote a polarization towards IFN-γ producing T_H1 cells, while IL-23, IL-6 and transforming growth factor-β (TGF-β) induce differentiation towards T_H17 cells that are characterized by the production of IL-17. iT_{REG}s are most likely induced by antigen presentation of immature, tolerogenic DCs which do not provide sufficient co-stimulatory signals or lack production of inflammatory cytokines. Simultaneously, the presence of TGF-β and of the immunosuppressive cytokine IL-10 leads to differentiation of naïve T-cells to iT_{REG}s (Maldonado and Von Andrian, 2010). These differential stimuli lead to the induction of distinct transcription factors in naïve T_H-cells resulting in their specific polarization. Activation of T-box transcription factor TBX21 (T-bet) leads to a T_H1 polarization, while FoxP3 induces T_{REG}s and RAR-related orphan receptor (Rorγt) triggers T_H17 polarization.

1.5.5 The role of the adaptive immune system in *H. pylori* infection

DCs have been shown to be present in the gastric epithelial layer also of uninfected individuals where they penetrate the epithelial surface enabling them to interact with *H. pylori* (Bimczok et al., 2010). In mouse models it could also be demonstrated that 6 h after infection, DCs are invading the inflammatory site (Kao et al., 2006; Necchi et al., 2009). Upon infection DCs take up and process *H. pylori* for antigen presentation and it is suggested that DC-SIGN (CD209) is capable of interacting with *H. pylori,* playing a potential role in phagocytosis (Kao et al., 2006; Kranzer et al., 2004). Contact with *H. pylori* further leads to DC activation and maturation. Activation is probably mediated via sensing of *H. pylori* by TLRs, putative candidates are TLR2, 4 and 9 (Bimczok et al., 2010) and maturation is reflected by the upregulation of cell-surface receptors such as CD80, CD83, CD86 and CD40 and the production of different cytokines like IL-12, IL-6, and TNF-α as well as the immunosuppressive IL-10 (Bimczok et al., 2010; Fehlings et al., 2012). The cytokine mixture produced by DCs, macrophages and other immune cells at the site of infection creates a milieu leading to a T_H1/T_H17 predominant lineage commitment of naïve T_H cells (Akhiani et al., 2004; Bimczok et al., 2010; Shi et al., 2010). Consequently, in the gastric mucosa of infected individuals, high levels of IFN-γ and IL-17 can be found (Aebischer et al., 2008). IFN-γ has been shown to be essential for controlling the infection (Sayi et al., 2009). Immunization of mice with *H. pylori* sonicate together with an adjuvant fosters a T_H1/T_H17-polarized immune response resulting in stronger inflammation and reduced bacterial load (Hitzler et al., 2011). Yet,

there is evidence that DCs, upon *H. pylori* infection via polarization of T-cells towards iT_{REGS}, a subset of T-cells showing immunosuppressive functions, seem to foster tolerogenic effects hampering the effective clearing of an infection (Hitzler et al., 2011; Müller et al., 2011; Zhang et al., 2010). So, both a pro-inflammatory and an anti-inflammatory polarization of T-cells has been described following *H. pylori* infection. Another subset of T-cells reported to be activated in various frequencies in infected hosts are natural killer T-cells (NKT) (O'Keeffe and Moran, 2008). These cells are activated via glycolipids bound to CD1d expressed on DCs or on epithelial cells which is followed by the production of large amounts of IFN-γ (Matsuda et al., 2000; Mattner et al., 2005; van de Wal et al., 2003). It has also been reported that upon infection with *H. pylori,* activation of natural killer cells (NK) takes place (Lindgren et al., 2010) which, upon co-stimulation with *H. pylori* and IL-12, start producing large amounts of IFN-γ (Lindgren et al., 2011; Yun et al., 2005). Yet, the role of NK and NKT-cells in *H. pylori* infection is controversial, as it could be shown that depletion of these cells in a mouse infection model does not alter the effectivity of the immune response (Sayi et al., 2009).

1.5.6 Impact of adaptive on innate immunity

In summary, infection with *H. pylori* initiates an immune response which depends on both the innate immunity of epithelial cells and on adaptive immunity. Upon infection, epithelial cells respond with production of AMPs (Bauer et al., 2012) and the secretion of IL-8. Cells of the innate immune system and DCs upon *H. pylori* detection start producing cytokines, leading to a T_H1/T_H17-polarized T-cell response (Akhiani et al., 2004; Bimczok et al., 2010) and the induction of several other T-cell subsets. The cytokine milieu which is then produced by adaptive immunity is characterized by high levels of pro-inflammatory cytokines like IFN-γ and IL-17. It has been proposed that these pro-inflammatory cytokines can activate the innate immunity of gastric epithelial cells (Albanesi et al., 2007; Joly et al., 2005), leading to enhanced production of AMPs (Menendez and Brett Finlay, 2007; Sørensen et al., 2005; Steubesand et al., 2009). This reinforcement of AMP production might play a critical role in infection with *H. pylori,* as Boughan et al. (2006) could show that bacterial load of infected mice increases when the induction of defensins is impaired in a NOD1 knockout model. Below, defensins and their exact role in *H. pylori* infection will be discussed in more detail. Figure 1.1 gives an overview of the immune reaction following *H. pylori* infection and putative crosstalk mechanisms of adaptive and innate immune response.

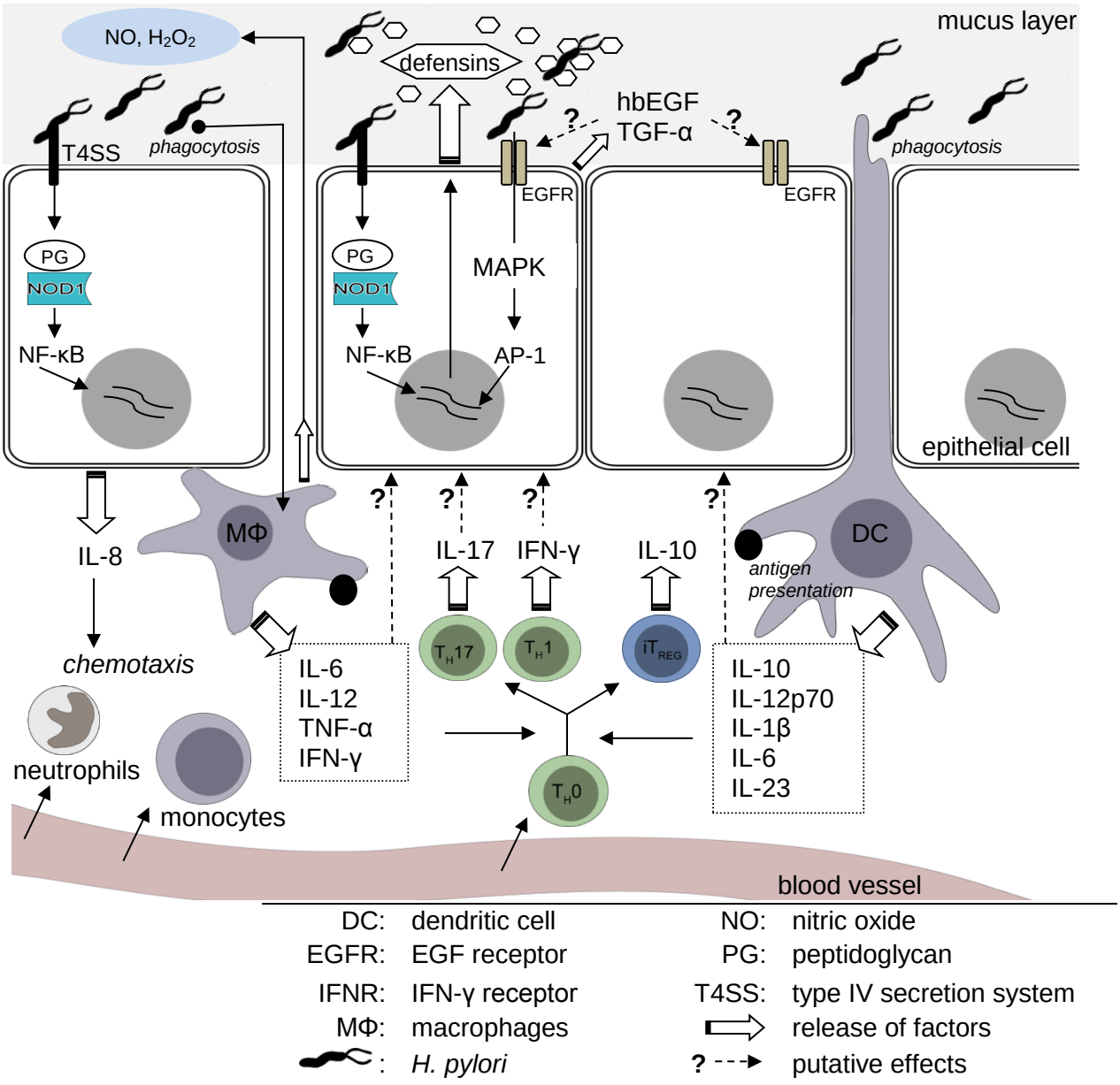

Figure 1.1 Schematic overview of the immune response following *H. pylori* infection. *H. pylori* is sensed by epithelial cells, which in turn start producing bactericidal substances like defensins. Furthermore, upon infection epithelial cells release chemotactic IL-8 which leads to invasion of neutrophils and monocytes to the site of infection. The latter can differentiate into macrophages or DCs. Macrophages and neutrophils release NO or H_2O_2 and actively kill *H. pylori*. DCs, neutrophils and macrophages show phagocytic activity, and upon sensing *H. pylori* start producing cytokines, creating an environment favoring a T_H1/T_H17 polarization of T-cells or the induction of iT_{REG}s. The pro-inflammatory cytokines like TNF-α, IL-1, IFN-γ or IL-17 released by immune cells might stimulate the epithelial layer and lead to expression of defensins or amplify their induction via paracrine effects. Additionally, upon infection, epithelial cells release mediators like hbEGF or TGF-α which in an autocrine or paracrine way stimulate the epithelial layer.

1.6 Anti-microbial response of the host

1.6.1 Anti-microbial peptides

AMPs are a large group of short peptides consisting of less than 100 amino acids that are expressed in a variety of organisms. They are produced by bacteria (then they are termed bactericins), fungi or by eukaryotic cells and pose an archaic defense mechanism of cells against pathogens (Wiesner and Vilcinskas, 2010). In recent years, the importance of AMPs in human disease has been discovered and now is under intensive research. AMPs that have been shown to be relevant in humans are the cathelicidins and the defensins (Jenssen et al., 2006). They show a broad spectrum of antimicrobial activity and are a crucial agent of the innate immune response. Defensins can be divided into three classes: Alpha and beta defensins, that are found in humans and theta defensins that only have been reported in macaque (Selsted and Ouellette, 2005). Defensins share common structural patterns: They are small cationic peptides of about 29 - 45 amino acids that form a tertiary structure, the so-called ‘defensin fold’ of 3 anti-parallel beta sheets linked by 3 disulfide bridges between 6 cysteine residues (Dhople et al., 2006; Selsted and Ouellette, 2005). Alpha defensin is mainly produced by Paneth cells and leucocytes that use defensins to kill phagocytized bacteria (Wiesner and Vilcinskas, 2010). Beta defensins can be further subdivided into different groups. Based on genetic analysis it is estimated that there are 25 different beta defensins (Selsted and Ouellette, 2005), but not all of them have been identified so far. Defensins differ in their expression profile. hBD-1 is constitutively expressed in epithelial cells, hBD-2 and hBD-3 can be induced by infectious stimuli (Dhople et al., 2006; O’Neil et al., 2000). Antimicrobial activity is mainly dependent on charge-mediated binding to bacterial membranes and its subsequent disruption (Wiesner and Vilcinskas, 2010). But the exact mechanisms are still under discussion and may be distinct in the different AMPs. One possible mechanism is the formation of pores via a perpendicular grouping of defensins, stretching through the whole cell membrane. Another model that is proposed is a formation of carpets made of AMPs and phospholipids that, when a critical amount of AMPs are present, leads to disruption of the cell membrane (Wiesner and Vilcinskas, 2010). Additionally, bactericidal activity of distinct defensins has been shown to be salt-sensitive (Bauer et al., 2012; Wiesner and Vilcinskas, 2010). As mentioned above, AMPs have been shown to be induced under *H. pylori* infection in gastric epithelial cells. Among the AMPs reported to be induced and bactericidal against

H. pylori are LL-37, hBD-2, hBD-4 and hBD-3 (Bauer et al., 2012; Grubman et al., 2010; Hase et al., 2003; O'Neil et al., 2000; Otte et al., 2009).

1.6.2 Human beta defensin 3

hBD-3 is expressed in a variety of epithelial cells of human tissues such as tonsils, trachea, urinary tract, skin or stomach (García et al., 2001; Jia et al., 2001). It has been shown that hBD-3 is induced in epithelial cells upon coming into contact with Gram-negative or Gram-positive bacteria or funghi and it exerts a strong bactericidal effect against a variety of pathogens (García et al., 2001; Harder et al., 2001; Steubesand et al., 2009). hBD-3 recently was shown to be highly active against *H. pylori* (Bauer et al., 2012; Kawauchi et al., 2006). Antimicrobial activity is blocked only at high, unphysiologic salt and plasma protein concentrations (García et al., 2001; Harder et al., 2001). Upon stimulation, hBD-3 is released as a 67 amino acid pre-peptide that is processed to the 45 amino acid mature form. hBD-3 is highly positively charged and has shown the capability of forming stable dimers or even multimers (Boniotto et al., 2003; Crovella et al., 2005). Besides its bactericidal activity, hBD-3 has immunomodulatory functions. It has been reported to be chemotactic for monocytes, as hBD-3 is capable of binding to the CCR6 receptor (Dhople et al., 2006; García et al., 2001). At the site of infection, hBD-3 leads to maturation of DCs and an increased expression of the co-stimulatory molecules CD40, CD80 and CD86 on DCs and monocytes via activation of TLR1 and TLR2 (Funderburg et al., 2007). Thus, hBD-3 seems to play an important role not only in killing pathogens but also in the crosstalk between adaptive and innate immune response. Furthermore, it has been shown to have a beneficial impact on wound healing by promoting epithelial cell migration and proliferation, partly because of a modulation of the EGFR (Hirsch et al., 2009).

1.6.3 Induction of human beta defensin 3

The exact mechanisms of induction of hBD-3 are still not fully understood. For up-regulation following infections, involvement of TLR2, 4, 5 and 9 has been described (Kawauchi et al., 2006; Menzies and Kenoyer, 2006; Scharf et al., 2010). Signaling pathways reported to play a role in induction of hBD-3 comprise AP-1 activation via MAPK pathways as well as the JAK/STAT pathway (Bauer et al., 2012; Boughan et al., 2006; Steubesand et al., 2009). There have been found to be AP-1, STAT1 and NF-κB binding sites in the promotor region of hBD-3 (Albanesi et al., 2007; Menzies and

Kenoyer, 2006). Yet induction of hBD-3 via an infective stimulus has been shown to be NF-κB- and NOD1-independent (Boughan et al., 2006; Grubman et al., 2010; Steubesand et al., 2009).

Other triggers of hBD-3 production besides direct contact with *H. pylori* or other pathogens are pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, IL-22 or mediators like histamine. (Albanesi et al., 2007; Harder et al., 2001; Ishikawa et al., 2009a; Joly et al., 2005; O'Neil et al., 2000; Pernet et al., 2003; Wolk et al., 2004). The most potent and most frequently reported cytokine to induce hBD-3 is the pro-inflammatory T_H1 cytokine IFN-γ (Albanesi et al., 2007; Joly et al., 2005). Induction of hBD-3 via IFN-γ was demonstrated to be JAK/STAT-dependent (Albanesi et al., 2007) and the combination of IFN-γ with either TNF-α or IL-1β had synergistic effects on the induction level (Joly et al., 2005). In contrast to activation via an infective stimulus, an involvement of NF-κB in the TNF-α/IFN-γ-dependent induction of hBD-3 could be shown in skin keratinocytes (Albanesi et al., 2007). At the same time, this IFN-γ-dependent induction of hBD-3 could be inhibited by T_H2 cytokines IL-4 and IL-13 (Albanesi et al., 2007). Another interesting finding is that treatment with supernatants (SN) of activated mononuclear cells in skin keratinocytes leads to an induction of hBD-3 (Howell et al., 2006; Sørensen et al., 2005). Here, a transactivation of the EGFR via EGFR-related peptides like TGF-α and hbEGF and a subsequent induction of hBD-3 is postulated (Sørensen et al., 2005). These data show that, according to the model proposed before, induction of hBD-3 in epithelial cells can be reinforced and modulated by the adaptive immune response.

1.6.4 Induction of human beta defensin 3 following *H. pylori* infection

hBD-3 is induced following *H. pylori* infection (George et al., 2003) and has been shown to be the most potent AMP, killing different *H. pylori* strains in vitro at physiological salt concentrations (Bauer et al., 2012; Kawauchi et al., 2006). So far, no bacterial factor could be identified that triggers induction of hBD-3. Independence of induction from VacA, CagA or CagE or from the presence of a *cag*PAI was demonstrated (Bauer et al., 2012; Boughan et al., 2006). Furthermore, heat-inactivated *H. pylori* was not capable of inducing hBD-3 (Bauer et al., 2012). Thus identifying the bacterial trigger is an important issue to be resolved.

For induction of hBD-3 following *H. pylori* infection, Kawauchi et al. (2006) proposed an involvement of the TLR4, which has been shown to be expressed on gastric epithelial cells *in vivo* (Schmausser et al., 2004). Moreover, it could be shown that activation of the EGFR and a subsequent signaling via the ERK1/2 MAPK pathway leading to AP-1 activation and to a lesser extent the JAK/STAT pathway are involved in the induction (Bauer et al., 2012; Boughan et al., 2006). The exact mechanisms leading to activation of the EGFR and the MAPK pathway still remain unknown. Boughan et al. (2006) proposed an induction of hBD-3 via transactivation of the EGFR via hbEGF released by infected epithelial cells (Boughan et al., 2006) and there have been several studies that showed a para- and autocrine transactivation of the EGFR following *H. pylori* infection (Boughan et al., 2006; Keates et al., 2001; Romano et al., 1998; Wallasch et al., 2002).

1.7 Project outline

An important role of hBD-3 against *H. pylori* has clearly been demonstrated and this study is designed to expand knowledge about the induction of hBD-3 in gastric epithelial cell lines following infection with *H. pylori* and the subsequent modulation by adaptive immunity. So the following issues will be addressed:

In a first step, triggers of hBD-3 expression in gastric epithelial cells upon *H. pylori* infection will be investigated. So far, induction was shown to be independent of a functional T4SS, the *cag*PAI status or other immunogenic factors like VacA or CagA. Subsequently, inducing mechanisms on epithelial cells will be addressed. Here, recent data demonstrated that induction of hBD-3 is dependent on activation of the EGFR which could be the result of an auto- or paracrine transactivation via EGFR-related peptides like TGF-α or hbEGF released by infected cells. Moreover, it could be shown in other epithelial cell culture models that induction of hBD-3 is not solely dependent on direct contact with pathogens but that it can also be evoked by a variety of pro-inflammatory cytokines or other factors like histamine. So far, gastric epithelial cells have not been tested for their reactivity towards these factors. Therefore, stimulants of hBD-3 identified in other studies will be tested for their ability to induce hBD-3 in gastric epithelial cells. The question derived from this experiment is whether the innate immune response can be shaped by the adaptive immunity in gastric epithelial cells. Here, a DC/T-cell co-cultivation model will be used to elucidate, in a first step, the effect of the adaptive immune response on innate immunity under *H. pylori* infection.

2. Materials and Methods

2.1 Chemicals

Acrylamide/bisacrylamide:	
Rotiphorese Gel 30; 37,5:1	Roth, Karlsruhe, Germany
Ammonium persulfate (APS)	Merck, Darmstadt, Germany
Bromphenol blue	Biomol, Hamburg, Germany
Bovine serum albumine (BSA)	Biomol, Hamburg, Germany
Complete protease inhibitor	Roche, Mannheim, Germany
Ficoll	GE Healthcare, NJ
Glycerol	Carl Roth, Karlsruhe, Germany
Glycine	Biomol, Hamburg, Germany
HCl	Merck, Darmstadt, Germany
Isopropanol	Emsure/Merck Millipore, Darmstadt, Germany
Methanol	Emsure/Merck Millipore, Darmstadt, Germany
Milkpowder: Sucofin	TSI, Zeven, Germany
N,N,N´,N´-tetrametylethylendiamin	Serva, Heidelberg, Germany
Sodium dodecyl sulfat (SDS)	Serva, Heidelberg, Germany
Sulfuric acid	Merck, Darmstadt, Germany
Tris-hydroxymethyl-aminomethane	Applichem, Darmstadt, Germany
Tryptan blue	Merck, Darmstadt, Germany
Tween 20	Merck, Darmstadt, Germany or Sigma

2.2 Antibiotics, materials for cell culture and bacterial culture

Brain heart infusion (BHI)	Becton Dickinson, Franklin Lakes, NJ, USA
Chloramphenicol	Sigma, Deisenhofen, Germany
Fetal calf serum (FCS):	
Lot#: 01412; 0710x	Biochrom, Berlin, Germany
Gentamycin	Sigma, Deisenhofen, Germany
Horse serum	Biochrom, Berlin, Germany
Nystatin	Merck, Darmstadt, Germany or Sigma
PBS	Gibco/Invitrogen, Paisley,UK
Remel GC Agar	Oxoid/ Thermo Fisher, Waltham, USA
RPMI 1640	Gibco/Life technologies, Paisley,UK

Streptomycin	Sigma, Deisenhofen, Germany
Trimethoprim	Serva, Heidelberg, Germany or Sigma
Trypsin-EDTA	Gibco/ Life technologies, Paisley,UK
Vancomycin	MP Biomedical, Santa Ana, CA, USA

2.3 Materials and kits

Cell culture dishes and multiwell plates	Corning, Tewksbury, MA, USA or TPP, Trasadingen, Switzerland
Cell viability assay:	
CellTiter-Glo Luminescent	Promega, Madison, USA
Concentrators: Vivaspin 6	Sartorius Stedim, Göttingen, Germany
Cuvettes, tubes, microtubes, pipette tips and pipets	Sarsted, Nümbrecht, Germany
E. coli LPS	Invivogen, San Diego, CA, USA
ECL developer reagent	Western Lightning/Perkin Elmer, USA
hBD-3 ELISA kit:	
Cat#: RHF 7771CK	Antigenix America, NY, USA
Human buffy coat	German Red Cross, Berlin, Germany
Hyperfilm ECL	Amesham/GE Healthcare, NJ
MACS equipment:	
midiMACS magnets	Miltenyi Biotec, Bergisch Gladbach, Germany
25 ms separation columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Microtiter plates: MaxiSorp F	Nunc/Thermo Scientific, Waltham, MA, USA
Microtubes	Sarsted, Nümbrecht, Germany
Protein ladder: PageRuler Plus	Fermentas/Thermo Scientific, Waltham, USA
Western blotting equipment:	
Mini PROTEAN	Bio-Rad, Hercules, CA, USA

2.4 Machines

Bacterial incubator: Thermo forma	Thermo Scientific, Waltham, MA, USA
Balances	Sartorius, Göttingen, Germany
Benches:	
Lamin Air	Holten/Thermo Scientific, MA, USA

HeraSafe	Heraeus/Thermo Scientific, MA, USA
Cell incubator: HERA cell 150	Heraeus/Thermo Scientific, MA, USA
Centrifuges:	
5810R, 5417C, 5804R, 5415R	Eppendorf, Hamburg, Germany
Developer machine: Optimax 2010	Protec, Oberstenfeld, GE
Luminometer:	
1420 Luminescence counter	Perkin Elmer, Waltham, USA
Milli-Q water filter	Merck Millipore, Darmstadt, Germany
pH-meter	Mettler-Toledo, Gießen, Germany
Photometer: DRV 2000	Hach-Lange, Berlin, Germany
Power supply:	
Power Pack P25	Biometra, Goettingen, Germany
Power Pack	Bio-Rad, Hercules, CA, USA
Semi-dry transfer machine	Biometra, Goettingen, Germany
Sonifier: sonifier 450	Branson, Danbury, CT, USA
Spectrometer: Spectramax 190	Molecular devices, Sunnyvale, USA

2.5 Recombinant cytokines, antibodies and microbeads

2.5.1 Recombinant cytokines

Cytokine	Company	Cat#	Lot#	Stock conc.	Reconstituted
rh IFN-γ	R&D Systems	285-IF	EA4008121	20 - 100 ng/µl	PBS
rh IL-22	Biomol	87326	2405	10 ng/µl	PBS 0.1% BSA
rh TGF-α	Peprotech	100-16A	060206	20 ng/µl	PBS 0.1% BSA
rh TNF-α	R&D Systmes	210-TA	AA2411031	10 ng/µl	PBS
rh IL-17A	Humanzyme	HZ-1113	0329100001	10 ng/µl	PBS
Histamine	Sigma	H7125	101057155	20 ng/µl	Milli-Q H_2O
hbEGF	Sigma	E4643	n.a.	10 ng/µl	PBS
rh IL-1α	Sigma	I2778	120M1226V	1 ng/µl	PBS
rh IL-4	R&D Systems	204-IL-010	AG2811051	n.a.	n.a.
GM-CSF	Bayer or Genzyme	n.a.	n.a.	n.a.	n.a.

Table 1 List of cytokines used in the experiments; Cat# = catalogue number, conc. = concentration, GM-CSF = granulocyte macrophage colony stimulating factor, Lot# = lot number, Milli-Q H_2O = Milli-Q-filtered H_2O, n.a. = information not available, rh = recombinant human.

2.5.2 Antibodies for western blot

Antibody reactivity	Source (clonality)	Company	Cat#	Lot#	Dilution	Coupled with
Anti-hBD-3	Rabbit (polyclonal)	Santa Cruz	Sc-30115	K0405 A1209	1 : 1,000	-
Anti-hBD-3	Mouse (polyclonal)	Sigma	SAB1401 766	10330	1 : 1,000	-
Anti-human β-Actin (clone AC-15)	Mouse (monoclonal)	Sigma	A5441	028K4826	1 : 3,000	-
Anti-rabbit IgG	Donkey (monoclonal)	Amersham	NA934	Different Lot#s	1 : 3,000	HRP
Anti-mouse IgG	Sheep (monoclonal)	Amersham	NA931	Different Lot#s	1 : 3,000	HRP

Table 2 Antibodies used for western blotting. Cat# = catalogue number, HRP = horseradish peroxidase, Lot# = lot number.

2.5.3 Antibodies for ELISA

Antibody reactivity	Source (clonality)	Company	Cat#	Lot#	Coupled with
Anti-hBD-3 capture	Rabbit (polyclonal)	Antigenix America	RHF 771CK	11	-
Anti-hBD-3 tracer	Rabbit (polyclonal)	Antigenix America	RHF 771CK	11	Biotin

Table 3 Antibodies used for ELISA. Cat# = catalogue number, Lot# = lot number.

2.5.4 Antibodies and microbeads for MACS sorting

Reactivity	Source	Company	Product#	Lot#	Coupled with
Anti-human HLA-DR Antibody	Mouse	BD Pharmingen	555811	03903	FITC
Anti-human CD14 microbeads	Mouse	Miltenyi	130-090-879	n.a.	Magnetic bead
Anti-FITC microbeads	n.a.	Miltenyi	120-000-293	5110617061	Magnetic bead

Table 4 Antibodies and microbeads used for magnetic-assisted cell sorting (MACS). FITC = fluorescein isothiocyanate, n.a. = information not available.

2.6 Cells

2.6.1 Cell lines

For the experiments the human gastric adenocarcinoma cell line MKN45 was used as standard cell line. MKN45 cells were purchased at DSMZ (ACC 409). This cell line was first isolated from a 62 year old woman and originates from a liver metastasis of a poorly differentiated gastric adenocarcinoma of the diffuse type. It shows low levels of endogenous epidermal growth factor (EGF) expression and transforming growth factor α (TGF-α) production and a low level of EGFR mRNA (Yokozaki, 2000). As a control cell line, MKN28 cells were used. This cell line originates from a metastatic lymph node of a well differentiated intestinal type gastric adenocarcinoma of a 70 year old female (Naito et al., 1984). Compared to MKN45 it shows higher levels of EGF and TGF-α (Yokozaki, 2000). Additionally the human gastric cancer cell line AGS, purchased at ATCC (CRL-1739), was used for the ELISA experiments. These cells originate from a 54 year old female suffering from a gastric adenocarcinoma.

2.6.2 Cell culture

Cell culture was performed under a working bench with a laminar flow to avoid contamination. Unless indicated otherwise, the following media were used for all experiments:

Standard culture medium:		Starvation medium:	
Gibco RPMI 1640		Gibco RPMI 1640	
L-Glutamine:	2.05 mM	L-Glutamine:	2.05 mM
hiFCS:	10%		

Washing of cells:

Gibco Dulbecco's Phosphate-Buffered Saline (DPBS), without calcium or magnesium

Cells were all grown in standard culture medium in 75 mm^2 culture flasks in the cell incubator at 37°C with 5% CO_2 in a water saturated atmosphere. They were split every second to third day when reaching 80 - 90% confluence. Medium, PBS and trypsin were pre-warmed to 37°C in a water bath prior to use. AGS cells were split at a ratio of 1 : 10, MKN28 and MKN45 at a ratio of 1 : 3. For splitting, cells were washed once with PBS. Subsequently, 1 ml of trypsin was added. Cells were incubated 5 min at 37°C with the trypsin until the cells detached from the flask. Trypsinized cells were resuspended in

RPMI supplemented with 10% hiFCS and split at the ratio mentioned above. Cells were cultured in a total volume of 12 ml medium and used for experiments from passage 5 to 20.

2.6.3 Seeding of cells

For performing experiments, cells were seeded in different formats of multi-well plates. On reaching 80 - 90% confluence in a 75 mm² flask (~$8x10^6$ cells), they were washed and trypsinized. Cells were then diluted in RPMI supplemented with 10% hiFCS and seeded in the wells. An exact overview of culturing and seeding conditions is given in table 5.

Cell line	Culture medium used	Passage used	Cell splitting	Cells seeded in 6 well	Cells seeded in 12 well	Cells seeded in 48 well
AGS	RPMI, HEPES[-], glutamine[+], 10% hiFCS	5 - 20	Every second day 1 : 10	~$2x10^5$ in 2 ml medium	~$1x10^5$ in 1 ml medium	~$2.5x10^4$ in 200 µl medium
MKN28	RPMI, HEPES[-], glutamine[+], 10% hiFCS	5 - 20	Every second day 1 : 3	~$4x10^5$ in 2 ml medium	~$2x10^5$ in 1 ml medium	~$5x10^4$ in 200 µl medium
MKN45	RPMI, HEPES[-], glutamine[+], 10% hiFCS	5 - 20	Every second day 1 : 3	~$4x10^5$ in 2 ml medium	~$2x10^5$ in 1 ml medium	~$5x10^4$ in 200 µl medium

Table 5 Cell culturing conditions, media used for culturing, the cell passages in which the cells were used, the ratio and frequency at which cells were split, as well as the number of cells seeded in different multiwell formats (6, 12, 48 wells) are given.

2.7 Bacteria

2.7.1 Bacterial strains

The P12 wild type (wt) strain was used as a standard for infections and P1wt strain served as a control in some experiments. In table 6, an overview over bacterial strains is given.

Strain	Type of strain	Isolate number	Origin	Resistances	Reference
P12wt	Type 1	888-0	Clinical isolate, University of Hamburg	Nystatin, trimetoprim, vancomycin	(Schmitt and Haas, 1994)
P1wt	Type 1	69A	Clinical isolate, University of Amsterdam	Nystatin, trimetoprim, vancomycin	(Haas et al., 1993)

Table 6 List of bacterial strains used in the experiments, the original number and origin of the clinical isolate and the resistances against antibiotics are given.

2.7.2 Bacterial culture

Standard agar plates:		Liquid culture:	
Gonococci (GC) Agar in H_2O:	36 mg/ml	Brain heart infusion in H_2O:	36%
Horse serum, inactivated:	10%	hiFCS:	10%
Vitamin-Mix:	1%		
Nystatin:	1 µg/ml		
Trimetoprim:	5 µg/ml		
Vancomycin:	10 µg/ml		

H. pylori strains were stored frozen at -80°C in a glycerol stock in aliquots. Bacteria were thawed and plated out on CG agar plates which were supplemented as mentioned above. Bacteria were grown for 3 days on a plate under microaerophilic conditions (5% CO_2, 4% O_2) at 37°C before first overplating. After 3 days, *H. pylori* was streaked out on fresh plates and cultured overnight. This was the earliest time point at which bacteria were used for experiments. The bacteria were subsequently replated every day and used for a maximum of one week.

2.7.3 Liquid culture

For creating a liquid culture, *H. pylori* was grown on a plate as described above for one day, resuspended in 10 ml BHI supplemented with 10% hiFCS and the OD was determined. Bacteria were grown in liquid culture overnight on a shaking platform under microaerophilic conditions at 37°C. Microaerophilc conditions on the shaker were

created by culturing bacteria in air-tight jars with oxygen absorbing CampyGen® sachets. Bacteria cultured overnight were washed and resuspended in RPMI and the CFU/ml was determined by measuring the OD.

2.7.4 Infection protocol

For infection, cells were pre-starved overnight in starvation medium and infection was also carried out under serum-free conditions. Bacteria were streaked out in a thin layer the day before infection on fresh agar plates. For infection, *H. pylori* was harvested with a swab and resuspended in 5 ml of serum-free RPMI and the optical density (OD) of the suspension was measured. For determining the number of bacteria in this suspension a calibration curve was used. This matches the OD of the suspension to the amount of bacteria. Therefore a standard curve needed to be set up. For this purpose a serial dilution of *H. pylori* suspensions was created and the OD of each dilution step was determined. The dilutions were then all plated out and the colony forming units per ml (CFU/ml) present in each dilution were counted and assigned an OD. With this calibration curve the CFU/ml of the bacterial suspension can be directly determined from its OD. The number of cells that were infected was calculated from the size of the wells and the confluence of cells. So the correct multiplicity of infection (MOI), which is the ratio of bacteria per cell, was determined using the following formula:

$$\mu l \text{ bacterial suspension} = \frac{\text{cell number x MOI}}{\text{CFU/ml}} \times 1{,}000$$

The calculated volume of bacterial suspension was directly added to the overnight starved cells without changing the medium.

2.8 Bacterial preparations

2.8.1 Creation of culture supernatants

Bacteria were grown in liquid culture as described above. The liquid culture was transferred to a 50 ml tube and spun down at 4,600 RPM for 5 min. The supernatants were taken and filtered with a 0.22 µm filter to remove bacteria. 25 µl of filtered supernatant was plated out as a test that bacteria were completely removed.

2.8.2 Gentamycin killed *H. pylori*

Bacteria were grown in liquid culture overnight and were subsequently transferred to a 50 ml tube and spun down at 4,600 RPM for 5 min. The bacteria were resuspended in BHI with 10% hiFCS and the CFU/ml was determined via the OD of the suspension.

Bacterial concentration was adjusted to a MOI of 100 according to the number of cells. Gentamycin at a concentration of 20 µg/ml was added and cells were placed in the incubator for 2 h. 25 µl of the gentamycin-treated suspension was plated out as a test that bacteria were completely killed.

2.8.3 Sonication of bacteria

For the creation of bacterial lysate, bacteria were sonicated. Sonication works via the application of high-frequency mechanical vibration to a suspension via a probe. The probe oscillates at ~20 kHz which leads to the formation of small vacuum cavities in the liquid that implode in microscopic shockwaves. This cavitation disrupts bacterial membranes and a lysate is created.

Bacteria were harvested from the plate, resuspended in 20 ml ice-cold PBS and spun down at 2,500 RPM for 25 min at 4°C. After centrifugation, the supernatant was discarded and the pellet resuspended in 7 ml ice-cold PBS containing complete protease inhibitor. The OD of the bacterial suspension was determined and the amount of bacteria was adjusted to $1x10^9$ CFU/ml. Bacterial suspension was placed in an ice bucket to prevent it from heating up during sonication. The sonifier “sonifier 450” from Branson was used which was cleaned with ethanol before use and the suspension was sonicated at output level 4 at intervals of 10 repeats à 20 seconds with a 10 second break in between to allow the sample to cool down. The sonicated sample was spun down for 25 min at 14,000 RPM and the supernatant was sterile-filtered with a 0.22 µM filter.

2.9 Cytokine treatment of cells

For treatment of cells with cytokines, cells were starved overnight. Cytokines were thawed directly before use and added to the cells. The multiwell plates were shaken after adding the cytokines and incubated in the cell culture incubator. An overview of the cytokines used is given in table 6. When cells were simultaneously infected and cytokine-treated, the infection protocol was performed as described above. Directly after infection, cytokines were added to the infected cells at their respective concentrations.

2.10 Protocol for assessing intraepithelial crosstalk

For the auto- and paracrine stimulation of MKN45 cells via epithelial cell supernatants, cells were infected with P12wt following standard infection protocol. After different time

points, supernatants of the cells were collected with a syringe and sterile-filtered with a 0.22 µM filter. For stimulation of untreated cells, these supernatants were added to uninfected, serum-starved MKN45 cells. Cells were lysed after different time points in Laemmli buffer and used for western blotting.

2.11 Western blot

2.11.1 Western blot protocols

For detecting hBD-3 by western blot, a protocol established by Bauer et al. (2012) was applied. In the course of this work, two different antibodies were used for detecting hBD-3. The first antibody, a rabbit polyclonal, was purchased from Santa Cruz, product number Sc-30115, the other antibody was a mouse polyclonal from Sigma, product number SAB1401766. The western blot protocol was adjusted according to each antibody. Differences in the protocols are listed in table 7. As seen in figure 2.1, the two antibodies showed comparable results.

Antibody (Cat#)	Lysis	SDS-PAGE	Transfer	Blocking solution	Antibody Incubation
Anti-hBD-3 Santa Cruz (Sc-30115)	RIPA-lysis or lysis directly in Laemmli buffer	Denaturing, discontinuous SDS-PAGE, TRIS/glycin buffer	Wet blot/ semi-dry blot	TBS-T 5% milk, 3% BSA	TBS-T 5% milk, 3% BSA
Anti-hBD-3 Sigma (SAB1401766)				TBS-T 3% BSA, filtered	TBS-T

Table 7 Comparison of different western blot protocols. Cat# = catalogue number.

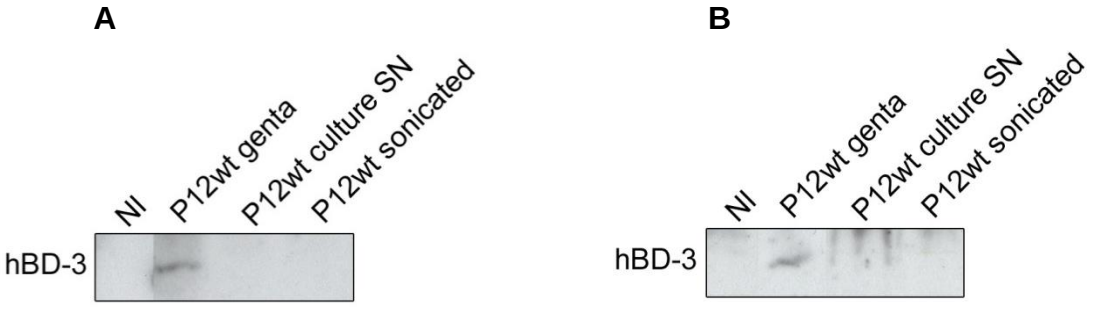

Figure 2.1 Comparison of anti-hBD-3 antibodies from (A) Santa Cruz and (B) Sigma. Samples from figure 3.1 (B) were run with two different blotting protocols and two different anti-hBD-3 antibodies. (A) shows the results for the Santa Cruz antibody and (B) the results for the Sigma antibody.

2.11.2 Methodical background of cell lysis and SDS-PAGE

In a first step, cells have to be lysed in order to detect intracellular proteins. Therefore, they are treated with Laemmli buffer or RIPA lysis buffer, containing anionic detergents like sodium dodecyl sulfate (SDS) or sodium deoxycholate that disrupt the cellular membrane. Simultaneously, SDS in the Laemmli buffer binds to the proteins leading to denaturation and masking of the proteins' charge. As a result proteins are all evenly negatively charged and now differ only in size. In the subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins start wandering towards the anode and are separated in a polyacrylamide gel according to their electrophoretic mobility. Here, the polyacrylamide running gel, a polymer of acrylamide and bisacrylamide adjusted to the pH of 8.8, acts as a filter where smaller proteins migrate faster and as a result proteins are arranged according to their size. In order to create sharp distinguishable bands, a discontinuous SDS-page is performed where proteins are stacked before separation. A stacking gel with low percentage polyacrylamide at pH of 6.8 is cast on top of the lower running gel. Stacking of the proteins is dependent on chloride and glycine found in the running buffer. Chloride ions, which are negatively charged and migrate rapidly, run in front of the proteins. The glycine, at pH 6.8, is almost neutrally charged, migrates more slowly, behind the proteins. Proteins are stacked in the electric field that is created between glycine and chloride ions. When the protein running front enters the running gel, the pH changes to 8.8 and glycin turns to anionic glycinate. It passes the proteins which are slowed down due to friction of the high percentage polyacrylamide running gel and the gradient of field strength between glycine and chloride is abolished and proteins start separating.

<u>Buffers for Cell lysis and SDS-PAGE</u>

RIPA buffer:

Tris:	25 mM
NaCl:	150 mM
Triton x-100:	1% (v/v)
Sodium doxycholat:	0.5% (w/v)
SDS	0.1% (w/v)
PMSF	1 mM

Adjusted to pH 7.6; complete protease inhibitor and 100 µM orthovanadate added

4x Laemmli buffer:

Tris:	250 mM

Adjusted to pH 6.8 with HCl

Glycerol:	5.47 M
SDS:	5.5 mM
Bromphenol:	Traces
β-mercaptoethanol:	713 mM

used diluted to 1x with Milli-Q H_2O

Upper buffer:		Lower buffer:	
Tris:	500 mM	Tris:	1.5 M
SDS:	14 mM	SDS:	14 mM
Adjusted pH 6,8 with HCl		Adjusted pH 8,8 with HCl	

SDS Running buffer:		10% APS:	
Glycine:	192 mM	APS:	10% (w/v)
Tris:	25 mM	in Milli-Q H_2O	
SDS:	3.46 mM		

2.11.3 Experimental procedure of cell lysis and SDS-PAGE

For lysis with RIPA buffer, all steps were performed on ice. Medium of cells was removed, cells were washed with ice-cold PBS and pre-cooled RIPA buffer was added. Cells were scraped into the buffer and transferred into pre-cooled microtubes where they were lysed for 30 min by vortexing all 10 min. Cellular debris was spun down and supernatant was isolated. 4x Laemmli buffer was added to the lysate in a volume that a final concentration of 1x was achieved.

Alternatively, cells were directly lysed with Laemmli buffer. Therefore, the culture medium of cells was discarded, 1x Laemmli buffer was directly added to the cells and the lysate was transferred to microtubes. All lysates were boiled and shaken at 95°C in a thermo shaker for 10 min and stored at -20°C.

For separation, Bio-Rad Mini-PROTEAN system was used with a Tris-glycin running buffer system and a discontinuous gel with a 3% stacking gel and a 17% running gel. The gels were prepared as shown in table 8. For running the SDS-PAGE, gels were placed in a mini tank and filled completely with fresh 1x SDS running buffer. In the first or last lane, 6 µl of a pre-stained protein ladder was loaded. The protein samples were thawed and loaded on the gels. For cells lysed in RIPA buffer, a total of 20 µl was loaded and for the cells directly lysed in Laemmli buffer 10 µl was used. The gels were run for 30 min at 60 V until the running front reached the running gel. Then voltage was increased to 120 V and gels ran for 120 min until the 17 kilodalton (kDa) and 10 kDa marker lanes were clearly separated at the end of the gel.

	17% Running gel	3% Stacking gel
Buffer	Lower buffer pH 8.8 24.69% (v/v)	Upper buffer pH 6.8 24% (v/v)
30% Acrylamide	56.78% (v/v)	9.9% (v/v)
Milli-Q H_2O	17.44% (v/v)	64.8% (v/v)
TEMED	0.074% (v/v)	0.2% (v/v)
10% APS	0.89% (v/v)	1% (v/v)

Table 8 Preparation of the running and staking gel for the SDS-PAGE.

2.11.4 Methodical background of protein transfer and immunostaining

For transfer, proteins via electroblotting are mobilized from the polyacyrlamide gel and via electric current transferred to a polyvinylidene difluoride (PVDF) membrane, ensuring minimized lateral diffusion. The technique can be performed either with a semi-dry machine or in a wet blot tank. The PVDF membrane shows unspecific binding of proteins based on hydrophobic and charge-mediated interactions. Protein bound to the membrane is exposed in a thin layer on the surface of the membrane and can be detected via antibodies. In order to minimize the unspecific protein binding capacity after transfer, the membrane is blocked with BSA or milk powder. The protein of interest can be detected by adding a specific primary antibody directed against the protein and a secondary horse radish peroxidase (HRP) coupled antibody directed against the primary antibody. When a developer reagent is added containing the chemiluminescent agent luminol the antibody bound HRP in the presence of H_2O_2 oxidizes luminol and in this process light is emitted. This can be detected by a film placed on top of the membrane. In order to ensure that a consistent amount of protein is loaded in different lanes a constitutively expressed protein is monitored as loading control. β-actin is a well-established and widely used control, and was chosen as internal loading control for this study.

<u>Buffers for western blot</u>

Semi-dry blot transfer buffer:		Wet blot transfer buffer:	
Tris:	48 mM	Tris-HCl:	25 mM
Glycine:	39 mM	Glycine:	192 mM
SDS:	1.3 mM	SDS:	0.1% (w/v)
Methanol:	10% (v/v)	Methanol:	10% (v/v)

Blocking buffer Sigma antibody:

1x TBS-T
BSA: 3% (w/v)
Stirred for 20 min and filtered with a 0.22 µM filter

Blocking buffer Santa Cruz antibody:

1x TBST
Milk powder: 5% (w/v)
BSA: 3% (w/v)

1x TBS-T:

Tris: 20 mM,
NaCl: 140 mM
Tween-20: 0.1% (v/v)

Stripping buffer:

Tris: 68.5 mM
Adjusted to pH 7.5 with HCl
SDS: 69 mM
β-mercaptoethanol: 142 mM

2.11.5 Experimental procedure of protein transfer

For semi-dry blotting, PVDF membrane and Whatman blotting paper were cut to the size of the gel. Blotting paper was soaked with 1x transfer buffer and the PVDF membrane was activated with methanol and subsequently equilibrated in filtered transfer buffer. The running gels from SDS-PAGE were also equilibrated in running buffer and a pile in the order: anode - 2 blotting papers - membrane - gel - two blotting papers - cathode was created on the semi dry blotting apparatus. The machine was assembled and the blotting ran for 90 min at 6 mAh per gel.

For wet blot, PVDF membrane and Whatman blotting paper were cut bigger than the gel and wet-blot transfer buffer was used to equilibrate membranes. A pile in the order: sponge - blotting paper - gel - membrane - blotting paper - sponge was created and placed in the tank so the membrane was facing the anode. The wet blot tank was filled with buffer and a cooling device with a temperature of - 80°C was added. Blots were run on a magnetic stirrer at 4°C overnight at 100 mA per gel.

When using the Santa Cruz Antibody, membranes after transfer were blocked in TBS-T with 3% BSA and 5% milk for 1 h. Then the membrane was incubated with the primary antibody that was diluted 1 : 1,000 in TBST with 3% BSA and 5% milk overnight on a roller platform at 4°C. For the Sigma antibody membranes after semi-dry blotting were blocked in TBS-T with 3% BSA for 1 h. Afterwards the membranes were washed in TBS-T for 10 minutes. The Sigma anti-hBD-3 antibody was diluted 1 : 1,000 in TBS-T without the addition of blocking proteins and incubated overnight on a roller platform at 4°C. After that membranes were washed for 30 minutes in TBS-T the washing buffer being exchanged every 10 minutes. Blots were incubated for 1 h with secondary antibody at a dilution of 1 : 3,000 in TBS-T at room temperature. Blots were

subsequently washed and placed in a transparent plastic booklet and 2 ml of freshly mixed developer reagent was added per blot. In the dark room, the signal was detected by placing a film on the membranes, exposure time was varied between 30 sec - 15 min according to signal intensity. Films were developed, fixed, scanned and labeled.

2.11.6 Experimental procedure of actin staining

Blots were cut in two parts between the 30 kDa and the 17 kDa marker band. The upper part of the membrane with larger proteins was incubated with the anti-actin antibody in a 1 : 3,000 dilution in TBS-T overnight at 4°C. The incubation with the corresponding secondary antibody and the protein detection were done according to the protocol mentioned above. Blots already incubated with another antibody prior to actin staining were stripped. For this purpose, membranes were activated in methanol and treated with stripping buffer at 50°C for 20 min. Membranes were washed and incubated with anti-actin antibody diluted 1 : 3,000 in TBS-T overnight at 4°C. Incubation with the secondary antibody and detection were done following the protocol mentioned above.

2.11.7 Quantification of blots

Lanes of blots were quantified by comparing mean grey values of different bands, using the program ImageJ. The grey value of a certain area of the blot comprising one single band was measured and from this measurement the background grey value of the blot was subtracted. The values of the hBD-3 bands were normalized to the corresponding grey values of the actin bands of the same sample. These normalized values were again normalized to the value of the untreated control.

2.12 Enzyme-linked immunosorbent assay

2.12.1 Methodical background of ELISA

The enzyme-linked immunosorbent assay (ELISA) detects proteins in a liquid sample with specific antibodies via development of color. In the sandwich ELISA used in this study, a specific capturing antibody directed against a defined antigen is attached to a plastic surface of a polystyrene microtiter plate. When adding a sample, the antigen is immobilized on the plate and can be detected by a biotin-coupled tracer antibody directed against the same antigen. Now, a sandwich consisting of the capture antibody, the specific antigen and the tracer antibody is created. In a next step, HRP coupled with

streptavidin is added which binds to the biotin of the tracer antibody. 3,3',5,5'-tetramethylbenzidine (TMB) is added as substrate of the HRP. TMB is oxidized via HRP and the oxidized product 3,3',5,5'-tetramethylbenzidine diimine has a blue color. The color change in the solution can be measured by spectrophotometry as an increase in the OD. In order to quantify the results a standard with defined protein concentrations is run with each ELISA. For this purpose, the OD of the samples can be compared to the OD of the standards.

2.12.2 Experimental procedure of ELISA

For ELISA an hBD-3 detection kit from Antigenix America (Product number: RHF771CK) was used. Tracer and capture antibodies, coating stabilizer, streptavidin coupled HRP, the hBD-3 protein standard and the developer reagents were provided with the kit.

Buffers for ELISA

Diluent:		Washing buffer:	
Tween-20 in PBS:	0.05% (v/v)	PBS	
BSA:	0.1% (w/v)	Tween-20:	0.05% (v/v)

For ELISA, Nunc Maxisorp 96 well plates with a flat bottom were used. For coating of the plate, capture antibody was diluted with 1x PBS to a concentration of 0.5 µg/ml. 100 µl was added to each well and the plate was sealed, incubated overnight at room temperature and subsequently washed and then blocked for 1 h. For performing ELISA, an hBD-3 standard twofold dilution series was created ranging from 2 ng/ml to 0.03125 ng/ml. Standard and samples were added in triplicate to the plate and incubated for 2 h at room temperature. After that, plates were washed 4 times with washing buffer which was done after each of the following steps. 100 µl of tracer antibody was added at a concentration of 0.2 µg/ml for 1.5 h. In a next step wells were washed and incubated with streptavidin-HRP conjugate diluted 1 : 1,000 for 30 min. Unbound streptavidin-HRP was washed away and TMB containing substrate solution mixed 1 : 1 with the oxidizing solution was put into the wells. The plate was put in a dark place and the color development was monitored. After ~10 minutes reaction was stopped with 100 µl of 2 N sulfuric acid. The plate was read at 450 nm with a spectrophotometer and data was analyzed using the SoftMaxPro software.

2.12.3 Creating and concentrating supernatants for ELISA

In order to bring supernatants to the detection range of the ELISA, they were concentrated via ultrafiltration. A higher volume of supernatant was needed and infection was carried out in 100 mm cell culture dishes in ~6 ml of medium. As it was important to avoid cell death in order not to interfere with the ELISA by cellular debris, cells were grown to a confluence of maximum 70% when starting the experiments and cells were not starved prior to infection. Before starting infection, cells were washed with serum-free medium and experiments were performed under serum-free conditions to avoid interference from serum proteins. After performing experiments, supernatants were collected and the exact volume was determined. For subsequent concentration, an ultrafiltration spin column was used. In this device samples are pressed by centrifugal force through a polyethersulfone (PES) membrane with a specific molecular weight cut off (MWCO) of 3,000 Da. All particles bigger than the MWCO of the membrane are concentrated in the upper part of the ultrafiltration spin column. Here, supernatants were concentrated ~10 times to a final volume of ~600 µl in a centrifuge at 4,000 RPM at 15°C for 1 - 2 h. The exact volume of the concentrated samples was noted in order to calculate the concentration factor. Concentrates were then stored at -80°C.

2.13 Co-cultivation of dendritic cells and T-cells

2.13.1 Methodical background

For mimicking the adaptive immunity in vitro, a DC/T-cell co-cultivation model was used following a well-established protocol (Fehlings et al., 2012; Lai et al., 2011; Romani et al., 1994; Sallusto and Lanzavecchia, 1994). The theoretical background of this method is that DCs can be differentiated from $CD14^+$ monocytes. Monocytes are isolated via MACS from peripheral blood monocytes (PBMCs). For MACS cells are specifically labeled with magnetic microbeads that are coupled with antibodies so they can be separated in magnetic columns, since labeled cells adhere to the columns while the unlabeled ones rinse through. The isolated monocytes, under the influence of IL-4 and GM-CSF, differentiate towards a DC-like phenotype (Fehlings et al., 2012; Sallusto and Lanzavecchia, 1994). These monocyte-derived DCs are either infected with *H. pylori,* leading to antigen presentation and cytokine production, or treated with LPS which is mainly an inducer of DC cytokine production (Fehlings et al., 2012; Kranzer et al., 2004). In a next step, T-cells are obtained from the CD14-depleted PBMCs via MACS by additional depletion of $HLA\text{-}DR^+$ cells. HLA-DR is expressed on macrophages, DCs,

B-cells, activated T-cells and NK cells. So these cells are removed after MACS sorting and untouched T-cells are isolated (Fehlings et al., 2012). These autologous $CD4^+$ T-cells are subsequently added to the activated DCs and co-cultivation of DCs and T-cells leads to T-cell proliferation (Fehlings et al., 2012).

2.13.2 Experimental procedure for differentiation of dendritic cells

PBMCs were isolated from human buffy coat and separated from erythrocytes, granulocytes and plasma via Ficoll density gradient centrifugation. Isolated PBMCs were washed and resuspended in 20 ml complete RPMI 1640 medium containing 2 mM L-glutamine, 25 mM HEPES, 10% hiFCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were counted and dead cells were excluded by trypan blue staining. PBMCs were resuspended in MACS buffer and incubated with CD14 microbeads for 15 min at 4°C, washed and after that put on the MACS separation columns. After rinsing through, unbound cells were washed out of the columns and the rinse through, depleted by $CD14^+$ cells, was stored in the cell culture incubator for 6 days in complete RPMI medium for subsequent isolation of T-cells. $CD14^+$ cells attached to the MACS columns were collected by removing the columns from the magnets and rinsing them. $CD14^+$ cells were seeded in 6 well plates in quantities of $3x10^6$ cells/well in 3 ml complete RPMI 1640 medium. 1,000 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 100 U/ml recombinant IL-4 were added to initiate the differentiation of the monocytes to DCs. Differentiation took 6 days and at days 2, 4 and 6, 200 µl of fresh medium containing 1,000 U/ml GM-CSF and 100 U/ml IL-4 was added to the cells. On day 6, immature DCs were harvested from the wells, counted and dead cells were excluded by trypan blue staining. DCs were washed and seeded in 12 well plates with $5x10^5$ cells/well in fresh, antibiotics free RPMI medium for infection. DCs were infected with P12wt MOI of 10, treated with *E. coli* LPS (100 ng/ml) or treated with medium as negative control and spun down to synchronize infection. After 1 h DCs had phagocytosed bacteria and subsequently infection was terminated to kill non-phagocytosed bacteria by washing cells and resuspending in antibiotics containing complete RPMI 1640 supplemented with 1,000 U/ml GM-CSF and 100 U/ml IL-4. Cells were seeded in 12 well plates and incubated for 24 h. In this period the DCs could process phagocytosed bacteria for antigen presentation.

2.13.3 Experimental procedure for T-cell isolation and co-cultivation

T-cells were isolated from $CD14^+$ depleted PBMCs by additional depletion of $HLA\text{-}DR^+$ cells. PBMCs were washed and resuspended at a ratio of $1x10^8$ cells/ml in MACS buffer. First, cells were incubated with FITC conjugated anti-HLA-DR antibodies for 15 min, washed and then incubated with anti-FITC-Beads. Cells were washed again, resuspended in MACS buffer and used for MACS sorting. The rinsed through fraction was now depleted of $HLA\text{-}DR^+$ cells and contained the T-cells. The latter were washed 2 times and resuspended in complete RPMI medium at a concentration of $2x10^6$ cells/ml. Autologous T-cells were added to the *H. pylori*- or LPS-pulsed DCs. This was performed in 24 well plates with $1x10^4$ DCs/well and $5x10^5$ T-cells so the ratio of DCs to T-cells was 1 : 50. The complete volume of the co-cultivation was 250 µl/ well. Cells were put into the cell culture incubator and after days 3 and day 6 supernatants were collected, sterile-filtered with a 0.22 µM filter and stored at -80°C.

2.13.4 Treatment of epithelial cells with DC/T-cell supernatants

For treatment of epithelial cells with DC/T-cell supernatants, MKN45 cells were seeded in 48 well plates at a density of ~$5x10^4$ cells/well. They were grown for one day in RPMI 1640 culture medium and then serum starved overnight in starvation medium. DC/T-cell supernatants were thawed and brought to 37°C. Starving medium was removed and 100 µl of different DC/T-cell supernatants were added to the wells without diluting. Cells tested for another condition were simultaneously infected with P12wt, MOI of 20. Infection was done following standard infection protocol. Cells were lysed after 24 h in 30 µl 1x Laemmli buffer.

2.14 Cellular viability assay

2.14.1 Methodical background of cellular viability assay

For quantifying cellular viability, a bioluminescence-based cell viability assay can be used, which measures the amount of adenosine triphosphate (ATP) in a cell culture system. This is an indicator of cellular viability, as the amount of ATP is directly proportional to the number of metabolically active cells. In order to quantify the amount of ATP present in one well, cells are lysed and ATP is released. In an enzymatic reaction, driven by the ATP released from the cells, luciferase activates luciferin which emits light as it decays. The intensity of the emitted light can be measured and so the intensity of the luminescence reaction is dependent on the cellular amount of ATP.

2.14.2 Experimental procedure of cellular viability assay

MKN45 cells were grown in 96 well cell culture plates and ~2,5x10^4 cells/well were seeded. Cells were grown for 1 day in RPMI with 10% hiFCS. Cells were then starved overnight with RPMI. Following this, cells under serum starved conditions were infected with P12wt or P1wt, infected and simultaneously treated with IFN-γ or only treated with IFN-γ. For determining the cellular viability, the commercially available CellTiter-Glo Luminescent Cell Viability Assay from Promega was used. The protocol was done following the manufacturer's recommendation. Cells after 48 h treatment were incubated with the CellTiter-Glo reagent. For this purpose, the substrate and the buffer were added to the wells and the plate was shaken for 2 min on a shaking platform to lyse cells. Luminescence was measured, 5 seconds/well. Background luminescence was determined in cell-free medium. Values were all normalized to the untreated control, which was set as 100% viability.

3. Results

3.1 hBD-3 induction upon *H. pylori* infection

3.1.1 Analysis of different bacterial components as hBD-3 inducers

Knowledge about bacterial factors which mediate hBD-3 expression remains fragmentary. In order to analyze the mechanisms which lead to *H. pylori*-mediated hBD-3 induction, different bacterial fractions were tested for their efficiency in inducing defensin expression in gastric epithelial cells. Therefore, MKN45 cells were incubated with bacterial preparations and analyzed for hBD-3 production. Even though it was shown by Bauer et al. (2012) that heat-inactivated *H. pylori* is not able to induce defensin expression, proteins are normally denatured under heat inactivation thereby increasing the possibility for wrong interpretations. Hence, *H. pylori* P12wt suspension was sonicated and the bacterial debris was spun down in order to isolate the soluble fraction. It contains a mixture of different *H. pylori* PAMPs like bacterial proteins, DNA, LPS and other possible factors that can trigger the innate immune response. The soluble fraction was sterilized by filtering and added to the cells. Furthermore, culture supernatants of an *H. pylori* overnight culture were sterilized by filtering and put on the cells. These broth culture supernatants were chosen as it has been reported that *H. pylori* is capable of shedding immunogenic factors into culture supernatants like OMVs (Viala et al., 2004a) or VacA (Cover and Blanke, 2005). In addition, *H. pylori* was incubated with 20 µg/ml gentamycin for 2 h in order to efficiently kill the bacteria for later induction assays. Killing efficacy was analyzed for all methods by checking bacterial growth on agar plates. Sonicated bacterial fractions and culture supernatants were sterile, though in gentamycin-treated preparation, minor growth of bacteria was still detectable (data not shown). MKN45 cells were treated for 6 h or 24 h with all preparations respectively (figure 3.1). As positive control, MKN45 cells were infected with *H. pylori* P12wt (MOI 100). Whereas, neither the soluble fraction of the sonicated bacteria, nor the culture supernatants induced any production of hBD-3, the gentamycin-treated bacteria showed a notable induction of hBD-3, though lower than untreated bacteria. This supports the notion that only viable *H. pylori* can induce the production of hBD-3 in gastric epithelial cells, yet a sonicate containing different PAMPs fails to do so.

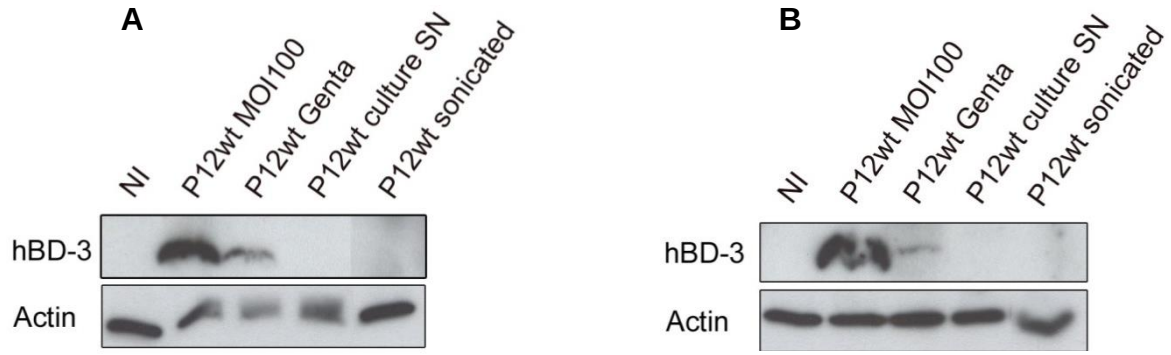

Figure 3.1 Induction of hBD-3 is dependent on viable *H. pylori*. MKN45 cells were incubated with 3 different bacterial preparations for 6 h (A) and 24 h (B). Preparations: P12wt, pre-treated with 20 µg/ml gentamycin (Genta), sterile-filtered culture supernatants of a *H. pylori* overnight liquid culture (culture SN), and sonicated *H. pylori* (sonicated). As positive control, cells were infected with P12wt (MOI 100). Uninfected cells were used as negative control (NI). Cells were lysed with Laemmli buffer and analyzed by western blot for hBD-3 induction. Actin serves as loading control.

3.1.2 Effect of bacterial load on hBD-3 induction

As it could be shown in repeated experiments that the induction is dependent on bacterial viability, the dependency of the level of induction on the amount of viable bacteria was tested. In order to do so, MKN45 cells and MKN28 cells were infected with the *H. pylori* P12wt strain for 24 h with MOI 20 or 100 (figure 3.2). Concordantly with previous findings MOI-dependent hBD-3 expression was clearly detectable by western blot in infected MKN45 and MKN28 cells 24 h post infection. Thus, the level of induction correlates with the load of viable bacteria. Furthermore, it can be seen that the overall induction level in MKN28 is lower than that observed in MKN45 cells.

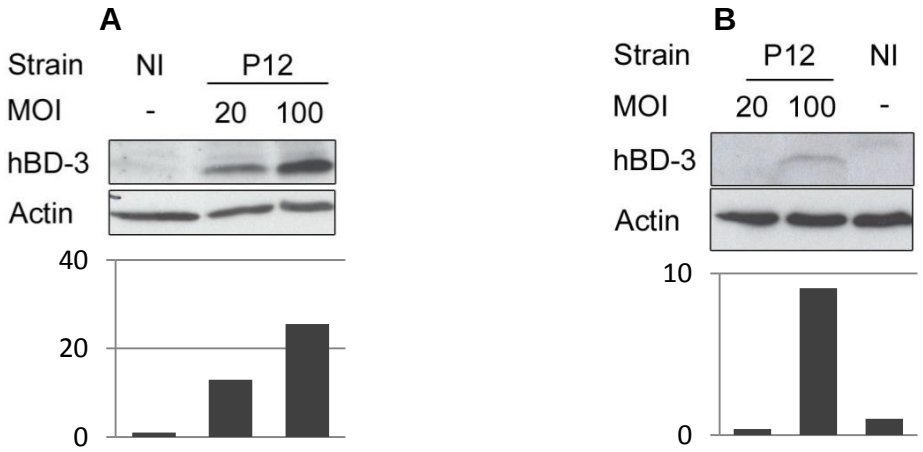

Figure 3.2 Level of hBD-3 induction is *H. pylori* MOI-dependent. Upper panel**:** MKN45 cells (A) or MKN28 cells (B) infected with *H. pylori* P12wt (MOI of 20 or 100) for 24 h. Cell lysates were analyzed for hBD-3 expression using western blot and an anti-hBD-3 antibody. Actin serves as loading control. Lower panel: Quantification of blots via ImageJ.

3.2 Effects of different stimulants on hBD-3 induction of gastric epithelial cells

3.2.1 Paracrine effect of *H. pylori*-triggered factors on hBD-3 induction

Since it could be shown that viable *H. pylori* induces hBD-3 expression, the intraepithelial mechanisms leading to the induction were addressed. A main receptor involved in the induction of hBD-3 is the EGFR (Bauer et al., 2012). It was proposed by Boughan et al. (2006) that an intraepithelial crosstalk via autocrine and paracrine mechanisms leads to the activation of the EGFR following *H. pylori* infection and a subsequent induction of hBD-3. A similar mechanism for the induction of hBD-3 has already been proven in skin keratinocytes (Sørensen et al., 2005). So the hypothesis was addressed that paracrine or autocrine stimuli deriving from infected cells lead to an induction of hBD-3 in uninfected gastric epithelial cells.

For the testing of paracrine stimulation, different conditions were applied. Here, MKN45 cells were infected with bacteria using different MOIs. Supernatants of infected cells were isolated and untreated MKN45 cells were subsequently incubated with respective supernatants (figure 3.3). The effects of putative paracrine stimuli on hBD-3 induction upon infection with *H. pylori* were tested by examining short-term infections with high MOI (A and B) as well as prolonged infections with lower MOI (C). To this end, MKN45 cells were infected with *H. pylori* MOI 200 for 30, 90 and 180 min (A, B) or MOI 20 and 100 for 24 h, respectively (C). Supernatants of infected cells were collected after indicated time points and sterile-filtered. Subsequently, supernatants were added to untreated MKN45 cells. hBD-3 induction in these supernatant-treated cells was assessed using western blot. Although infected cells which were used to create supernatants showed hBD-3 expression, no induction could be observed in any of the conditions where supernatants were applied. Altogether, the data indicates that in this experimental setup, no paracrine stimuli derived from epithelial cells can be identified to be involved in *H. pylori*-mediated hBD-3 induction.

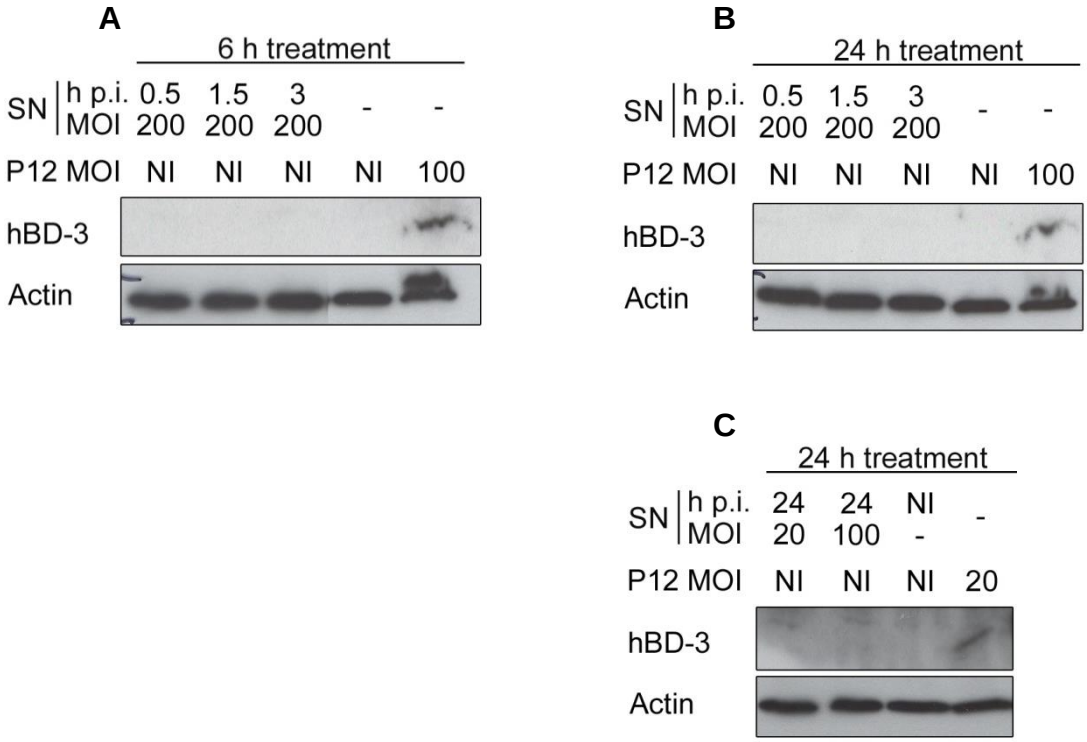

Figure 3.3 Analysis of hBD-3 induction as a response to putative paracrine effects of *H. pylori*-induced cytokines from epithelial cells. For the preparation of different supernatants (SN), MKN45 cells were infected with P12wt (MOI 200) for 0.5, 1.5 or 3 h (h p.i.), (A and B) or P12wt (MOI 20 or 100) for 24 h (C). Supernatants were collected and added to uninfected (NI) MKN45 cells. Cells were incubated with respective supernatants for 6 h (A) or 24 h (B and C). As positive control, cells were infected with MOI of 100 for 6 h (A) or 24 h (B). Cells which were used for supernatant production were also analyzed for hBD-3 induction (C). Cells were lysed and analyzed by western blotting using an anti-hBD-3 antibody. Actin was monitored as loading control.

3.2.2 Effect of different cytokines on hBD-3 induction in MKN45 cells

The induction of hBD-3 in different cell lines has been shown to be mediated by autocrine and/or paracrine mechanisms (Boughan et al., 2006; Steubesand et al., 2009). The paracrine induction of hBD-3 mediated via e.g. pro-inflammatory cytokines (Albanesi et al., 2007; Joly et al., 2005; Wolk et al., 2004) implicates a possible modulation of innate immune response by adaptive immunity. As the reactivity of gastric epithelial cells to different stimuli is not yet clearly understood the capacity of cytokines and other ligands to induce hBD-3 in gastric epithelial cells was investigated. For this purpose, factors and cytokines were chosen for analyses that have been described before inducing hBD-3.

The effect of histamine was examined as it could be shown that histamine can induce hBD-3 in epithelial cells (Ishikawa et al., 2009a) and it has been reported to be

produced by enterochromaffin-like cells during infection with *H. pylori* (Kidd et al., 1997). Additionally the pro-inflammatory cytokine TNF-α was included in the experiment as it is also a known inducer of hBD-3 in keratinocytes (Albanesi et al., 2007; Joly et al., 2005), and is released by macrophages upon infection with *H. pylori* (Wilson and Crabtree, 2007). Since T-cell cytokines like IFN-γ and IL-17 have been reported to be upregulated during *H. pylori* infections, they were chosen for analysis as well. With regard to IFN-γ, the induction effect on hBD-3 is known in keratinocytes (Albanesi et al., 2007; Joly et al., 2005), for IL-17 induction capacity has so far only been shown for other AMPs (Liang et al., 2006). Moreover, IL-22 was included because it was reported to stimulate hBD-3 expression in keratinocytes (Wolk et al., 2004) and could be shown to be induced in mice infected with *Helicobacter felis* (Obonyo et al., 2011). The pro-inflammatory cytokine IL-1α that has only been reported to induce hBD-2 (O'Neil et al., 2000) was included as well. Other factors that were tested in this experiment were EGFR-related peptides. In the previous experimental setup it was demonstrated that supernatants of infected epithelial cells could not induce hBD-3. Yet, as activation of the EGFR is a crucial step in the induction of hBD-3 (Bauer et al., 2012), the effect of high concentrations of different recombinant EGFR ligands on hBD-3 production was tested. TGF-α has been reported to induce hBD-3 in a para- and autocrine way via an activation of the EGFR in keratinocytes (Sørensen et al., 2005). Furthermore, hbEGF was shown to be released during *H. pylori* infection and to transactivate the EGFR (Wallasch et al., 2002) and therefore these two EGFR-related peptides were included in the investigation. Figure 3.4 gives an overview of the effect of different stimuli on hBD-3 production in MKN45 cells.

MKN45 cells were treated with different concentrations of recombinant cytokines for 24 h. As a control, cells were either infected with P12wt or left untreated. Whereas infection assays lead to varying hBD-3 induction, figure 3.4 demonstrates no detection of hBD-3 induction when cells are treated with any of the cytokines listed.

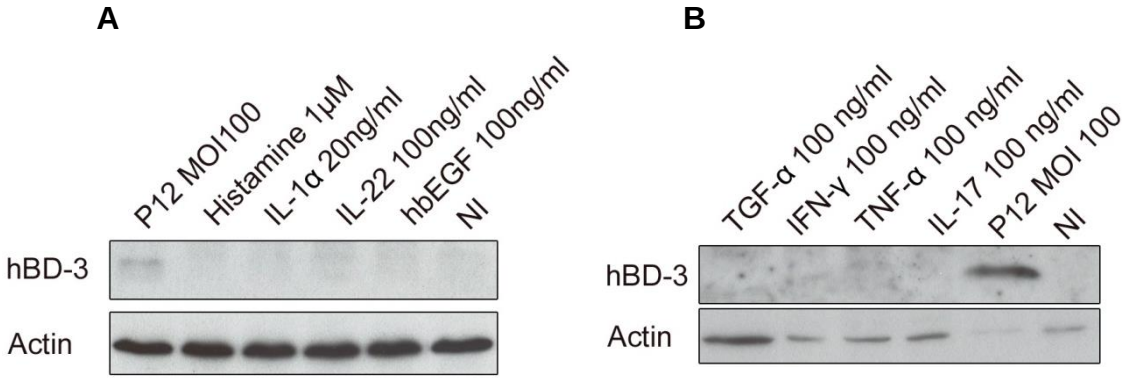

Figure 3.4 Analysis of hBD-3 induction in MKN45 cells in presence of different cytokines. MKN45 cells were treated with different cytokines at indicated concentrations for 24 h. As positive control, cells were simultaneously infected with P12wt (MOI 100). As negative control, untreated cells were used (NI). Cells were lysed and analyzed for hBD-3 induction using western blot. Actin serves as a loading control.

3.2.3 Effect of different cytokines on hBD-3 induction in MKN28 cells

To exclude a cell line-dependent effect, the experiment was repeated with MKN28 cells (figure 3.5). Here, the same result as that with MKN45 cells was observed. Only infection produced a clear and strong induction of hBD-3. Since it has been reported that the combination of different cytokines leads to a stronger induction (Joly et al., 2005), IFN-γ was combined with IL-22 or TNF-α at a concentration of 100 ng/ml in order to enhance the effects (figure 3.5 (C)). Nevertheless, combination of cytokines did not lead to an induction. This leads to the conclusion that cytokines alone are not able to induce hBD-3 in gastric epithelial cells.

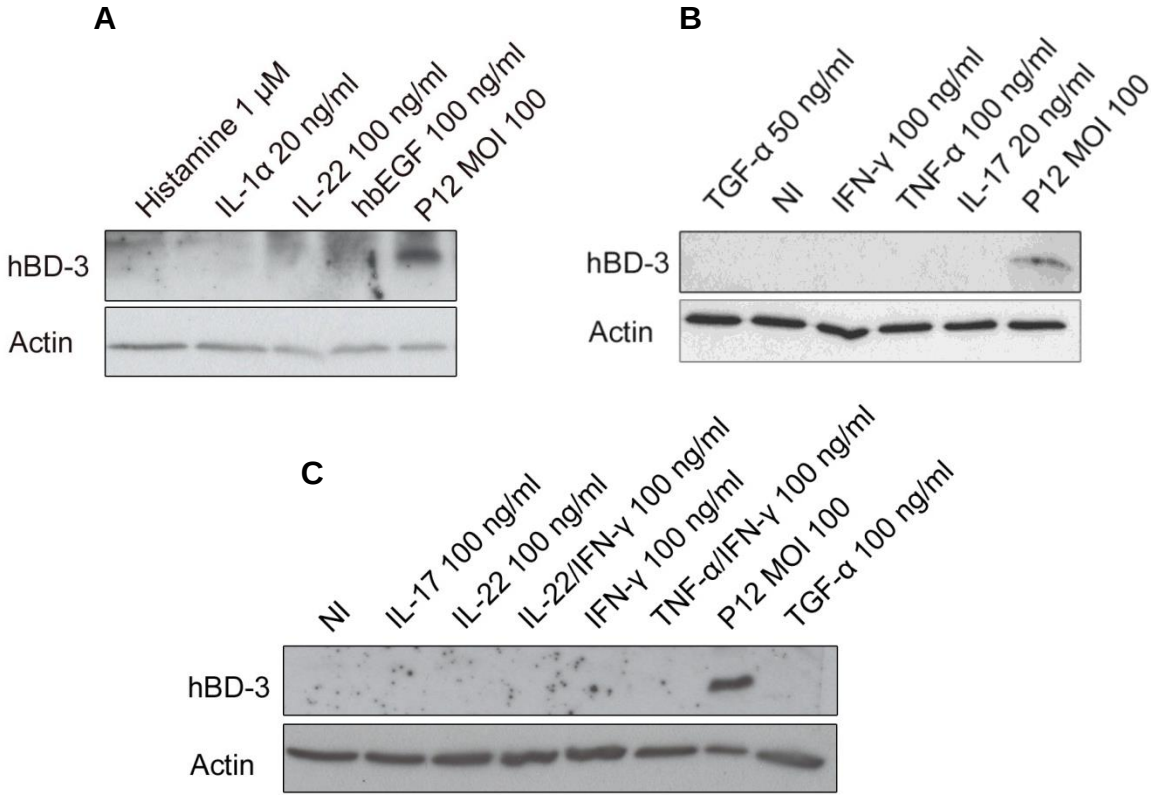

Figure 3.5 Analysis of hBD-3 induction in MKN28 cells in presence of different cytokines. MKN28 cells were treated 24 h with different cytokines (A, B) at indicated concentrations. Furthermore, the effect of a combination of IL-22 or TNF-α with IFN-γ was tested and with an increased concentration of TGF-α and IL-17 (C). As positive control, cells were simultaneously infected with P12wt MOI 100. Untreated cells were used as negative control (NI). Cells were subsequently lysed and analyzed by western blot for hBD-3 induction. Actin serves as a loading control.

3.3 Analysis of stimulants enhancing hBD-3 induction upon *H. pylori* infection

3.3.1 Time course of *H. pylori* infection and treatment with cytokines

As cytokine treatment of MKN45 cells alone did not show an induction of hBD-3, an additional stimulus could be important for maximal hBD-3 induction. Therefore, *H. pylori* infection was combined with the treatment of IFN-γ. IFN-γ is known to be strongly produced upon *H. pylori* infections (Aebischer et al., 2008; Bimczok et al., 2010) and it is known to induce hBD-3 (Albanesi et al., 2007; Joly et al., 2005). Although, no hBD-3 induction could be observed when MKN45 cells were treated with IFN-γ alone (figure 3.4), simultaneous infection and IFN-γ treatment was assessed. MKN45 cells were infected with P12wt (MOI 20) in the presence or absence of IFN-γ for 72 h. Every 24 h, cells were lysed and hBD-3 production was monitored via western blot. The results are shown in figure 3.6 (A). Interestingly, hBD-3 induction was clearly enhanced in infected

cells when simultaneously treated with IFN-γ. In contrast to that, in cells simultaneously infected and treated with histamine, no difference was detectable (B).

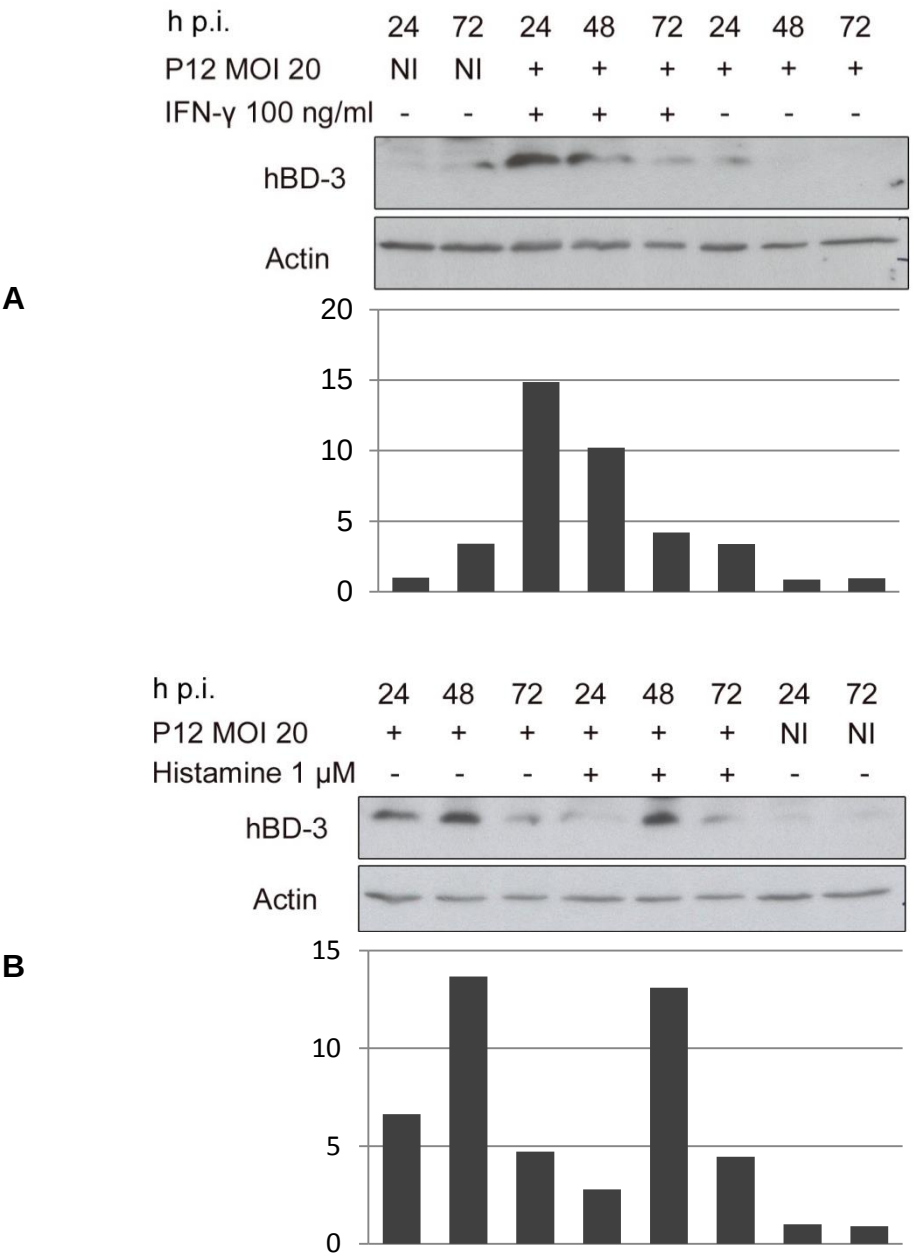

Figure 3.6 Impact of simultaneous infection with *H. pylori* and cytokine treatment on hBD-3 production in MKN45 cells. Upper panel**:** MKN45 cells were infected with *H. pylori* P12wt (MOI 20) for 24, 48 and 72 h. Cells were either only infected or simultaneously treated with (A) IFN-γ (100 ng/ml) or (B) Histamine (1 µM). As a negative control, cells were left untreated (NI). Cells were subsequently lysed and analyzed by western blot for hBD-3 induction. Actin serves as a loading control. Lower panel: Blots were quantified using ImageJ.

Since Bauer et al. (2012) showed *H. pylori*-dependent inhibition of hBD-3 expression after prolonged infections, 48 h and 72 h time points were analyzed as well in order to

determine if there could be any effects, circumventing *H. pylori*-mediated hBD-3 inhibition. In the 72 h time course of infection with MOI 20, it could be observed that hBD-3 is suppressed after prolonged infection, which is in line with the findings of Bauer et al. (2012). The peak of induction varied in different experiments between 24 h p.i. (A) and 48 h p.i. (B). In addition, blot (A) shows that *H. pylori*-mediated inhibition of the hBD-3 production cannot be prevented in IFN-γ-treated cells but slight hBD-3 expression was still detectable after 72 h.

3.3.2 Simultaneous *H. pylori* infection and treatment with IFN-γ

Here, the effect of IFN-γ on hBD-3 expression upon infection with *H. pylori* was further investigated. Therefore, MKN45 cells were infected with *H. pylori* P12wt with different MOIs (20 and 100). The infection was done in the presence or absence of 100 ng/ml IFN-γ. Concordantly with previous results, IFN-γ strongly increased hBD-3 expression in *H. pylori*-infected cells (figure 3.7), but treatment with IFN-γ alone did not. These results also demonstrate that the effect is independent of any MOI, since simultaneous treatment enhanced the effect in samples with MOI 20 or 100. Quantification (bar diagrams) of the protein bands emphasizes the observation.

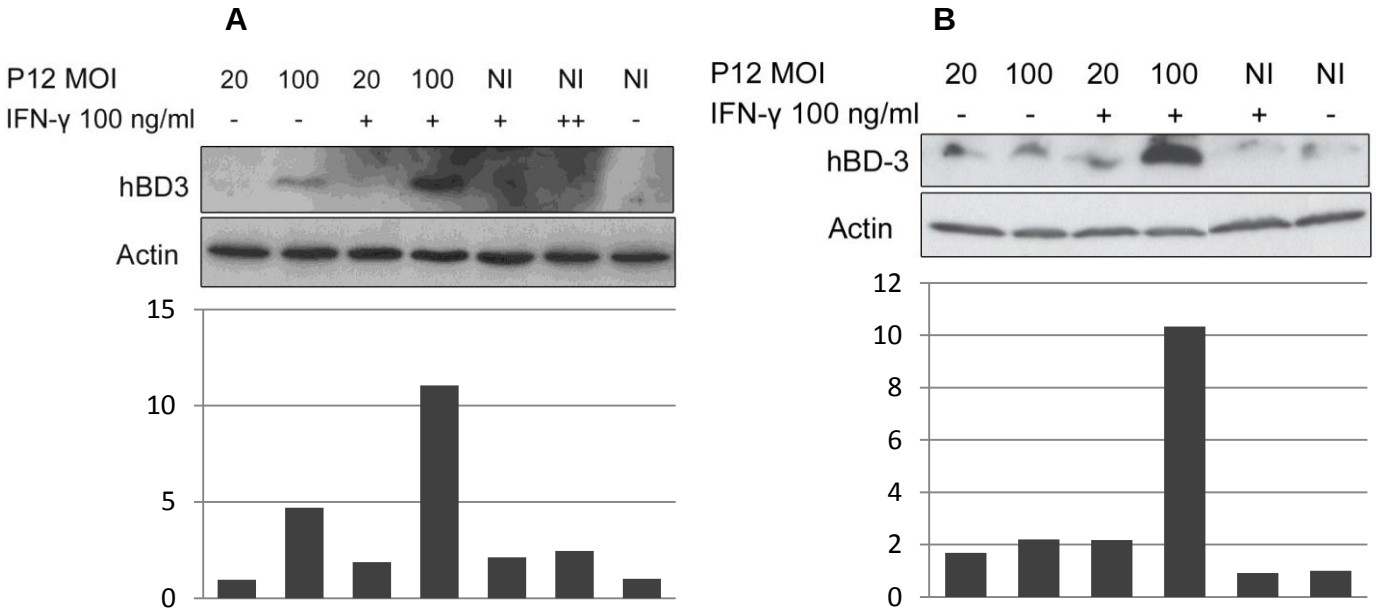

Figure 3.7 IFN-γ strongly stimulates hBD-3 induction in *H. pylori*-infected MKN45 cells. Upper panel: MKN45 cells were infected with *H. pylori* P12wt (MOI 20 or 100) for 6 h (A) or 24 h (B). Cells were either simultaneously treated with IFN-γ (100 ng/ml) or only infected. Additionally, cells were left untreated (NI) or only treated with 100 ng/ml IFN-γ (+), or 200 ng/ml (++). Cells were lysed and used for the detection of hBD-3 by western blot. Actin serves as a loading control. Lower panel: Quantification of the blots was done by using ImageJ.

Having shown the effect of simultaneous infection and IFN-γ treatment in MKN45 cells, the same experiment was repeated in MKN28 cells in order to prove that the effect is not restricted to a single cell line. The enhancing effect of IFN-γ on *H. pylori*-stimulated hBD-3 induction was also detectable in MKN28 cells (figure 3.8), thereby underlining that IFN-γ acts independent of any cell line-specific effects.

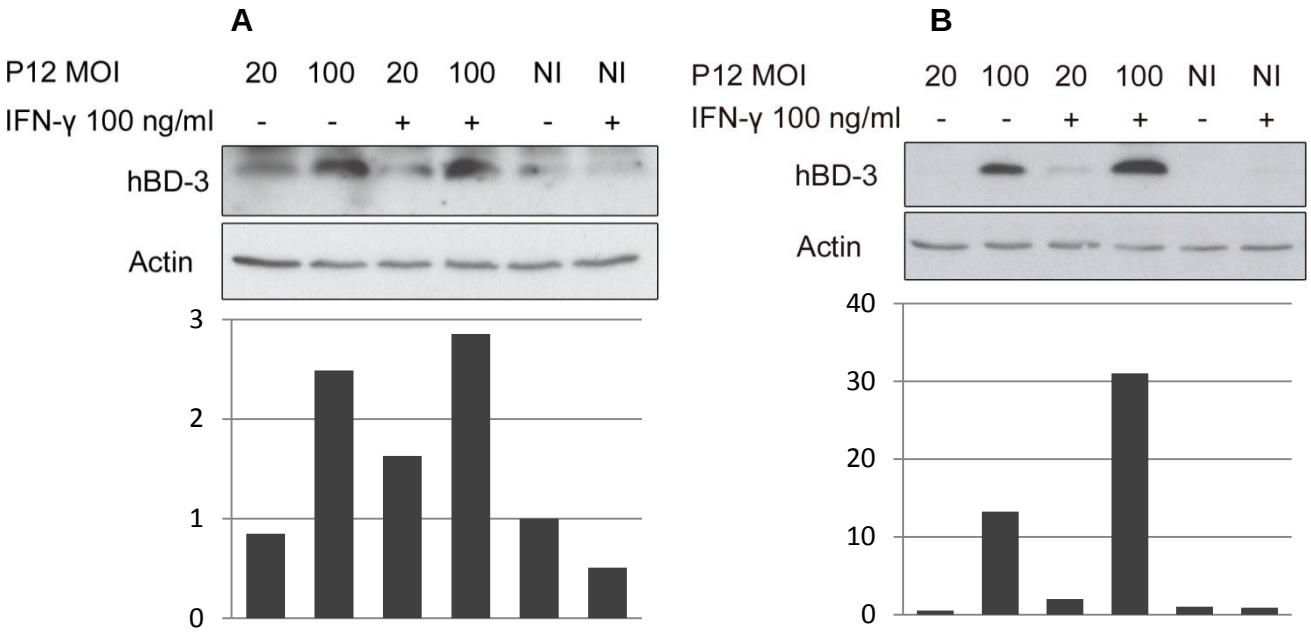

Figure 3.8 IFN-γ strongly stimulates hBD-3 induction in *H. pylori*-infected MKN28 cells. Upper panel: MKN28 cells were infected with *H. pylori* P12wt (MOI 20 or 100) for 6 h (A) or 24 h (B). Cells were either simultaneously treated with 100 ng/ml IFN-γ (+) or infected only. Additionally, cells were left untreated (NI) or just treated with IFN-γ without infection. Cells were lysed and used for the detection of hBD-3 by western blot. Actin serves as a loading control. Lower panel: Quantification of the blots was done by using ImageJ.

It has been demonstrated that infection in combination with IFN-γ enhanced the hBD-3 induction in gastric epithelial cells. In a further experiment, the dependence of this effect on viable bacteria was assessed. Therefore MKN45 cells were simultaneously treated with sonicated bacterial lysate and IFN-γ. Figure 3.9 shows that bacterial lysate alone or in combination with IFN-γ and TNF-α did not lead to an induction of hBD-3, underlining that viable bacteria are required as inducing trigger.

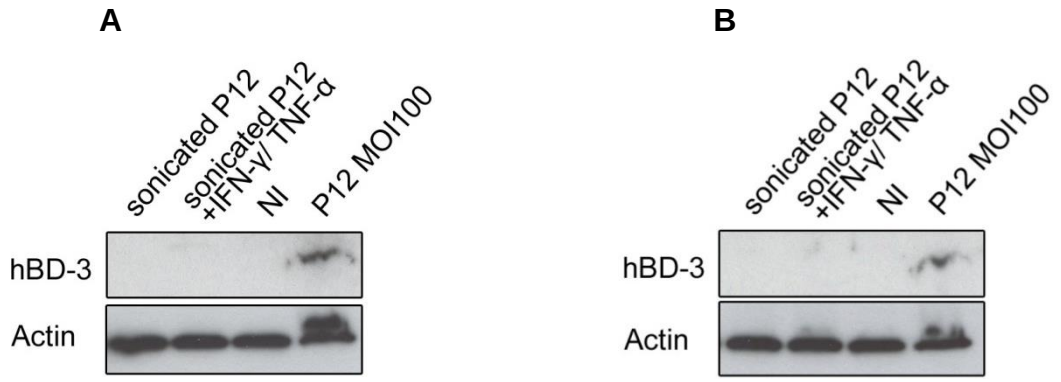

Figure 3.9 Non-viable *H. pylori* does not induce hBD-3 in combination with IFN-γ and TNF-α. MKN45 cells were treated for 6 h (A) or 24 h (B) with sonicated P12wt with or without the addition of IFN-γ (100 ng/ml)/TNF-α (50 ng/ml). Further cells were left untreated (NI) or infected with P12wt MOI 100. Cells were lysed and used for the detection of hBD-3 by western blot. Actin serves as a loading control.

3.4 Confirmation of enhancing effects of IFN-γ on *H. pylori*-dependent hBD-3 production via ELISA

To confirm the enhancing effect of IFN-γ on *H. pylori*-dependent hBD-3 induction and to analyze if hBD-3 is released in the supernatant after cytokine treatment, an ELISA experiment was performed. Initially, MKN45 cells were infected with P12wt and treated with IFN-γ for 24 h. The supernatants were analyzed for hBD-3 by ELISA. However, induction could not be detected in the supernatants in any condition tested (data not shown). As the sensitivity of the ELISA could be below the detection level of secreted hBD-3, supernatants were concentrated prior to ELISA analysis.

The concentrated supernatant was then used for ELISA measurement and the following conditions were tested 6 h and 24 h after treatment: supernatants of infected cells (P12wt MOI 20), supernatants of cells treated with IFN-γ (100 ng/ml) and supernatants of simultaneously infected and cytokine-treated cells (figure 3.10). 6 h after treatment, minor amounts of hBD-3 were detectable in the supernatants of infected and both infected plus IFN-γ-treated cells. Concordantly with the western blot results (figure 3.4), hBD-3 was not detectable in supernatants of cells treated with IFN-γ alone. In contrast to the 6 h time point, hBD-3 was clearly detectable in supernatants of infected and both infected and IFN-γ-treated cells after 24 h. Similar to the 6 h time point measurements, IFN-γ treatment alone did not induce any detectable hBD-3 amounts in cellular supernatants after 24 h.

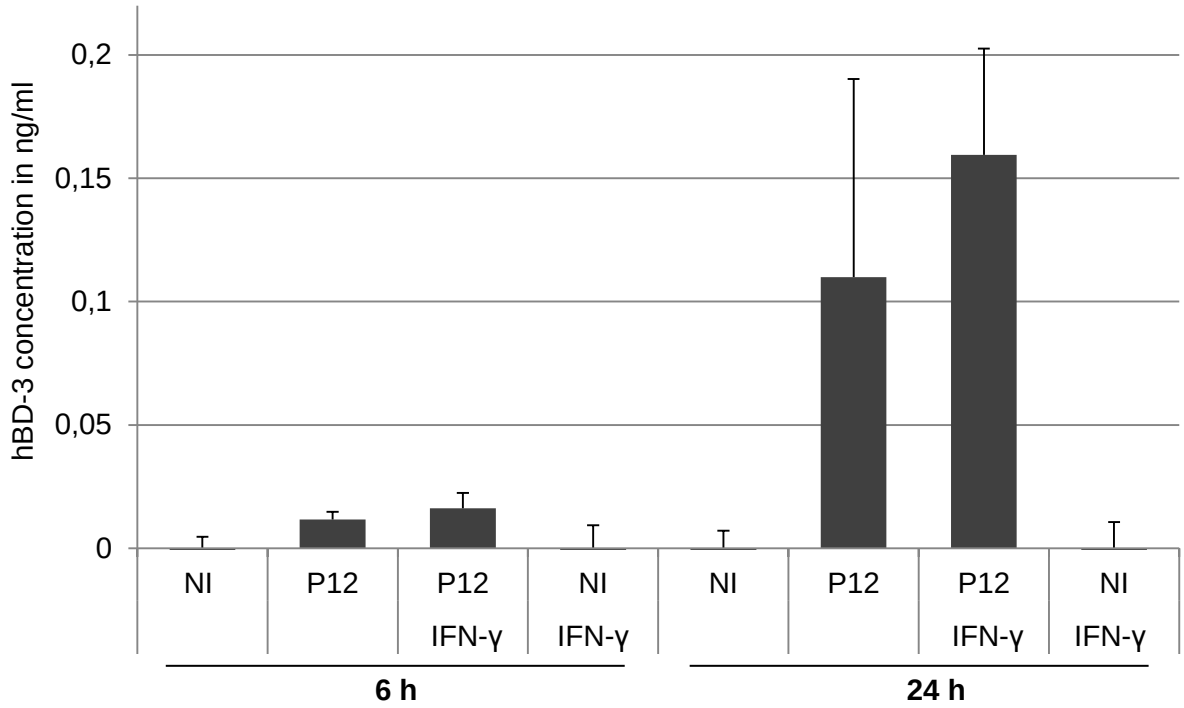

Figure 3.10 ELISA of IFN-γ-treated and P12wt-infected MKN45 cells. MKN45 cells were infected with P12wt MOI 20, infected and simultaneously treated with IFN-γ (100 ng/ml), just treated with IFN-γ 100 ng/ml or left untreated (NI). Supernatants were collected after 6 h or 24 h. Concentrated supernatants were then used to perform an ELISA to detect the hBD-3 induction. Error bars indicate SD of two independent experiments.

Standard variations between biological replicates in MKN45 cells were quite high for samples of infected cells. In wells infected for 24 h, a high cell-death rate was observed under the microscope and it was strongly enhanced when cells were simultaneously treated with IFN-γ. This fact could account for the elevated variability detected. A reason for the varying results could be the high rate of cell death in infected MKN45 samples that was observed via microscope after 24 h. This effect was strongly enhanced when cells were simultaneously treated with IFN-γ.

Since microscopical analysis of IFN-γ-treated plus infected AGS cells revealed a lower level of cell death, an additional ELISA experiment was performed with this cell type. Moreover, another bacterial strain, the P1wt strain was used, to exclude strain-specific effects. AGS cells were infected for 24 h with *H. pylori* P1wt (MOI 100), infected and IFN-γ-treated (100 ng/ml) or only treated with IFN-γ (100 ng/ml) respectively. Supernatants were concentrated via ultrafiltration and used for ELISA to detect hBD-3 induction. Results are shown in figure 3.11

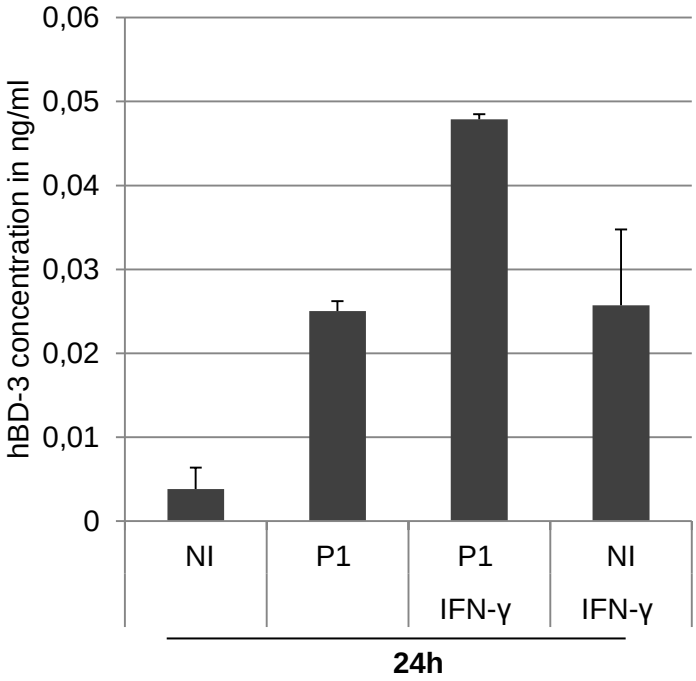

Figure 3.11 ELISA of IFN-γ-treated and P1wt-infected AGS cells for 24 h. AGS cells were only infected with P1wt MOI 100, infected and simultaneously treated with IFN-γ (100 ng/ml) or just treated with IFN-γ 100 ng/ml. Supernatants were collected after 24 h and concentrated 10x via ultrafiltration. The concentrated supernatants were then used to detect hBD-3 via ELISA. Error bars indicate SD of 2 independent experiments.

In contrast to MKN45 cells, not only infection of AGS cells with P1wt leads to an upregulation of hBD-3 after 24 h but also treatment with IFN-γ alone. Yet the overall detectable amount of hBD-3 was much lower in AGS cells. Concordantly with previous western blot results with MKN45 cells, combination of infection and IFN-γ treatment leads to an increased release of hBD-3 into the supernatant. The data clearly show cell line-specific differences in cytokine responses, thereby underlining the necessity to test more than only one cell line to confirm observations.

3.5 Impact of IFN-γ on cellular viability in infected MKN45 cells

The microscopic observation of enhanced cell death in MKN45 cells simultaneously infected and IFN-γ-treated was quantified by a cell viability assay. First, cells were either treated with IFN-γ (100 ng/ml), infected with P12wt (MOI 20) or simultaneously infected and treated with IFN-γ. Additionally, P1wt was used to exclude strain-specific effects. In figure 3.12 the viability of cells after 24 h (A) and 48 h (B) of treatment is given. Results were all normalized to uninfected and untreated cells. The data demonstrates that IFN-γ treatment of uninfected cells did not induce cell death after 24 h or 48 h and infection

with P12wt and P1wt induced moderate cell death after 24 h that was more pronounced after 48 h. Indeed, this effect could be strongly enhanced when cells were simultaneously infected and treated with IFN-γ indicating an effect of this cytokine on the cellular viability, but only in combination with *H. pylori* infection.

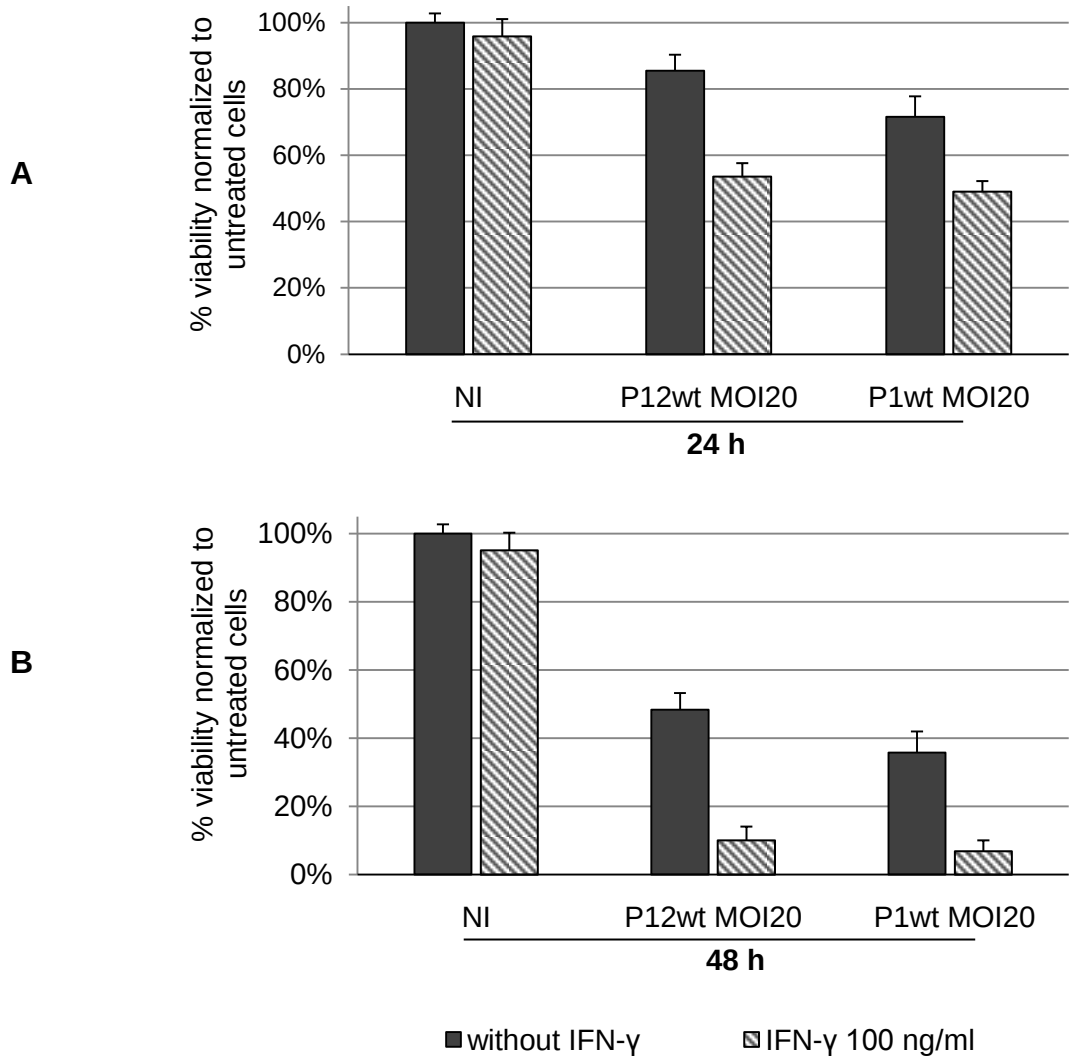

Figure 3.12 Effect of IFN-γ on cell survival of infected MKN45 cells after 24 h (A) and 48 h (B) post infection. MKN45 cells were either untreated (NI), treated with IFN-γ (100 ng/ml), infected with P12wt MOI 20 or P1wt MOI 20 or simultaneously infected and treated with IFN-γ for 24 h (A) or 48 h (B). After 48 h cellular viability was tested via a luminescence-based cell viability assay. Cellular viability was normalized to untreated cells. Error bars indicate SD of 2 independent experiments.

3.6 Cross-talk of innate immunity with adaptive immunity

3.6.1 Effects of DC/T-cell supernatants on uninfected cells

Since it could be shown that cytokines are able to modulate hBD-3 expression upon *H. pylori* infection, putative cross-talk between innate and adaptive immune responses was analyzed. Based on the hypothesis that immune cells can influence hBD-3 expression of epithelial cells via various cytokines, the influence of DCs and T-cells on hBD-3 expression was investigated in first orienting experiments.

It has been shown that infection with *H. pylori* leads to an activation of DCs and a subsequent T-cell response accompanied by the polarization of T_H0 cells to T_H1 (Bimczok et al., 2010). Interestingly, T_H1 cells are characterized by the production of large amounts of IFN-γ, the cytokine for which modulating effects on *H. pylori*-mediated hBD-3 expression could be demonstrated in this study. In an experimental layout it was attempted to remodel the crosstalk of adaptive and innate immunity *in vitro*. Following a well-established protocol, DCs were created from PBMCs via the addition of GM-CSF and IL-4 to isolated PBMCs for 6 days. In order to induce DC activation and antigen presentation, DCs were subsequently infected with *H. pylori* for one hour or activated with *E. coli* LPS. Subsequently, gentamycin was added to kill non-phagocytosed *H. pylori*. After 24 h, autologous T-cells were added. Activated DCs and naïve T-cells were incubated for 3 or 6 days allowing the secretion of cytokines by DCs and activation and secretion of cytokines by T-cells. Supernatants of these co-cultivations were sterile-filtered to remove all cells or other contaminants. Figure 3.13 shows a schematic overview over the process.

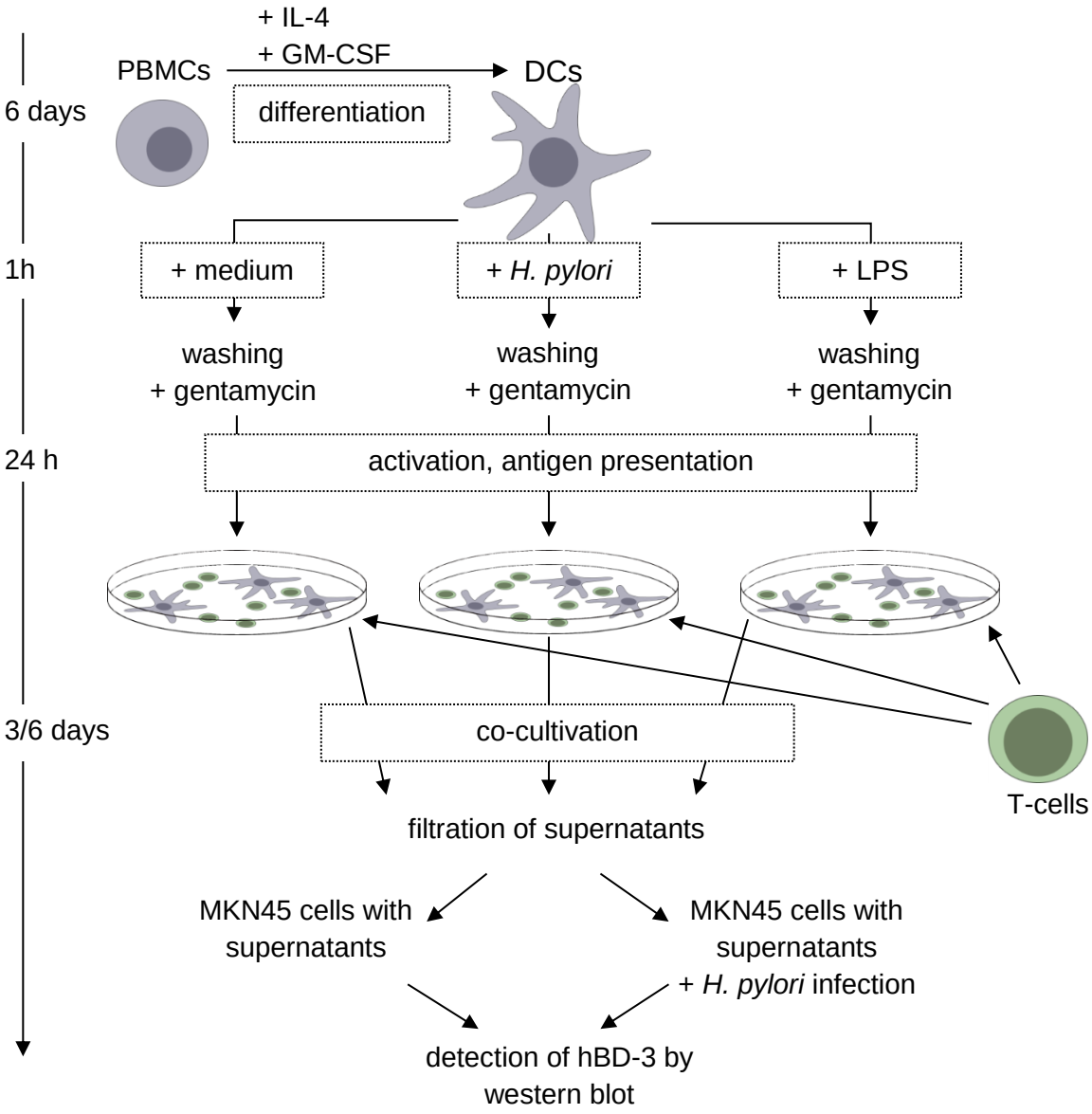

Figure 3.13 Schematic overview of the creation and differential activation of DCs, the co-cultivation with T-cells and the subsequent stimulation of epithelial cells

The sterile-filtered DC/T-cell supernatants were used to treat uninfected, serum starved MKN45 cells for 24 h. The results show 2 independent experiments each with a DC/T-cell set from independent donors. hBD-3 expression was analyzed by western blot. As can be seen in figure 3.14 for Donors 428 (A) and 3 (B), unstimulated DCs/T-cells as well as *H. pylori*-activated DCs/T-cells supernatants did not lead to an induction of hBD-3. In contrast to that, supernatants from LPS-activated DCs/T-cells from both donors lead to an induction. For Donor 3, supernatants were collected 3 and 6 days after co-incubation which showed a similar pattern of induction in both cases, albeit to a lower degree in the supernatants collected after 3 days. As positive control cells were infected

with *H. pylori* P12wt (figure 3.14 (B)). Taken together, it can be stated that only LPS-activated DCs stimulate T-cells to release factors that are able to induce hBD-3 in epithelial cells in the absence of an additional infective stimulus.

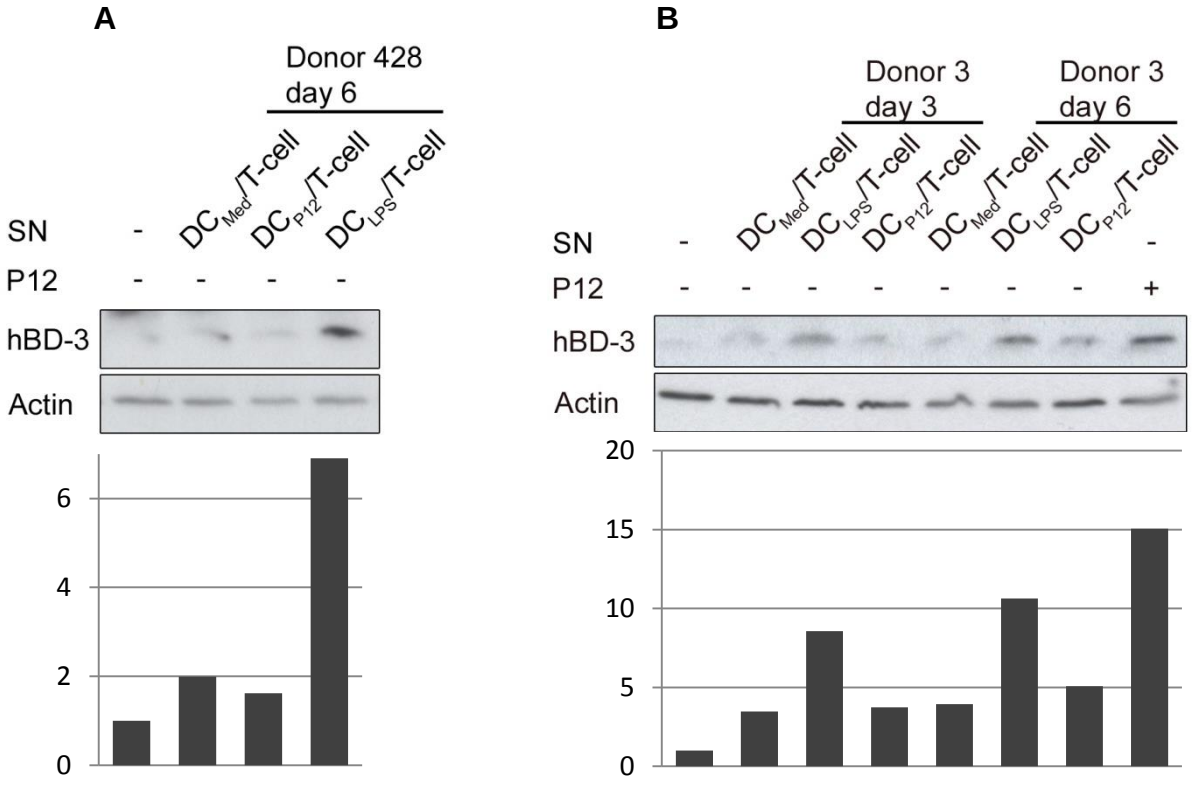

Figure 3.14 Induction of hBD-3 via cytokines of activated T-cells in MKN45 cells. Upper panel: MKN45 cells were treated with supernatants of a DC/T-cell co-cultivation. DCs were infected with P12wt (DC_{P12}) or, as a positive control treated with *E. coli* LPS (DC_{LPS}). As a negative control, DCs were just treated with medium (DC_{Med}). Activated DCs were then co-cultivated with T-cells. Supernatants of these co-cultivations were collected after 6 days (A), or 3 and 6 days (B) and sterile-filtered. These supernatants were added to fresh uninfected, serum starved MKN45 cells for 24 h. Cells were lysed and the lysate was used for western blotting and the detection of hBD-3 induction. Actin serves as a loading control. Lower panel: Quantification of the blots was performed by using ImageJ.

3.6.2 Effects of DC/T-cell supernatants on infected cells

It could be shown in previous experiments that simultaneous infection and treatment with IFN-γ leads to an enhanced induction of hBD-3. So the same setting was tested with the immune cell supernatants. In a further experiment, MKN45 cells were treated with supernatants of the co-cultivation and simultaneously infected with P12wt. The results of figure 3.15 (A) and (B) show two independent experiments each with supernatants of different donors. As demonstrated in previous experiments, infection alone leads to an induction of hBD-3 in this experiment and the treatment of IFN-γ enhanced this effect. Interestingly, when the cells were infected and treated with supernatant of LPS activated DCs/T-cells the level of induction was unchanged or lower compared to samples incubated with the supernatant of unstimulated DCs. Intriguingly, hBD-3 induction was strongly reduced in cells infected and treated with the supernatants of *H. pylori*-activated DCs/T-cells. Taken together, these data show on the one hand that LPS-stimulated DC/T-cells under infection with *H. pylori* do not enhance upregulation of hBD-3 and on the other that *H. pylori*-pulsed DC/T-cells inhibit induction.

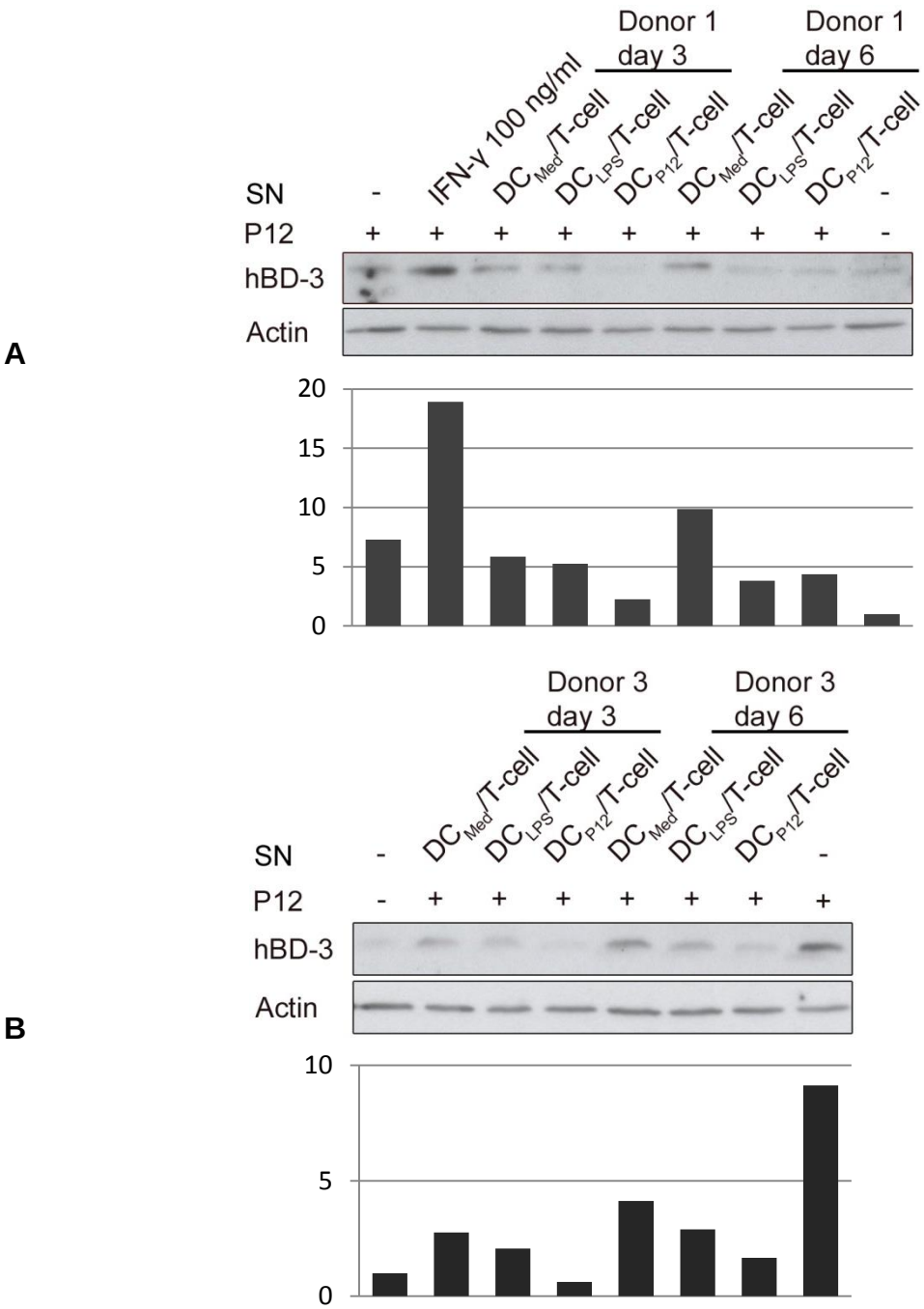

Figure 3.15 Induction of h-BD3 via cytokines of activated T-cells in MKN45 cells and simultaneous infection with P12wt. Upper panel: MKN45 cells were treated with supernatants of DC/T-cells co-cultivation (SN) of two independent donors (A and B). DCs were infected with P12wt (DC_{P12}) or treated with *E. coli* LPS (DC_{LPS}) or as negative control just treated with medium (DC_{Med}). Activated DCs were then co-cultivated with the T-cells for 3 days or 6 days. Supernatants of the co-cultivation were then added to MKN45 cells and simultaneously infected with P12wt MOI 20 for 24 h (+). In addition, cells were just infected with P12wt MOI 20, infected with P12wt MOI 20 and treated with IFN-γ (100 ng/ml) or left untreated. Cells were lysed and the lysate was used for western blotting for the detection of hBD-3. Actin serves as a loading control. Lower panel: Quantification of the blots was performed by using ImageJ.

4. Discussion

Infection with *H. pylori* is widespread and poses a major threat to human health as it is the leading cause for the development of a number of severe gastric diseases including the development of gastric cancer. It has been shown that the innate immunity of gastric epithelial cells plays a significant role in activating and directing the immune response (Selsted and Ouellette, 2005) and in recent years it could be demonstrated that epithelial cells actively counteract *H. pylori* via production of AMPs (Bauer et al., 2012; George et al., 2003). The defensin hBD-3 has recently been described to have the most potent activity against *H. pylori* (Bauer et al., 2012). Aim of the present study was to investigate beta defensin induction in gastric epithelial cells by systematically applying knowledge gathered in observations in other epithelial cells. Therefore, inducibility of hBD-3 by different bacterial stimuli and the epithelial crosstalk as well as the influence of adaptive immunity on the induction following an infection was examined. This study could show that bacterial viability is a crucial signal that is needed for an effective induction of hBD-3 following an infection. Furthermore, the induction and modulation of this induction through different cytokines and factors and the influence of adaptive immunity on the expression of this particular AMP in gastric epithelial cells were tested. IFN-γ as a marker cytokine of the T_H1-polarized adaptive immunity could be determined as an important modulator of hBD-3 expression and it was shown to simultaneously reduce cellular viability in infected cells. Additionally, it was demonstrated that *H. pylori* inhibits the influence of the adaptive immune system on the innate immunity.

Initially, the question was addressed as to which bacterial stimulus is needed to activate the epithelia's hBD-3 response. It could be shown that only viable bacteria are capable of inducing hBD-3 in gastric epithelial cells and that the level of induction is dependent on the number of viable bacteria. To test induction of hBD-3 via a bacterial stimulus which is not dependent on viability, different bacterial preparations like sonified bacterial lysate and bacterial culture supernatants were used. Yet, none of these triggers led to a production of hBD-3 that was detectable in western blots. Moreover, gentamycin-treated *H. pylori* was applied to the cells. These bacteria showed a strongly diminished viability and induced hBD-3, albeit to a much weaker degree compared to the infection with the untreated bacteria. This finding underlines the notion that bacterial viability is a crucial factor for the induction level of hBD-3 in gastric epithelial cells. It has been shown before by Bauer and colleagues (2012) that heat-killed bacteria are not capable of

inducing hBD-3 and that induction is MOI-dependent. This is in line with reports showing that in oral epithelial cells, induction of hBD-3 could only be observed when cells were incubated with viable bacteria (Feucht et al., 2003), underlining the role of bacterial viability as a stimulus for hBD-3 induction. In contrast to that, Harder et al. (2001) showed in their study that induction of hBD-3 via non-viable bacteria can be achieved but this was dependent on the genus of bacteria as well as the bacterial strain. A heat-inactivated clinical isolate of *Pseudomonas aeruginosa* could induce hBD-3 in keratinocytes. Strikingly, another heat-inactivated *Pseudomonas aeruginosa* strain or *Staphylococcus aureus* did not lead to an induction in these cells.

In view of these results, the question arises why gastric epithelial cells fail to react to non-viable *H. pylori* and what is the mechanism by which viable *H. pylori* is sensed leading to an infection-dependent upregulation of hBD-3. The innate immune system is activated by sensing PAMPs which are conserved microbial structures like bacterial DNA, LPS or peptidoglycan. Distinct PRR on the epithelial cells do react to corresponding PAMPs. So far, the only extracellularly exposed PRR which has been described to mediate upregulation of hBD-3 upon sensing *H. pylori* is TLR4, since hBD-3 expression was diminished when TLR4 was inhibited (Kawauchi et al., 2006). Conversely, it has not been proven so far that an isolated stimulation of TLR4 leads to an induction. Another intracellular PRR that senses *H. pylori* is NOD1 (Grubman et al., 2010). It binds peptidoglycan of *H. pylori* when delivered to the cytoplasm via the T4SS or via OMVs (Kaparakis et al., 2010; Viala et al., 2004a). However the role of NOD1 in the induction of hBD-3 is controversial, since some authors describe an involvement of NOD1 (Grubman et al., 2010), whereas others see hBD-3 induction as being completely independent from NOD1 (Boughan et al., 2006). Still the question arises why *H. pylori* lysates that contain a varying numbers of PAMPs addressing different receptors and pathways fail to induce hBD-3. It is well described that *H. pylori* has evolved disguising mechanisms thereby impairing the innate immune reaction by e.g. modulating its PAMPs in order to prevent them from being recognized by PRRs (Müller et al., 2011). *H. pylori*'s LPS for example, which is a ligand to TLR2 or TLR4, only has a weak endotoxic activity (Lepper et al., 2005; Muotiala et al., 1992). This could explain why lysates or inactivated *H. pylori* containing several PAMPs lead to neither a sufficient activation of the TLRs and/or other PRRs nor a subsequent induction of hBD-3. Furthermore, this would explain the discrepancies between this and other infection

models that show that broth culture supernatant as well as heat-inactivated *Legionella pneumophilae* (Scharf et al., 2010) or whole cell sonicate of *Mycobacterium leprae* (Cogen et al., 2012) are capable of inducing hBD-3. These pathogens express PAMPs that are not modified as they are in *H. pylori,* so they lead to an effective immune activation and induction of defensins. Another explanation could be that *H. pylori* does not release immunogenic factors into the supernatant which has already been demonstrated for flagellin (Gewirtz et al., 2004). This could provide an explanation for the impaired activation of innate immunity by conditioned supernatants. It has to be taken in account as well that most of the results showing an induction via non-viable bacteria were gathered in other cell lines than those used in this study. Different cell lines could react differently towards infection due to higher expression of TLRs and other PRRs. The experiment with *Legionella* for example was performed in airway epithelial cells that are known to express high levels of TLRs (Koff et al., 2008). In contrast to that, MKN45 cells have been described to express low amounts of TLR2, 4 and 5 (Smith et al., 2003) making them less sensitive to TLR-dependent stimulation.

Considering these findings the conclusion can be drawn that other stimuli than classical PAMPs are needed to elicit an hBD-3 response from gastric epithelial cells. It has been described before that the innate immunity upon *H. pylori* infections is directed against a functioning T4SS that is only found in viable bacteria (Viala et al., 2004a). Yet, Bauer et al. (2012) and Boughan et al. (2006) could show that cagPAI negative strains are also capable of inducing hBD-3 which displays an innate immune reaction that is independent of the cagPAI. Recently, a mechanism by which the strength of the immune reaction can be adapted to the level of the microbial threat was described (Blander and Sander, 2012). It was proposed that for example different PAMPs simultaneously activating multiple PRRs lead to a more severe reaction. In addition, it could be demonstrated in macrophages that bacterial viability can be sensed which leads to a modulation of the intensity of the immune reaction thereby demonstrating viability as a crucial factor for pathogenicity (Sander et al., 2011). It is tempting to speculate that similar mechanisms might be found in epithelial cells, yet this issue has not been addressed so far. According to this model, viable bacteria could induce hBD-3 as they lead to an upregulation of the immune response.

Having shown that only viable *H. pylori* induces hBD-3 in gastric epithelial cells, the intraepithelial mechanisms that lead to the induction were assessed. This induction upon infection has been described to be mainly dependent on a cascade of EGFR activation and a subsequent signaling via the JNK/ERK1/2 MAPK pathways and the JAK/STAT pathway (Bauer et al., 2012; Boughan et al., 2006). Nevertheless, signaling pathways that bridge the initial sensing of the pathogen with the subsequent activation and phosphorylation of the EGFR or the putative activation of other pathways are not fully understood. Intraepithelial crosstalk with soluble factors released by epithelial cells is discussed. Boughan et al. (2006) proposed an autocrine and paracrine transactivation of the EGFR following *H. pylori* infection leading to the activation of the MAPK pathway and a subsequent expression of hBD-3. It has been demonstrated before that infection with *H. pylori* leads to the phosphorylation of the EGFR via soluble factors (Keates et al., 2001; Romano et al., 1998; Wallasch et al., 2002). One of the transactivating factors that have been identified is hbEGF that, upon infection with *H. pylori,* is cleaved by metalloproteinases and released. Subsequently, hbEGF binds to the EGFR and induces its activation. This mechanism of transactivation has not been definitely linked to the induction of hBD-3 in gastric epithelial cells, whereas hbEGF in other cell culture models has been described to activate hBD-3 (Steubesand et al. 2009). The EGFR-related peptide TGF-α was reported to be a hBD-3 inducer as well (Sørensen et al., 2005). For assessing this crosstalk model *in vitro,* the working hypothesis was proposed that transactivating factors are released by infected cells into the supernatant. These conditioned supernatants in turn should then be able to upregulate hBD-3 in uninfected cells. Surprisingly, none of the experiments that were performed for the analysis of an intraepithelial crosstalk indicated any effect of supernatants of infected cells on uninfected cells. Stimulating cells with high concentrations of recombinant EGFR-related peptides TGF-α and hbEGF did not cause hBD-3 production either. The results suggest that the above mentioned intraepithelial crosstalk is in general not the main signaling pathway leading to an induction of hBD-3 and demonstrates that at least for *H. pylori* infection another stimulus is needed. This notion might be underlined by a finding of Keates et al. (2001) who reported that blocking of the EGFR before stimulation with EGF inhibits ERK1/2 activation completely. When the EGFR was blocked during infection with *H. pylori,* a basal activation of the ERK1/2 pathway, which represents the main MAPK pathway reported to be important for hBD-3 induction (Boughan et al.,

2006), could still be observed. This indicates an additional activation mechanism of the ERK1/2 signaling pathway upon infection with viable *H. pylori* independent of EGFR activation. Bauer et al. (2012a) could also demonstrate that despite the inhibition of the EGFR, an induction of hBD-3, albeit strongly diminished, could still be observed. Here, an additional induction of hBD-3 via JAK/STAT signaling was shown. What is more, Romano et al. (1998) demonstrated that *H. pylori,* besides the induction of EGFR-related peptides following infection, simultaneously blocks their proliferative impact on epithelial cells in a so far unidentified mechanism, indicating an inhibiting effect of soluble *H. pylori* factors on signaling events of EGFR-related peptides. So the exact function of the EGFR in the induction of hBD-3 and the signaling events leading to its activation following *H. pylori* infection need to be critically analyzed. Another issue that has to be taken in account with this experiment is that the concentrations of paracrine stimulating factors that may be found directly on top of the cell layer can be sufficient to lead to an upregulation of hBD-3 in neighboring cells, but the experimental setup allows only the incubation with highly diluted released factors when supernatants of infected cells are added to unstimulated cells which might diminish induction. Furthermore, upon infection, several different factors are released by epithelial cells (Romano et al., 1998), so stimulating with a single recombinant EGFR-related peptide might not provide a sufficient stimulus for hBD-3 induction. Taking in account these technical limitations, experiments with highly concentrated supernatants are needed to confidently exclude or verify an intraepithelial crosstalk.

In order to assess the reactivity of the gastric epithelium to non-microbial stimuli in more detail, another way of stimulating hBD-3 in gastric epithelial cell was chosen. For this purpose, other factors that have previously been reported to promote hBD-3 production were tested for their induction capacity in gastric epithelial cells. The factors comprised the pro-inflammatory cytokines IL-22, IL-17, TNF-α, IL-1α as well as IFN-γ and histamine. None of these factors led to an expression of hBD-3 that was measurable by western blot. In this experiment also EGFR-related peptides were tested and, as discussed before, didn’t lead to an induction. As an additional approach a combination of IL-22 and TNF-α with IFN-γ that previously has been reported to enhance induction (Joly et al., 2005) was tested. The combination of two stimuli didn’t cause a clearly measurable production of hBD-3 either. A conclusion that can be drawn from this is that a stimulus via one or two cytokines is not sufficient to induce hBD-3 to the level that can

be detected by western blot. The observation that a combination of TNF-α, IFN-γ, IL-1β, and IL-6 leads to induction of hBD-3 on RNA level in keratinocytes (Howell et al., 2006) underlines the need of multiple simultaneous stimuli for upregulation. Furthermore, also cell line-dependent effects can be anticipated. Keratinocytes for example have been shown to be highly reactive upon stimulation with IFN-γ and TNF-α, which could not be observed in epithelial cells of the small intestine (Ou et al., 2009).

As cytokines alone were insufficient to induce hBD-3 in gastric epithelial cells the modulating effect of these cytokines on the upregulation during infection was assessed and *H. pylori* infection was coupled with cytokine treatment. Interestingly, it could be shown that IFN-γ had a strongly enhancing effect on hBD-3 induction in *H. pylori*-infected gastric epithelial cells. To confirm the effect of IFN-γ, the experiment was repeated with infection at different MOIs and simultaneous IFN-γ treatment and the effect was shown to be MOI-independent. Moreover, the experiment was repeated in a different gastric cancer cell line. Here, similar results could be observed, excluding a cell line-dependent effect and confirming this effect to have a great biological relevance. Intriguingly, the induction was again dependent on viable bacteria, as sonicated bacterial lysate in combination with IFN-γ and TNF-α did not induce hBD-3, again underlining viability as an important stimulus. In addition, the enhanced induction of hBD-3 in infected cells stimulated with IFN-γ was also confirmed by ELISA by showing similar results as seen in western blot experiments.

So *H. pylori*-dependent induction of hBD-3 can be enhanced by IFN-γ, suggesting a modulation of innate immunity by pro-inflammatory cytokines. Interestingly, it is known, that IFN-γ is released upon *H. pylori* infection by cells of adaptive immunity like T_H1-polarized $CD4^+$ T-cells and an effective immune response against *H. pylori* is dependent on the production of IFN-γ at the site of infection (Sayi et al., 2009). IFN-γ is the only member of the type II interferon family and is important for natural resistance against predominantly intracellular and viral infections. IFN-γ functions mainly via activation of JAK/STAT signaling which targets genes leading to antiviral activities in cells, enhanced antigen presentation, inhibition of cell proliferation and induction of apoptosis (Schroder et al., 2004). IFN-γ elicits an important function in the modulation of the innate immune system and hBD-3 was shown to be induced in different epithelial cells upon treatment with IFN-γ (García et al., 2001; Joly et al., 2005) with the promoter region of hBD-3

having a STAT1 binding site (Albanesi et al., 2007). The enhancing effect of IFN-γ on the induction of hBD-3 upon infection can be discussed with regard to different mechanisms. The stronger hBD-3 expression might be due to a combination of two different stimuli: the induction of hBD-3 via infection with *H. pylori* mediated mainly via an activation of the EGFR (Bauer et al., 2012; Boughan et al., 2006) which is reinforced via IFN-γ acting as a second stimulus that additionally activates induction via the JAK/STAT pathway. Furthermore, there is another mechanism by which IFN-γ could reinforce hBD-3 production. IFN-γ can lead to phosphorylation and activation of the EGFR via Src family kinases (Burova et al., 2007) and release of the transactivating EGFR-related peptide TGF-α (Uribe et al., 2002). So this enhanced activation of the EGFR represents another way of reinforcing hBD-3 expression under infection with *H. pylori.*

Interestingly, the amount of hBD-3 decreased again after 72 h of infection even under the treatment with IFN-γ which might indicate a blocking of the IFN-γ effect. Concordantly, it has been reported that *H. pylori* is capable of inhibiting the IFN-γ signaling via a blockade of STAT1 in MKN45 cells (Mitchell et al., 2004). Another mechanism by which *H. pylori* blocks hBD-3 induction is mediated via the CagA-dependent SHP-2 activation. The CagA/SHP-2 complex has previously been reported to inhibit the hBD-3 induction via blockage of the EGFR. As the IFN-γ effect might be due to a modulation of the EGFR, the inhibition of this receptor would also diminish the IFN-γ effect. Interestingly, SHP-2 has also been reported to negatively regulate the JAK/STAT pathway thereby inhibiting IFN-γ signaling in mouse fibroblasts (You et al., 1999) which could therefore putatively be an additional way of a SHP-2-dependent inhibition of the IFN-γ-mediated hBD-3 expression.

As mentioned above, ELISA experiments confirmed previous findings of IFN-γ enhancing the release of hBD-3 from epithelial cells following infection. Here, the maximum amount of hBD-3 detected via ELISA in the supernatant following stimulation was about 150 pg/ml in MKN45 cells. AGS cells that were used as a control showed markedly lower maximum concentrations of about 50 pg/ml upon treatment. This concentration of a single AMP could not be sufficient for killing higher numbers of bacteria, as the concentration of hBD-3 needed *in vitro* to create a bactericidal effect against *H. pylori* was in the µM concentration range (Bauer et al., 2012; Harder et al.,

2001). Yet, in an *in vivo* situation the preconditions are different. Upon infection several AMPs are secreted simultaneously. This combination has been shown to lead to an increased antimicrobial activity (Hase et al., 2003) and compared to an *in vitro* setup where incubation time with hBD-3 is usually limited to a short time, *in vivo* there is a permanent exposure to different AMPs. Furthermore, the biological niche of *H. pylori* in the stomach is a thin layer close to the epithelium (juxtamucosa) or attached to the cells (Schreiber et al., 2004). Here, secreted AMPs could be present at much higher concentrations as they are diluted much less. So AMPs exert their effect on bacteria that are located close to the epithelial layer. The concentrations of AMPs that can be found *in vivo* in the biological niche of *H. pylori* have not been determined so far. An additional observation that was made when performing ELISAs, besides a differing amount of secreted hBD-3 in the particular cell line used, was an IFN-γ-dependent upregulation of hBD-3 which was only observable in AGS cells. This finding is in contrast to previous results. It has to be taken in account that AGS cells are a different cancer cell line that might express other receptor populations and show a signal transduction distinct from MKN45 cells. Interestingly, it has been reported that AGS cell lines can be infected with parainfluenza virus type 5, which leads to degradation of STAT1 (Young et al., 2007), resulting in an IFN-signaling defect. However, IFN-γ has been shown to signal via STAT1-independent pathways (Gough et al., 2008). In STAT1 deficient cells for example, IFN-γ has been demonstrated to induce compensatory signaling via STAT3 (Qing and Stark, 2004). Here, a link to hBD-3 induction might be established as hBD-3 has been shown to be inducible via STAT3 (Ishikawa et al., 2009b). Another explanatory approach could be based on the reports mentioned above that IFN-γ can lead to transactivation of the EGFR (Burova et al., 2007) and on the findings showing an IFN-γ mediated activation of AP1 via ERK1/2 in a STAT1-independent manner (Gough et al., 2008). These alternative signaling cascades that might be only active in STAT1 deficient AGS cells could give implications for an IFN-γ-dependent induction of hBD-3. Yet the exact mechanisms leading to induction need to be determined. Another reason for these differing results in AGS cells can be seen in the technical limitations of ELISA. When performing ELISAs a high variability of results in biological replicates was observed. Technical limitations of ELISAs detecting hBD-3 have been described before: In other studies the transcriptional upregulation of hBD-3 did not correlate with the amount of protein detected in ELISA (Bauer et al., 2013;

Garreis et al., 2010). No conclusive explanation for this observation has been provided so far but unspecific reactivity of the antibodies used in ELISA could be a reason for these observations.

Furthermore, in this study cell death was observed by microscope when infecting MKN45 cells for prolonged time. This effect was enhanced when IFN-γ was added but could not be observed to the same degree in AGS or MKN28 cells, indicating a differing reaction towards treatment. This microscopically observed effect of IFN-γ on enhancing cell death in MKN45 cells during infection could be confirmed by a viability assay. Infections that were done in the presence of IFN-γ showed strongly enhanced cell death compared to cells that were only infected. In contrast, IFN-γ treatment alone did not have an influence on cellular viability. This impact of IFN-γ on gastric epithelial cells has been addressed before in other studies showing similar results. Takagi et al. (2000) and Watanabe et al. (2000) could show that IFN-γ treatment in combination with *H. pylori* infection induces apoptosis in MKN45 cells, but infection or IFN-γ treatment alone does not. Watanabe et al. (2000) additionally could demonstrate that the enhanced cytotoxic effect of IFN-γ together with *H. pylori* infection is most likely dependent on viable bacteria, as LPS in combination with IFN-γ did not lead to enhanced cell death. The mechanisms of IFN-γ enhancing cell death have been closer investigated in the study of Hayashi et al. (1997). There it was described that MKN45 cells express high levels of Fas receptors (FasR) on their surface. FasR upon binding of its ligand FasL is trimerized and leads to activation of caspase 8 and induction of apoptosis. The level of FasR was enhanced when MKN45 cells were treated with IFN-γ, but the treatment alone did not induce apoptosis (Hayashi et al., 1997). Yet addition of FasL lead to enhanced cell death in IFN-γ-pre-treated cells. Interestingly, this study also showed that MKN28 cells did not react with cell death towards IFN-γ treatment. This is in line with observations made in this study where it could be seen microscopically that IFN-γ in combination with *H. pylori* infection did not greatly induce cell death in MKN28 cells. This model was further reinforced by a study of Shimada et al., (2002) who showed that in human gastric biopsies taken from ulcer sites, the expression of IFN-γ and soluble FasL is enhanced and the epithelial cells show higher rates of apoptosis and the expression of pro-apoptotic proteins. This effect was reversed when H*. pylori* was eradicated. Altogether these studies and findings of the present study show that the combination of pro-inflammatory cytokines, especially IFN-γ, and *H. pylori* infection lead

to enhanced cell death in epithelial cells. This effect could have implications on the turnover of the epithelial layer. At the site of infection, invading immune cells start producing IFN-γ. In combination with *H. pylori,* this leads to enhanced rates of apoptosis in the epithelial layer and this could therefore be a possible mechanism for the development of gastric ulcer and one factor leading to the development of gastric atrophy. So IFN-γ can be seen on the one hand as a pivotal signaling molecule in the immune response against *H. pylori,* reinforcing the innate immunity and on the other it plays an important role in the development of *H. pylori*-related gastric injuries. The dual role of reinforcing the immune response and affecting cellular turnover has also been described in a mouse model, where only mice producing IFN-γ could mount an effective immune reaction with a stronger inflammation and simultaneously elevated levels of IFN-γ were clearly associated with the development of gastric atrophy and other *H. pylori*-induced pathologies (Sayi et al., 2009).

By showing that IFN-γ influences the induction of hBD-3 in gastric epithelial cells and exerts additional important functions during infection with *H. pylori,* the question of the origin of this cytokine became the focus of interest. As mentioned above, IFN-γ is a cytokine produced by the adaptive immune response following *H. pylori* infection (Hafsi et al., 2004; Sayi et al., 2009) and it is the marker cytokine of the T_H1-polarized adaptive immune response that is prominent in *H. pylori*-infected patients. This T_H1-polarized immune reaction is known to activate the innate immunity of tissues like the skin (Cogen et al., 2012). For first orienting experiments delineating the influence of the adaptive immune response on the hBD-3 production in gastric epithelial cells, an *in vitro* model system was created. Resident or invading DCs have been shown to be critical regulators of the adaptive immune reaction following an infection with *H. pylori* (Hafsi et al., 2004) but their exact role still is controversial. So for remodeling the adaptive immunity, a DC/T-cell co-incubation model was used. By adding the supernatant of activated immune cells on infected or non-infected gastric epithelial cells, the effect of invading immune cells following infection was mimicked. In the experiments that were performed, it could be demonstrated that supernatant of LPS-activated DC/T-cells is capable of inducing hBD-3 even in the absence of an infective stimulus. Surprisingly, *H. pylori*-pulsed DCs didn't show an induction of hBD-3. The inducibility of hBD-3 in epithelial cells via LPS-activated immune cells is in line with the findings of Sørensen et al. (2005) who could demonstrate that mononuclear blood leukocytes stimulated with

LPS induce hBD-3 in keratinocytes. However, the effect of an upregulation of hBD-3 in uninfected epithelial cells treated with supernatant of LPS-stimulated DC/T-cells seems to be contradictory to previous findings, showing that cytokines without the stimulus of viable bacteria are not capable of inducing hBD-3 in gastric epithelial cells. Yet, in these supernatants a mixture of many stimulating factors and cytokines can be found activating a variety of different signaling pathways that in combination can lead to an induction. This notion can be underlined by an observation of Sørensen et al. (2005) showing that hBD-3 induction via supernatants of activated leukocytes could be markedly reduced when inhibiting single stimuli like IL-1 and IL-6 by blocking their respective receptor. However, stimulating the cells with either IL-1 or IL-6 directly didn't lead to an induction. This indicates that these stimuli are maybe acting in combination with others as an active part of the cytokine mixture produced by immune cells but alone are not sufficient for an effective induction of hBD-3. The exact hBD-3 inducing factors that are released upon LPS treatment in the DC/T-cell co-incubation model still need to be determined.

Another interesting finding was that LPS-activated DC/T-cell supernatants did not enhance induction of hBD-3 upon infection. What is more, supernatants of *H. pylori*-pulsed DCs were not only incapable of inducing hBD-3 in epithelial cells but clearly elicited an inhibiting effect on the *H. pylori*-mediated hBD-3 expression. Several studies proposed different mechanisms by which *H. pylori* modulates DC activation. Wang et al. (2010) reported that *H. pylori* hampers the activation of DCs by blocking antigen presentation and the secretion of cytokines. In addition to that, a soluble factor of *H. pylori* has been described that actively inhibits IL-12 production of DCs thereby blocking T_H1 polarization of T-cells which leads to a diminished IFN-γ production (Kao et al., 2006). Mitchell et al. (2007) demonstrated that treatment of DCs with *H. pylori* leads to an impaired T-cell activation and cytokine production. Inhibition of immune activation by *H. pylori* could explain the lack in induction of hBD-3. Another tentative explanation could be that DCs upon challenge with *H. pylori* more efficiently induce $FoxP3^+$ $iT_{REG}s$ that create tolerance towards *H. pylori* infection and hamper differentiation of IFN-γ producing T_H1 cells (Hitzler et al., 2011). Concomitant, explaining the inhibiting effects of the *H. pylori*-activated DCs/T-cell supernatant DCs have become the focus of interest because it is reported that infection of DCs with *H. pylori* leads to a different cytokine profile compared to that observed in LPS-stimulated DCs (Fehlings et al., 2012; Kranzer

et al., 2005). The anti-inflammatory cytokine IL-10 has been reported to be released in greater amounts by *H. pylori*-infected DCs (Fehlings et al., 2012; Kranzer et al., 2004; Mitchell et al., 2007). It has been observed that IL-10 inhibits the induction of hBD-3 in keratinocytes that are stimulated with activated PBMC supernatants (Howell et al., 2006). This could explain the inhibiting effect of *H. pylori*-activated DC/T-cell supernatants on the induction of hBD-3 upon infection. Furthermore iT_{REGs} that, as mentioned above, can be induced by *H. pylori*-pulsed DCs are also characterized by the production of IL-10 which could be another explanation for the inhibiting effects. It has to be taken in account that these DC/T-cell supernatants might also elicit a bactericidal effect and thereby diminish bacterial viability and therewith hamper *H. pylori* triggered hBD-3 induction which could explain why LPS-stimulated DC/T-cell supernatants did not enhance induction of hBD-3.

Yet in this first orienting model that was chosen in this study the polarization of T-cells and whether DCs or T-cells were the source of cytokines, were not investigated.
Alltogether, it could be demonstrated that supernatants from DC/T-cells representing the adaptive immunity can shape and reinforce the innate immunity of gastric epithelial cells. *H. pylori* seems to interfere with this crosstalk by modulating the activation and polarization of DCs and T-cells. This *H. pylori*-specific mechanism which represses the innate immune system could represent another immune evasion mechanism of *H. pylori,* enabling the establishment of a persistent infection. Interestingly, DCs have been discussed previously to induce tolerance to *H. pylori* infection, thereby hampering an effective clearing of the pathogen (Hitzler et al., 2011).

Another facet of hBD-3 that besides its bactericidal effect has to be taken in account is its immunomodulatory function. Against the background of its regulation by the adaptive immunity it is interesting that hBD-3 does not only function as an antimicrobial agent but simultaneously is an important factor for the orchestration of the adaptive immunity. It can recruit immune cells like monocytes (García et al., 2001) and DCs (Ferris et al., 2013) to the site of infection and leads to their maturation (Funderburg et al., 2007). The maturation of a subset of DCs under the influence of hBD-3 has been shown to induce DCs that foster the polarization of $CD4^+$ T-cells towards an IFN-γ producing phenotype (Ferris et al., 2013). So this could be an implication for a positive feedback loop with IFN-γ enhancing the expression of hBD-3 which in turn induces the production of IFN-γ.

This putative crosstalk between the adaptive and the innate immunity underlines the close linkage and the interdependence of these two systems.

This study was designed to test different factors regarding their impact on the hBD-3 expression in gastric epithelial cells generating an impression of their responsiveness thereby creating a hypothesis for the regulation of the innate immune system. Summing up the findings of this study it can be stated that the effective induction of hBD-3 on protein level in gastric epithelial cells seems to be dependent on the simultaneous activation of multiple different signaling pathways. The addition of single factors does not lead to an induction. In the course of the study it became apparent that the main inducers of hBD-3 in gastric epithelial cells are viable bacteria. So the question is how this induction pattern can be explained. In several studies, diverse stimuli and factors have been described to induce hBD-3 in a multitude of different epithelial cells. Furthermore, there are a multitude of signaling pathways that participate in the induction and modulation of this particular defensin. This reactivity pattern supports the notion that the secretion of hBD-3 is an archaic and unspecific host cell reaction mechanism to microbial threats. Therefore, induction of the same protein via many redundant signaling pathways and stimuli is important. On top of that, in different cell lines a distinct reactivity and level of induction towards the same stimuli can be observed, indicating cell line-dependent effects. Generally it seems that organs or tissues like skin, airway epithelium or tonsils, which are exposed to microbes more frequently show much higher expression of hBD-3 (García et al., 2001; Harder et al., 2001; Joly et al., 2005). The reason for this discrepancy in the reactivity of different cells might be due to the microbial threat these cells have to face. The stomach is normally exposed to fewer microbes and physiological features such as the low pH and the mucus layer effectively protect the epithelial layer and prevent most microbes from colonizing and establishing an infection. This might be a reason why the reactivity towards inducing stimuli is less pronounced. However, in this study it could be shown that the innate immunity of gastric epithelial cells is reactive towards the pro-inflammatory cytokine IFN-γ upon infection. Additionally, supernatants from activated immune cells lead to the expression of hBD-3 also in the absence of an infective stimulus. The role of epithelial cells in the immune reaction and their exact modulation and inhibition has to be closely investigated as they can actively participate in the killing of *H. pylori* by releasing bactericidal substances. So the regulation of hBD-3, the effector molecule of the innate immunity, via IFN-γ, a

marker cytokine of the T_H1-polarized adaptive immune reaction and via supernatants of immune cells indicates a paracrine modulation of the innate immunity via the adaptive immunity. What is more, this study revealed that *H. pylori* actively counteracts the reinforcement of the innate immune response, although mechanisms leading to this inhibition remain elusive, but a crucial role of DCs in this process can be anticipated. Therefore, cells of the adaptive immunity like DCs and certain T-cell subsets at the site of infection are critically modulating the innate immunity. This might lead to an altered immune response that enables *H. pylori* establishing a chronic infection. Together with the finding that IFN-γ critically interferes with the homeostasis of the epithelial barrier by inducing cell death, this interplay might be important for the susceptibility of a host towards acquiring an infection and might be a crucial factor determining the clinical outcome. In figure 4.1 an overview of the findings of this study and a model of the interplay of adaptive and innate immunity is given.

When performing the experiments, some technical limitations showed up that have to be taken in account. The main readout system that was used was western blotting. This technique proves that induction resulted in an expression of the protein. Yet the western blot technique is limited in its sensitivity so slight inductions or changes in the level of induction that might be seen under treatment with single factors just could not be detected. For most of the results in this study, a control with infected cells is given to compare expression levels with the level observed following infection. The ELISA assay, also used in several studies, showed high variations in repeated experiments in this study and therefore could not be used as main readout system but rather was used as a control. Problems that showed up in the assessment of intraepithelial crosstalk were discussed previously.

In future work different issues should be addressed. This study revealed that epithelial cells are reactive towards viable *H. pylori* and react towards infection by producing hBD-3. Hence it needs to be determined how viable bacterial can be sensed by epithelial cells and how this in turn leads to the induction of hBD-3. For this purpose, cellular receptors sensing the pathogen as well as the subsequent signaling pathways need to be determined. It could further be shown that this hBD-3 induction can be modulated by the adaptive immunity with IFN-γ as a potent stimulus. This study implies a crosstalk of adaptive and innate immune response under infection with *H. pylori,* and simultaneously

an inhibition of this modulation by *H. pylori* could be demonstrated. Here, it is important to investigate more closely the exact mechanisms of this crosstalk and the role of epithelial cells in the clearance of bacteria for a better understanding of the immune response initiated following infection with *H. pylori* as this could open novel therapeutic strategies addressing this crosstalk.

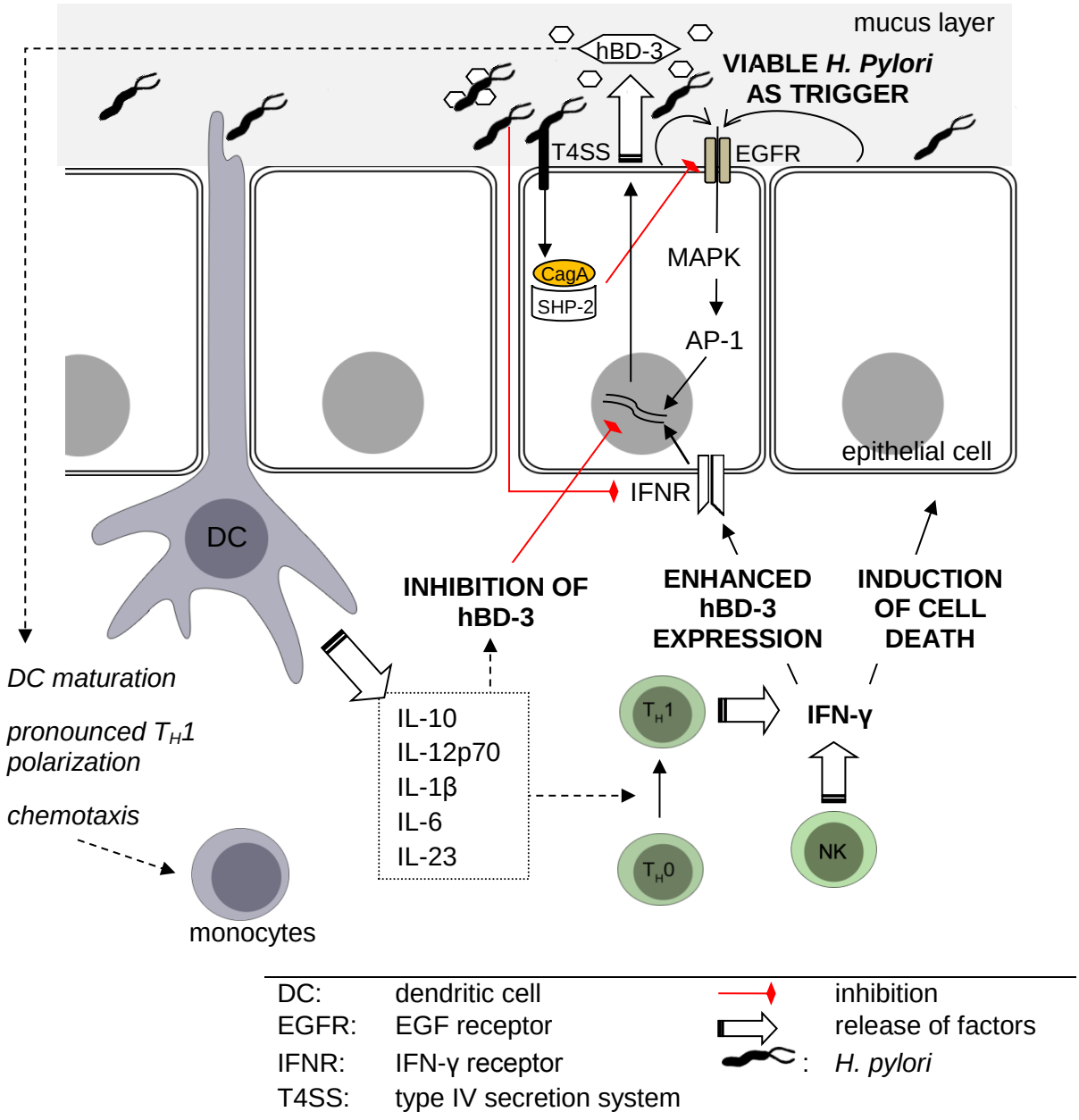

Figure 4.1 Graphical abstract of induction and modulation of hBD-3 expression following *H. pylori* infection. Upon infection viable bacteria are sensed by gastric epithelial cells and via a so far unidentified pathway, the EGFR is phosphorylated followed by an expression of hBD-3, killing *H. pylori*. This induction can be reinforced by IFN-γ, which is known to be released by activated T_H1-cells or other T-cell subsets. Besides modulating hBD-3 induction, IFN-γ leads to enhanced cell death in infected cells. Other biological functions of hBD-3 could also play an important role in the course of infection. hBD-3 is chemotactic to monocytes and leads to maturation of DCs and enhanced polarization towards IFN-γ producing T_H1 cells. However, *H. pylori* interferes with this induction of hBD-3 by blocking EGFR signaling in a CagA-dependent manner and by interfering with IFN-γ signaling. Furthermore *H. pylori* seems to modulate the above mentioned DC/T-cell activation and its stimulating effect on the epithelial layer. In a so far unknown mechanism, DCs and T-cells, upon infection with *H. pylori,* inhibit expression of hBD-3 in gastric epithelial cells.

5. Literature

Aebischer T, Bumann D, Epple HJ, et al. Correlation of T cell response and bacterial clearance in human volunteers challenged with Helicobacter pylori revealed by randomised controlled vaccination with Ty21a-based Salmonella vaccines. Gut, 2008;57:1065–1072.

Akhiani AA, Schön K, Franzén LE, Pappo J, and Lycke N. Helicobacter pylori-specific antibodies impair the development of gastritis, facilitate bacterial colonization, and counteract resistance against infection. Journal of immunology, 2004;172:5024–5033.

Albanesi C, Fairchild HR, Madonna S, et al. IL-4 and IL-13 negatively regulate TNF-alpha- and IFN-gamma-induced beta-defensin expression through STAT-6, suppressor of cytokine signaling (SOCS)-1, and SOCS-3. J Immunol, 2007;179:984–992.

Allison CC, Kufer T a, Kremmer E, Kaparakis M, and Ferrero RL. Helicobacter pylori induces MAPK phosphorylation and AP-1 activation via a NOD1-dependent mechanism. Journal of immunology, 2009;183:8099–8109.

Amedei A, Cappon A, Codolo G, et al. The neutrophil-activating protein of Helicobacter pylori promotes Th1 immune responses. The Journal of clinical investigation, 2006;116:1092–1101.

Appelmelk BJ, Negrini R, Moran AP, and Kuipers EJ. Molecular mimicry between Helicobacter pylori and the host. Trends in microbiology, 1997;5:70–73.

Appelmelk BJ, Monteiro MA, Martin SL, Moran AP, and Vandenbroucke-Grauls CM. Why Helicobacter pylori has Lewis antigens. Trends in microbiology, 2000;8:565–570.

Backert S, and Clyne M. Pathogenesis of Helicobacter pylori infection. Helicobacter, 2011;16 Suppl 1:19–25.

Backert S, Ziska E, Brinkmann V, et al. Translocation of the Helicobacter pylori CagA protein in gastric epithelial cells by a type IV secretion apparatus. Cellular microbiology, 2000;2:155–164.

Backert S, Schwarz T, Miehlke S, et al. Functional analysis of the cag pathogenicity island in Helicobacter pylori isolates from patients with gastritis, peptic ulcer, and gastric cancer. Infect Immun, 2004;72:1043–1056.

Banerjee A, and Gerondakis S. Coordinating TLR-activated signaling pathways in cells of the immune system. Immunology and cell biology, 2007;85:420–424.

Bauer B, and Meyer TF. The Human Gastric Pathogen Helicobacter pylori and Its Association with Gastric Cancer and Ulcer Disease. Ulcers, 2011;2011:1–23.

Bauer B, Pang E, Holland C, et al. The Helicobacter pylori Virulence Effector CagA Abrogates Human β-Defensin 3 Expression via Inactivation of EGFR Signaling. Cell host & microbe, 2012;11:576–586.

Bauer B, Wex T, Kuester D, Meyer T, and Malfertheiner P. Differential expression of human beta defensin 2 and 3 in gastric mucosa of Helicobacter pylori-infected individuals. Helicobacter, 2013;18:6–12.

Beigier-Bompadre M, Moos V, Belogolova E, et al. Modulation of the CD4+ T-cell response by Helicobacter pylori depends on known virulence factors and bacterial cholesterol and cholesterol α-glucoside content. The Journal of infectious diseases, 2011;204:1339–1348.

Bhattacharyya A, Pathak S, Datta S, et al. Mitogen-activated protein kinases and nuclear factor-kappaB regulate Helicobacter pylori-mediated interleukin-8 release from macrophages. Biochem J, 2002;368:121–129.

Bimczok D, Clements RH, Waites KB, et al. Human primary gastric dendritic cells induce a Th1 response to H. pylori. Mucosal immunology, 2010;3:260–269.

Bizzozero G. Ueber die schlauchförmigen Drüsen des Magendarmkanals und die Beziehungen ihres Epithels zu dem Oberflächenepithel der Schleimhaut Dritte Mittheilung. Archiv für Mikroskopische Anatomie, 1893;82–152.

Blander JM, and Sander LE. Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. Nature Reviews Immunology, 2012;12:215–225.

Boncristiano M, Paccani SR, Barone S, et al. The Helicobacter pylori vacuolating toxin inhibits T cell activation by two independent mechanisms. J Exp Med, 2003;198:1887–1897.

Boniotto M, Antcheva N, Zelezetsky I, et al. A study of host defence peptide beta-defensin 3 in primates. The Biochemical journal, 2003;374:707–714.

Boughan PK, Argent RH, Body-Malapel M, et al. Nucleotide-binding oligomerization domain-1 and epidermal growth factor receptor: critical regulators of beta-defensins during Helicobacter pylori infection. The Journal of biological chemistry, 2006;281:11637–11648.

Burova E, Vassilenko K, Dorosh V, Gonchar I, and Nikolsky N. Interferon gamma-dependent transactivation of epidermal growth factor receptor. FEBS letters, 2007;581:1475–1480.

Busiello I, Acquaviva R, Di Popolo A, et al. Helicobacter pylori gamma-glutamyltranspeptidase upregulates COX-2 and EGF-related peptide expression in human gastric cells. Cellular microbiology, 2004;6:255–267.

Cogen AL, Walker SL, Roberts CH, et al. Human beta-defensin 3 is up-regulated in cutaneous leprosy type 1 reactions. PLoS neglected tropical diseases, 2012;6:e1869.

Correa P. A human model of gastric carcinogenesis. Cancer research, 1988;48:3554–3560.

Cover TL, and Blanke SR. Helicobacter pylori VacA, a paradigm for toxin multifunctionality. Nat Rev Microbiol, 2005;3:320–332.

Crabtree JE, Taylor JD, Wyatt JI, et al. Mucosal IgA recognition of Helicobacter pylori 120 kDa protein, peptic ulceration, and gastric pathology. Lancet, 1991;338:332–335.

Crovella S, Antcheva N, Zelezetsky I, et al. Primate beta-defensins--structure, function and evolution. Current protein & peptide science, 2005;6:7–21.

Cullen TW, Giles DK, Wolf LN, et al. Helicobacter pylori versus the host: remodeling of the bacterial outer membrane is required for survival in the gastric mucosa. PLoS pathogens, 2011;7:e1002454.

Dhople V, Krukemeyer A, and Ramamoorthy A. The human beta-defensin-3, an antibacterial peptide with multiple biological functions. Biochimica et biophysica acta, 2006;1758:1499–1512.

Dorer MS, Sessler TH, and Salama NR. Recombination and DNA repair in Helicobacter pylori. Annual review of microbiology, 2011;65:329–348.

Dundon WG, Nishioka H, Polenghi A, et al. The neutrophil-activating protein of Helicobacter pylori. 2002;550:545–550.

Eaton KA, Suerbaum S, Josenhans C, and Krakowka S. Colonization of gnotobiotic piglets by Helicobacter pylori deficient in two flagellin genes. Infection and immunity, 1996;64:2445–2448.

Fehlings M, Drobbe L, Moos V, et al. Comparative analysis of the interaction of Helicobacter pylori with human dendritic cells, macrophages, and monocytes. Infection and immunity, 2012;80:2724–2734.

Ferris LK, Mburu YK, Mathers AR, et al. Human beta-defensin 3 induces maturation of human langerhans cell-like dendritic cells: an antimicrobial peptide that functions as an endogenous adjuvant. The Journal of investigative dermatology, 2013;133:460–468.

Feucht EC, DeSanti CL, and Weinberg A. Selective induction of human beta-defensin mRNAs by Actinobacillus actinomycetemcomitans in primary and immortalized oral epithelial cells. Oral microbiology and immunology, 2003;18:359–363.

Fischbach W. Long term outcome of patients with gastric marginal zone B cell lymphoma of mucosa associated lymphoid tissue (MALT) following exclusive Helicobacter pylori eradication therapy: experience from a large prospective series. Gut, 2004;53:34–37.

Funderburg N, Lederman MM, Feng Z, et al. Human -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. Proc Natl Acad Sci U S A, 2007;104:18631–18635.

García JR, Jaumann F, Schulz S, et al. Identification of a novel, multifunctional beta-defensin (human beta-defensin 3) with specific antimicrobial activity. Its interaction with plasma membranes of Xenopus oocytes and the induction of macrophage chemoattraction. Cell and tissue research, 2001;306:257–264.

Garreis F, Schlorf T, Worlitzsch D, et al. Roles of human beta-defensins in innate immune defense at the ocular surface: arming and alarming corneal and conjunctival epithelial cells. Histochemistry and cell biology, 2010;134:59–73.

Gebert B, Fischer W, Weiss E, Hoffmann R, and Haas R. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. Science, 2003;301:1099–1102.

George JT, Boughan PK, Karageorgiou H, and Bajaj-Elliott M. Host anti-microbial response to Helicobacter pylori infection. Mol Immunol, 2003;40:451–456.

Gewirtz AT, Yu Y, Krishna US, et al. Helicobacter pylori flagellin evades toll-like receptor 5-mediated innate immunity. The Journal of infectious diseases, 2004;189:1914–1920.

Gobert AP, McGee DJ, Akhtar M, et al. Helicobacter pylori arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. Proceedings of the National Academy of Sciences of the United States of America, 2001;98:13844–13849.

Goh K-L, Chan W-K, Shiota S, and Yamaoka Y. Epidemiology of Helicobacter pylori infection and public health implications. Helicobacter, 2011;16 Suppl 1:1–9.

Gong M, Ling SSM, Lui SY, Yeoh KG, and Ho B. Helicobacter pylori gamma-glutamyl transpeptidase is a pathogenic factor in the development of peptic ulcer disease. Gastroenterology, 2010;139:564–573.

Gough DJ, Levy DE, Johnstone RW, and Clarke CJ. IFNgamma signaling-does it mean JAK-STAT? Cytokine & growth factor reviews, 2008;19:383–394.

Grubman A, Kaparakis M, Viala J, et al. The innate immune molecule, NOD1, regulates direct killing of Helicobacter pylori by antimicrobial peptides. Cell Microbiol, 2010;12:626–639.

Haas R, Meyer TF, and Van Putten JP. Aflagellated mutants of Helicobacter pylori generated by genetic transformation of naturally competent strains using transposon shuttle mutagenesis. Molecular microbiology, 1993;8:753–760.

Hafsi N, Voland P, Schwendy S, et al. Human dendritic cells respond to Helicobacter pylori, promoting NK cell and Th1-effector responses in vitro. Journal of immunology, 2004;173:1249–1257.

Haque M, Hirai Y, Yokota K, et al. Lipid profile of Helicobacter spp.: presence of cholesteryl glucoside as a characteristic feature. J Bacteriol, 1996;178:2065–2070.

Harder J, Bartels J, Christophers E, Schroder JM, and Schro J. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. The Journal of biological chemistry, 2001;276:5707–5713.

Hase K, Murakami M, Iimura M, et al. Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against Helicobacter pylori. Gastroenterology, 2003;125:1613–1625.

Hayashi H, Tatebe S, Osaki M, et al. Expression of Fas antigen and its mediation of apoptosis in human gastric cancer cell lines. Japanese journal of cancer research : Gann, 1997;88:49–55.

Higashi H, Tsutsumi R, Muto S, et al. SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. Science, 2002;295:683–686.

Hirsch T, Spielmann M, Zuhaili B, et al. Human beta-defensin-3 promotes wound healing in infected diabetic wounds. The journal of gene medicine, 2009;11:220–228.

Hitzler I, Oertli M, Becher B, Agger EM, and Müller A. Dendritic cells prevent rather than promote immunity conferred by a helicobacter vaccine using a mycobacterial adjuvant. Gastroenterology, 2011;141:186–96, 196.e1.

Hofreuter D, Odenbreit S, and Haas R. Natural transformation competence in Helicobacter pylori is mediated by the basic components of a type IV secretion system. Molecular microbiology, 2001;41:379–391.

Howell MD, Boguniewicz M, Pastore S, et al. Mechanism of HBD-3 deficiency in atopic dermatitis. Clinical immunology, 2006;121:332–338.

Hoy B, Löwer M, Weydig C, et al. Helicobacter pylori HtrA is a new secreted virulence factor that cleaves E-cadherin to disrupt intercellular adhesion. EMBO reports, 2010;11:798–804.

Huang JQ, Zheng GF, Sumanac K, Irvine EJ, and Hunt RH. Meta-analysis of the relationship between cagA seropositivity and gastric cancer. Gastroenterology, 2003;125:1636–1644.

Ishii KJ, Koyama S, Nakagawa A, Coban C, and Akira S. Host innate immune receptors and beyond: making sense of microbial infections. Cell host & microbe, 2008;3:352–363.

Ishikawa T, Kanda N, Hau CS, Tada Y, and Watanabe S. Histamine induces human beta-defensin-3 production in human keratinocytes. Journal of dermatological science, 2009a;56:121–127.

Ishikawa T, Kanda N, Hau CS, Tada Y, and Watanabe S. Histamine induces human beta-defensin-3 production in human keratinocytes. J Dermatol Sci, 2009b;56:121–127.

Jenssen H, Hamill P, and Hancock REW. Peptide antimicrobial agents. Clinical microbiology reviews, 2006;19:491–511.

Jia HP, Schutte BC, Schudy A, et al. Discovery of new human beta-defensins using a genomics-based approach. Gene, 2001;263:211–218.

Joly S, Organ CC, Johnson GK, McCray JPB, and Guthmiller JM. Correlation between [beta]-defensin expression and induction profiles in gingival keratinocytes. Molecular Immunology, 2005;42:1073–1084.

Kandulski A, Selgrad M, and Malfertheiner P. Helicobacter pylori infection: a clinical overview. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver, 2008;40:619–626.

Kao JY, Rathinavelu S, Eaton KA, et al. Helicobacter pylori-secreted factors inhibit dendritic cell IL-12 secretion: a mechanism of ineffective host defense. American journal of physiology. Gastrointestinal and liver physiology, 2006;291:G73–G81.

Kaparakis M, Turnbull L, Carneiro L, et al. Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. Cellular Microbiology, 2010;12:372–385.

Kawauchi K, Yagihashi A, Tsuji N, et al. Human beta-defensin-3 induction in H. pylori-infected gastric mucosal tissues. World J Gastroenterol, 2006;12:5793–5797.

Keates S, Sougioultzis S, Keates a C, et al. cag+ Helicobacter pylori induce transactivation of the epidermal growth factor receptor in AGS gastric epithelial cells. The Journal of biological chemistry, 2001;276:48127–48134.

Kidd M, Miu K, Tang LH, et al. Helicobacter pylori lipopolysaccharide stimulates histamine release and DNA synthesis in rat enterochromaffin-like cells. Gastroenterology, 1997;113:1110–1117.

Kim K-M, Lee S-G, Kim J-M, et al. Helicobacter pylori gamma-glutamyltranspeptidase induces cell cycle arrest at the G1-S phase transition. Journal of microbiology, 2010;48:372–377.

Kimura M, Goto S, Wada A, et al. Vacuolating cytotoxin purified fromHelicobacter pyloricauses mitochondrial damage in human gastric cells. Microbial Pathogenesis, 1999;26:45–52.

Kirschner DE, and Blaser MJ. The dynamics of Helicobacter pylori infection of the human stomach. Journal of theoretical biology, 1995;176:281–290.

Koff JL, Shao MXG, Ueki IF, and Nadel J a. Multiple TLRs activate EGFR via a signaling cascade to produce innate immune responses in airway epithelium. American journal of physiology. Lung cellular and molecular physiology, 2008;294:L1068–75.

Kranzer K, Eckhardt A, Aigner M, et al. Induction of maturation and cytokine release of human dendritic cells by Helicobacter pylori. Infection and immunity, 2004;72:4416–4423.

Kranzer K, Söllner L, Aigner M, et al. Impact of Helicobacter pylori virulence factors and compounds on activation and maturation of human dendritic cells. Infection and immunity, 2005;73:4180–4189.

Kuck D, Kolmerer B, Iking-Konert C, et al. Vacuolating cytotoxin of Helicobacter pylori induces apoptosis in the human gastric epithelial cell line AGS. Infect Immun, 2001;69:5080–5087.

Kuipers EJ, Pena AS, Van Kamp G, et al. Seroconversion for Helicobacter pylori. Lancet, 1993;342:328–331.

Kuipers EJ, Pérez-Pérez GI, Meuwissen SG, and Blaser MJ. Helicobacter pylori and atrophic gastritis: importance of the cagA status. Journal of the National Cancer Institute, 1995;87:1777–1780.

Kwok T, Zabler D, Urman S, et al. Helicobacter exploits integrin for type IV secretion and kinase activation. Nature, 2007;449:862–866.

Lai C-H, Wang H-J, Chang Y-C, et al. Helicobacter pylori CagA-mediated IL-8 induction in gastric epithelial cells is cholesterol-dependent and requires the C-terminal tyrosine phosphorylation-containing domain. FEMS microbiology letters, 2011;323:155–163.

Lepper PM, Triantafilou M, Schumann C, Schneider EM, and Triantafilou K. Lipopolysaccharides from Helicobacter pylori can act as antagonists for Toll-like receptor 4. Cellular microbiology, 2005;7:519–528.

Leunk RD, Johnson PT, David BC, Kraft WG, and Morgan DR. Cytotoxic activity in broth-culture filtrates of Campylobacter pylori. J Med Microbiol, 1988;26:93–99.

Liang SC, Tan X-Y, Luxenberg DP, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. The Journal of experimental medicine, 2006;203:2271–2279.

Lindgren A, Yun CH, Lundgren A, et al. CD8- natural killer cells are greatly enriched in the human gastrointestinal tract and have the capacity to respond to bacteria. Journal of innate immunity, 2010;2:294–302.

Lindgren A, Pavlovic V, Flach C-F, Sjöling A, and Lundin S. Interferon-gamma secretion is induced in IL-12 stimulated human NK cells by recognition of Helicobacter pylori or TLR2 ligands. Innate immunity, 2011;17:191–203.

Maldonado RA, and Von Andrian UH. How tolerogenic dendritic cells induce regulatory T cells. Advances in immunology, 2010;108:111–165.

Marshall BJ, and Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet, 1984;1:1311–1315.

Matsuda JL, Naidenko O V, Gapin L, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J Exp Med, 2000;192:741–754.

Mattner J, Debord KL, Ismail N, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature, 2005;434:525–529.

Menendez A, and Brett Finlay B. Defensins in the immunology of bacterial infections. Current opinion in immunology, 2007;19:385–391.

Menzies BE, and Kenoyer A. Signal transduction and nuclear responses in Staphylococcus aureus-induced expression of human beta-defensin 3 in skin keratinocytes. Infection and immunity, 2006;74:6847–6854.

Mitchell DJ, Huynh HQ, Ceponis PJM, Jones NL, and Sherman PM. Helicobacter pylori disrupts STAT1-mediated gamma interferon-induced signal transduction in epithelial cells. Infection and immunity, 2004;72:537–545.

Mitchell P, Germain C, Fiori PL, et al. Chronic exposure to Helicobacter pylori impairs dendritic cell function and inhibits Th1 development. Infection and immunity, 2007;75:810–819.

Moese S, Selbach M, Kwok T, et al. Helicobacter pylori induces AGS cell motility and elongation via independent signaling pathways. Infection and immunity, 2004;72:3646–3649.

Moran AP. Relevance of fucosylation and Lewis antigen expression in the bacterial gastroduodenal pathogen Helicobacter pylori. Carbohydrate research, 2008;343:1952–1965.

Müller A, Oertli M, and Arnold IC. H. pylori exploits and manipulates innate and adaptive immune cell signaling pathways to establish persistent infection. Cell communication and signaling : CCS, 2011;9:25.

Muotiala A, Helander IM, Pyhälä L, Kosunen TU, and Moran AP. Low biological activity of Helicobacter pylori lipopolysaccharide. Infection and immunity, 1992;60:1714–1716.

Naito Y, Kino I, Horiuchi K, and Fujimoto D. Promotion of collagen production by human fibroblasts with gastric cancer cells in vitro. Virchows Archiv. B, Cell pathology including molecular pathology, 1984;46:145–154.

Nakayama M, Kimura M, Wada A, et al. Helicobacter pylori VacA activates the p38/activating transcription factor 2-mediated signal pathway in AZ-521 cells. J Biol Chem, 2004;279:7024–7028.

Naumann M, Wessler S, Bartsch C, et al. Activation of activator protein 1 and stress response kinases in epithelial cells colonized by Helicobacter pylori encoding the cag pathogenicity island. The Journal of biological chemistry, 1999;274:31655–31662.

Necchi V, Candusso ME, Tava F, et al. Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by Helicobacter pylori. Gastroenterology, 2007;132:1009–1023.

Necchi V, Manca R, Ricci V, and Solcia E. Evidence for transepithelial dendritic cells in human H. pylori active gastritis. Helicobacter, 2009;14:208–222.

Nishioka H, Baesso I, Semenzato G, et al. The neutrophil-activating protein of Helicobacter pylori (HP-NAP) activates the MAPK pathway in human neutrophils. European journal of immunology, 2003;33:840–849.

O'Keeffe J, and Moran AP. Conventional, regulatory, and unconventional T cells in the immunologic response to Helicobacter pylori. Helicobacter, 2008;13:1–19.

O'Neil D a, Cole SP, Martin-Porter E, et al. Regulation of human beta-defensins by gastric epithelial cells in response to infection with Helicobacter pylori or stimulation with interleukin-1. Infection and immunity, 2000;68:5412–5415.

Obonyo M, Rickman B, and Guiney DG. Effects of myeloid differentiation primary response gene 88 (MyD88) activation on Helicobacter infection in vivo and induction of a Th17 response. Helicobacter, 2011;16:398–404.

Odenbreit S, Püls J, Sedlmaier B, et al. Translocation of Helicobacter pylori CagA into Gastric Epithelial Cells by Type IV Secretion. Science, 2000;287:1497–1500.

Oertli M, Noben M, Engler DB, et al. Helicobacter pylori γ-glutamyl transpeptidase and vacuolating cytotoxin promote gastric persistence and immune tolerance. Proceedings of the National Academy of Sciences of the United States of America, 2013;110:3047–3052.

Otte J-M, Neumann HM, Brand S, et al. Expression of beta-defensin 4 is increased in human gastritis. European journal of clinical investigation, 2009;39:126–138.

Ou G, Baranov V, Lundmark E, Hammarström S, and Hammarström M-L. Contribution of intestinal epithelial cells to innate immunity of the human gut--studies on polarized

monolayers of colon carcinoma cells. Scandinavian journal of immunology, 2009;69:150–161.

Parkin DM. The global health burden of infection-associated cancers in the year 2002. International journal of cancer. Journal international du cancer, 2006;118:3030–3044.

Parsonnet J, Friedman GD, Vandersteen DP, et al. Helicobacter pylori infection and the risk of gastric carcinoma. The New England journal of medicine, 1991;325:1127–1131.

Perez-Perez GI, Rothenbacher D, and Brenner H. Epidemiology of Helicobacter pylori infection. Helicobacter, 2004;9 Suppl 1:1–6.

Perez-Perez GI, Olivares AZ, Foo FY, et al. Seroprevalence of Helicobacter pylori in New York City populations originating in East Asia. J Urban Health, 2005;82:510–516.

Pernet I, Reymermier C, Guezennec A, et al. Calcium triggers beta-defensin (hBD-2 and hBD-3) and chemokine macrophage inflammatory protein-3 alpha (MIP-3alpha/CCL20) expression in monolayers of activated human keratinocytes. Exp Dermatol, 2003;12:755–760.

Petersson C, Forsberg M, Aspholm M, et al. Helicobacter pylori SabA adhesin evokes a strong inflammatory response in human neutrophils which is down-regulated by the neutrophil-activating protein. Medical microbiology and immunology, 2006;195:195–206.

Pham KT, Weiss E, Jiménez Soto LF, et al. CagI is an essential component of the Helicobacter pylori Cag type IV secretion system and forms a complex with CagL. PloS one, 2012;7:e35341.

Qing Y, and Stark GR. Alternative activation of STAT1 and STAT3 in response to interferon-gamma. The Journal of biological chemistry, 2004;279:41679–41685.

Raymond J, Thiberg JM, Chevalier C, et al. Genetic and transmission analysis of Helicobacter pylori strains within a family. Emerg Infect Dis, 2004;10:1816–1821.

Romani N, Gruner S, Brang D, et al. Proliferating dendritic cell progenitors in human blood. The Journal of experimental medicine, 1994;180:83–93.

Romano M, Ricci V, Di Popolo A, et al. Helicobacter pylori upregulates expression of epidermal growth factor-related peptides, but inhibits their proliferative effect in MKN 28 gastric mucosal cells. The Journal of clinical investigation, 1998;101:1604–1613.

Sallusto F, and Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. The Journal of experimental medicine, 1994;179:1109–1118.

Sander LE, Davis MJ, Boekschoten M V, et al. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. Nature, 2011;474:385–389.

Sayi A, Kohler E, Hitzler I, et al. The CD4+ T cell-mediated IFN-gamma response to Helicobacter infection is essential for clearance and determines gastric cancer risk. Journal of immunology, 2009;182:7085–7101.

Scharf S, Vardarova K, Lang F, et al. Legionella pneumophila induces human beta defensin-3 in pulmonary cells. Respiratory research, 2010;11:93.

Schmausser B, Andrulis M, Endrich S, et al. Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in Helicobacter pylori infection. Clinical and experimental immunology, 2004;136:521–526.

Schmitt W, and Haas R. Genetic analysis of the Helicobacter pylori vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. Molecular microbiology, 1994;12:307–319.

Schreiber S, Konradt M, Groll C, et al. The spatial orientation of Helicobacter pylori in the gastric mucus. Proceedings of the National Academy of Sciences of the United States of America, 2004;101:5024–5029.

Schreiber S, Bücker R, Groll C, et al. Rapid loss of motility of Helicobacter pylori in the gastric lumen in vivo. Infection and immunity, 2005;73:1584–1589.

Schroder K, Hertzog PJ, Ravasi T, and Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. Journal of leukocyte biology, 2004;75:163–189.

Schwarz S, Morelli G, Kusecek B, et al. Horizontal versus familial transmission of Helicobacter pylori. PLoS pathogens, 2008;4:e1000180.

Schweitzer K, Sokolova O, Bozko PM, and Naumann M. Helicobacter pylori induces NF-kappaB independent of CagA. EMBO reports, 2010;11:10–11.

Selbach M, Moese S, Hauck CR, Meyer TF, and Backert S. Src is the kinase of the Helicobacter pylori CagA protein in vitro and in vivo. J Biol Chem, 2002;277:6775–6778.

Selsted ME, and Ouellette AJ. Mammalian defensins in the antimicrobial immune response. Nature immunology, 2005;6:551–557.

Shi Y, Liu X-F, Zhuang Y, et al. Helicobacter pylori-induced Th17 responses modulate Th1 cell responses, benefit bacterial growth, and contribute to pathology in mice. Journal of immunology, 2010;184:5121–5129.

Shimada M, Ina K, Kyokane K, et al. Upregulation of mucosal soluble fas ligand and interferon-gamma may be involved in ulcerogenesis in patients with Helicobacter pylori-positive gastric ulcer. Scandinavian journal of gastroenterology, 2002;37:501–511.

Smith MF, Mitchell A, Li G, et al. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells. The Journal of biological chemistry, 2003;278:32552–32560.

Sørensen OE, Thapa DR, Rosenthal A, et al. Differential regulation of beta-defensin expression in human skin by microbial stimuli. Journal of immunology, 2005;174:4870–4879.

Stein M, Bagnoli F, Halenbeck R, et al. c-Src/Lyn kinases activate Helicobacter pylori CagA through tyrosine phosphorylation of the EPIYA motifs. Molecular microbiology, 2002;43:971–980.

Steubesand N, Kiehne K, Brunke G, et al. The expression of the beta-defensins hBD-2 and hBD-3 is differentially regulated by NF-kappaB and MAPK/AP-1 pathways in an in vitro model of Candida esophagitis. BMC immunology, 2009;10:36.

Stingl K, Altendorf K, and Bakker EP. Acid survival of Helicobacter pylori: how does urease activity trigger cytoplasmic pH homeostasis? Trends Microbiol, 2002;10:70–74.

Suerbaum S, Smith JM, Bapumia K, et al. Free recombination within Helicobacter pylori. Proceedings of the National Academy of Sciences of the United States of America, 1998;95:12619–12624.

Szabo I, Brutsche S, Tombola F, et al. Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of Helicobacter pylori is required for its biological activity. Embo J, 1999;18:5517–5527.

Takagi a, Watanabe S, Igarashi M, et al. The effect of Helicobacter pylori on cell proliferation and apoptosis in gastric epithelial cell lines. Alimentary pharmacology & therapeutics, 2000;14 Suppl 1:188–192.

Tan S, Tompkins LS, and Amieva MR. Helicobacter pylori usurps cell polarity to turn the cell surface into a replicative niche. PLoS pathogens, 2009;5:e1000407.

Tonkic A, Tonkic M, Lehours P, and Mégraud F. Epidemiology and diagnosis of Helicobacter pylori infection. Helicobacter, 2012;17 Suppl 1:1–8.

Uemura N, Okamoto S, Yamamoto S, et al. Helicobacter pylori infection and the development of gastric cancer. The New England journal of medicine, 2001;345:784–789.

Uribe JM, McCole DF, and Barrett KE. Interferon-gamma activates EGF receptor and increases TGF-alpha in T84 cells: implications for chloride secretion. American journal of physiology. Gastrointestinal and liver physiology, 2002;283:923–931.

Viala J, Chaput C, Boneca IG, et al. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol, 2004a;5:1166–1174.

Viala J, Chaput C, Boneca IG, et al. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nature immunology, 2004b;5:1166–1174.

Wada A, Yamasaki E, and Hirayama T. Helicobacter pylori vacuolating cytotoxin, VacA, is responsible for gastric ulceration. J Biochem, 2004;136:741–746.

Van de Wal Y, Corazza N, Allez M, et al. Delineation of a CD1d-restricted antigen presentation pathway associated with human and mouse intestinal epithelial cells. Gastroenterology, 2003;124:1420–1431.

Wallasch C, Crabtree JE, Bevec D, et al. Helicobacter pylori-stimulated EGF receptor transactivation requires metalloprotease cleavage of HB-EGF. Biochemical and biophysical research communications, 2002;295:695–701.

Wang Y-H, Gorvel J-P, Chu Y-T, Wu J-J, and Lei H-Y. Helicobacter pylori impairs murine dendritic cell responses to infection. PloS one, 2010;5:e10844.

Watanabe S, Takagi a, Koga Y, Kamiya S, and Miwa T. Helicobacter pylori induces apoptosis in gastric epithelial cells through inducible nitric oxide. Journal of gastroenterology and hepatology, 2000;15:168–174.

Weeks DL, Eskandari S, Scott DR, and Sachs G. A H+-gated urea channel: the link between Helicobacter pylori urease and gastric colonization. Science, 2000;287:482–485.

Wiesner J, and Vilcinskas A. Antimicrobial peptides: the ancient arm of the human immune system. Virulence, 2010;1:440–464.

Wilson KT, and Crabtree JE. Immunology of Helicobacter pylori: insights into the failure of the immune response and perspectives on vaccine studies. Gastroenterology, 2007;133:288–308.

Wolk K, Kunz S, Witte E, et al. IL-22 increases the innate immunity of tissues. Immunity, 2004;21:241–254.

Wunder C, Churin Y, Winau F, et al. Cholesterol glucosylation promotes immune evasion by Helicobacter pylori. Nat Med, 2006;12:1030–1038.

Yamaoka Y. Importance of in clinical presentation, gastric inflammation, and mucosal interleukin 8 production. Gastroenterology, 2002;123:414–424.

Yamaoka Y, Kwon DH, and Graham DY. A M(r) 34,000 proinflammatory outer membrane protein (oipA) of Helicobacter pylori. Proceedings of the National Academy of Sciences of the United States of America, 2000;97:7533–7538.

Yokozaki H. Molecular characteristics of eight gastric cancer cell lines established in Japan. Pathology international, 2000;50:767–777.

You M, Yu DH, and Feng GS. Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated Jak/STAT pathway. Molecular and cellular biology, 1999;19:2416–2424.

Young DF, Carlos TS, Hagmaier K, Fan L, and Randall RE. AGS and other tissue culture cells can unknowingly be persistently infected with PIV5; a virus that blocks interferon signalling by degrading STAT1. Virology, 2007;365:238–240.

Yun CH, Lundgren A, Azem J, et al. Natural killer cells and Helicobacter pylori infection: bacterial antigens and interleukin-12 act synergistically to induce gamma interferon production. Infect Immun, 2005;73:1482–1490.

Zhang M, Liu M, Luther J, and Kao JY. Helicobacter pylori directs tolerogenic programming of dendritic cells. Gut microbes, 2010;1:325–329.

Schistosomes, liver flukes and Helicobacter pylori. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. IARC monographs on the evaluation of carcinogenic risks to humans / World Health Organization, International Agency for Research on Cancer, 1994;61:1–241.

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Ich, Lennart Pfannkuch, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Human beta defensin 3 induction in gastric epithelial cells under *Helicobacter pylori* infection and cytokine-dependent modulation“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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