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LTβR-mediated alternative NF-κB signaling executes *H. pylori*-induced gastric pathology independent of CagA

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Abbreviation list

α	alpha
β	beta
κ	kappa
BabA	Blood group antigen-binding adhesin
bp	basepair
Cag	Cytotoxin associated gene
CD	cluster of differentiation
CCL	chemokine (C-C motif) ligand
CCR	chemokine receptor
CO_2	Carbon dioxide
CXCL	chemokine (C-X-C motif) ligand
d	day
DC	dendritic cell
DNA	deoxyribonucleic acid
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram
gGT	gamma-Glutamyltranspeptidase
h	hour(s)
H. pylori	*Helicobacter pylori*
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
i.p.	intraperitoneal
k	kilo
kb	kilobase
l	liter
LB	Lucia broth
LIGHT	lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes; TNFSF14
LN	lymph node
LPS	Lipopolysaccharide
LTα	Lymphotoxin α
LTβ	lymphotoxin β
LTβR	lymphotoxin β receptor
mm	millimeter
M	molar
MALT	Mucosa-associated lymphoid tissue

µ	micro
mg	milligram
MHC	major histocompatibility complex
min	minute(s)
MOI	Multiplicity of infection
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
OD	Optical density
PAI	Pathogenicity island
PAMP	Pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PFA	paraformaldehyde
p.i.	post infection
pH	Pondus Hydrogenii
PRR	pattern recognition receptor
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
s	second
SD	standard derivation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLO	secondary lymphoid organ
TEMED	Tetramethyldiamine
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TNFR	tumor necrosis factor
Treg	regulatory T cell
UreA/B	urease subunit A/B
WHO	World Health Organization
wt	wild-type

1 Summary

Gastric cancer is stated the third leading cause of cancer-related deaths worldwide, mostly due to lack of specific early clinical symptoms and therefore late diagnosis. A critical factor contributing to carcinogenesis is chronic tissue inflammation, which is, in the case of gastric cancer, associated with persistent colonization of the gastric mucosa with the bacterium *Helicobacter (H.) pylori*. Approximately 50% of the human population is infected with *H. pylori*, of which 10% develop clinical symptoms. Canonical NF-κB signaling plays a major role during both acute and chronic inflammatory responses and has been already well documented to drive development in *H. pylori*-mediated gastric pathology. Pathway activation is induced by conserved bacterial components, such as *H. pylori* lipopolysaccharides or peptidoglycan, which are recognized by the innate pattern recognition receptors Toll-like receptor (TLR) 2 and 4 or via TLR5, respectively. In addition, *H. pylori* possesses an array of virulence factors including the type 4 secretion system T4SS (encoded by cagPAI) and CagA,, which gets translocated via T4SS into the host epithelial cell thereby initiating an inflammatory response. Canonical NF-κB signaling plays a major role in acute and chronic inflammatory responses and is already well documented to drive development in *H. pylori*-mediated gastric pathology. Thus far, however, a functional role of alternative / non-canonical NF-κB signaling in this context has remained elusive. Through analyses of gastric patient biopsy samples, *in vitro* experiments with gastric cancer cell lines infected with distinct *H. pylori* clinical isolates and an *in vivo* infection model with a human pathogenic *H. pylori* strain, this study provides evidence that *H. pylori* infection induces the alternative NF-κB signaling pathway. Alternative NF-κB signaling initiation was triggered via ligation of the LTβR with lymphotoxin (LT), whose expression was upregulated via the canonical NF-κB activation in a cagPAI-dependent but CagA-independent manner in gastric epithelial cells following infection. Importantly, LTβR triggering by LT occurred both in cis and trans, thus leading to activation of alternative NF-κB signaling in both infected and bystander gastric

epithelial cells. This LT-driven feed-forward loop induced a broadened pro-inflammatory chemokine milieu and also the expression of the pathway-specific cytokine LIGHT, which promoted ongoing inflammation independently of canonical NF-κB. Blocking of LTβR signaling *in vivo* suppressed *H. pylori*-driven gastritis mediated by inflammatory and epithelial cells but increased bacterial load, whereas LTβR-stimulated activation of the signaling pathway exacerbated pathology via LIGHT and reduced bacterial colonization. The study at hand identifies LTβR-mediated alternative NF-κB signaling as an important secondary signaling cascade upon initial canonical NF-κB activation that drives *H. pylori*-induced chronic gastritis, thereby setting the basis for progression towards gastric cancer.

2 Zusammenfassung

Das Magenkarzinom ist die dritthäufigste Form eines durch Krebsleiden verursachten Todes weltweit, hauptsächlich begründet durch eine späte Diagnose aufgrund fehlender Früherkennungssymptome. Ein entscheidender Faktor, der zur Krebsentstehung beiträgt, ist eine langanhaltende Entzündung des Gewebes. Im Magen wird diese Entzündung durch das Bakterium *Helicobacter pylori* induziert mit dem etwa 50% der Erdbevölkerung infiziert sind, von denen etwa 10% klinische Symptome entwickeln. Der kanonische NF-κB Signalweg spielt in akuter und chronischer Entzündung eine Schlüsselrolle und ist bereits im Detail in Hinblick auf eine *H. pylori*-induzierte Pathologieentwicklung im Magen beschrieben. Die Aktivierung erfolgt durch konservierte bakterielle Komponenten wie beispielsweise bakterielles Lipopolysachharid oder Peptidoglycan, die jeweils durch die Mustererkennungsrezeptoren Toll-like Rezeptor (TLR) 2 und 4 oder TLR5 des Wirts erkannt werden. Zusätzlich verfügt *H. pylori* über verschiedene Virulenzfaktoren, unter anderem das T4 Sekretionssystem (T4SS) (kodiert auf der Cag-Pathogenitätsinsel (cagPAI)), sowie CagA, den das Pathogen in Epithelzellen transloziert und somit die Entzündungsantwort auslöst.

Die Rolle des alternativen NF-κB Signalwegs ist bisher größtenteils ungeklärt. Durch die Analyse von humanen Magenbiopsien, *in vitro* Studien in Magenkrebs-Zelllinien mit verschiedenen klinischen *H. pylori* Isolaten und ein *in vivo* Mausmodell mit dem human *H. pylori* PMSS-1 Stamm bestätigten *H. pylori*-vermittelte Aktivierung des alternativen NF-κB Signalwegs über den Lymphotoxin β Rezeptor (LTβR). Die Infektion mit *H. pylori* führte zu einer Hochregulierung des Liganden LT spezifisch über den kanonische NF-κB Signalweg, unabhängig vom Virulenzfaktor CagA aber abhängig von cagPAI. Die durch Interaktion von LT und LTβR vermittelte Aktivierung des alternativen NF-κB Signalwegs in Magenepithelzellen in cis und trans.

Obwohl die gegen *H. pylori* gerichtete Immunantwort durch den kanonischen NF-κB Signalweg induziert wird, verstärkt der alternative NF-κB Signalweg die pro-inflammatorische Chemokinantwort der Magenepithelzellen in *cis* und *trans* und im weiteren Verlauf durch Hochregulierung des LTβR-spezifischen Liganden LIGHT auch unabhängig vom kanonischen NF-κB Signalweg. Eine Blockierung des LTβR Signalwegs *in vivo* unterstützt die *H. pylori*-induzierte Gastritis, führt jedoch zu einer erhöhten Anzahl von Bakterien. Im Gegensatz dazu konnte die Bakterienkolonisierung reduziert werden durch verstärkte LTβR Signalwegaktivierung.

Die vorliegende Doktorarbeit zeigt, dass *H. pylori*-ausgelöste alternative Signalwegaktivierung über den LTβR einen wichtige Kaskade darstellt, die sekundär nach kanonischer NF-κB Signalwegaktivierung die chronische Gastritis vorantreibt, die sich letztendlich zu Magenkrebs verschlimmern kann.

3 Introduction

3.1 *Helicobacter pylori*

3.1.1 Gastric cancer

Of all diagnosed cancers worldwide, an amount of 6.8% brings stomach cancer on the 5th place on the most common cancer's list in 2012. However, a 5-year relative survival rate of only 30% (Japan excluded) shifts gastric cancer on the 3rd place concerning cancer mortality (Ferlay et al., 2015). The umbrella term stomach cancer comprises a subset of 5 different malignant gastric pathologies, with Gastric Adenocarcinoma (GC) being the most abundant one (95%). Further forms are the Non-Hodgkin lymphomas including MALT lymphoma (<5%), as well as gastrointestinal stromal tumors (1-3%) and to a lesser extent Leiomyosarcoma and Carcinoid tumors (both <1%). Due to its heterogeneous pathology, GC is further defined by anatomical location (cardia and non-cardia) and histology. The cardiac form of gastric cancer could be linked to gastro-esophageal reflux and several other factors such as smoking, diet or *Helicobacter pylori* colonization.

3.1.2 The stomach

Liver, pancreas, small and large intestine and the stomach constitute the gastrointestinal tract which regulates the digestion. The stomach in particular is a muscular organ, located between the esophagus and the small intestine on the left side of the abdomen. Anatomically the stomach is divided into the regions cardia, fundus, corpus and antrum with each of them composed of several tissue layers. The outermost layer, the so called serosa is a fibrous membrane, which isolates the inner gastric layers from the remaining body interior and also covers the muscularis propria and underlying submucosa. The submucosa is a layer of connective tissue harboring glands that secrete digestive hormones, nerve cells, blood vessels and also lymph glands. The

underlying mucosa, which is the epithelial layer of the stomach, also consists of numerous glands, which secrete digestive enzymes as well as products essential for tissue protection. The specialized cells providing the necessary environment of a functional stomach include 1) mucus secreting cells to protect the gastric epithelium from the acidic environment of the stomach; 2) chief cells, which secrete the pre-active form of pepsin (named pepsinogen), an enzyme necessary for denaturation of proteins from absorbed nutrients and 3) parietal cells that produce hydrochloric acid, which provides the acidic gastric milieu of a low pH of 2, thereby killing harmful bacteria and pathogenic factors that might be in the chyme.

3.1.3 *Helicobacter pylori*

During food intake, various different species of bacteria get distributed in the gastric lumen. The acidic milieu ensures that most of these species are not capable to survive. The spiral-shaped pathogen *Helicobacter pylori* is unique in that it is perfectly adjusted to the low oxygenated as a result of an over 60000 yearlong co-evolution with humans (Blaser, 1998; Falush et al., 2003; Ghose et al., 2002). This Gram-negative bacterium of 2 to 4 µm length, which selectively colonizes the mucus of the gastric lumen, is carried by more than 50% of humankind. Infection with *H. pylori* provokes a coexisting chronic inflammatory response in its host, although infection remains subclinical in the majority of infected individuals. *H. pylori* possesses 3 to 5 flagella localized on one end of the bacterial rod thereby guaranteeing polar and screw-like motility necessary for penetration of the bacterium through the mucin layer that covers the lumen of the stomach (Chan et al., 1994). Movement of *H. pylori* is highly dependent on the surrounding tissues' pH value, which needs to be above 4 for an efficient motility of the bacterium (Miller-Podraza et al., 1999). Since the human stomach presents in its antral part a higher pH compared to the gastric corpus, initial colonization of *H. pylori* occurs in the gastric antrum (Weeks et al., 2000). The suitable pH value to enable colonization is provided by *H. pylori* itself through production of the enzyme urease (Eaton et al., 1991; Eaton et al., 1992), which

hydrolyzes urea to ammonium followed by binding of hydrogen ions that are present in the stomach which finalizes in the formation of ammonia (NH_3) and CO_2 (Bauerfeind et al., 1997; Marshall et al., 1990). The maintenance of a neutral pH enveloping the bacterium enables its survival in the acidic gastric environment (Hawtin et al., 1990; Perez-Perez et al., 1992). Colonization of the stomach with *H. pylori* typically occurs during childhood via oral-to oral and/or fecal-to-oral transmission from *H. pylori* colonized individuals (Ernst et al., 2000; Everhart, 2000). Without proper antibiotic treatment the bacterium establishes a long-term chronic active gastritis (Brown, 2000; Perry et al., 2006), thereby significantly increasing the risk of further site-specific diseases, such as peptic ulcer disease (~10%), mucosa-associated lymphoid tissue (MALT) lymphoma (<0.1%) or gastric adenocarcinoma (1-3%) (Peek et al., 2006).

3.1.4 Pathogenesis of *H. pylori*

Co-evolution of thousands of years between the pathogen and its host established equilibrium between bacterium and host factors that guarantees the survival of both species. However, long-term colonization of *H. pylori* might lead to severe pathology, including gastric cancer (GC), which justified the 1994 set designation of *H. pylori* as class I carcinogen by the WHO. Due to the lack of early specific symptoms, diagnosis of GC is often made very late (mean age men: 50.4 years, women: 47.7 years) (Correa et al., 2007; Hatakeyama, 2004), i.e. at a stage when the cancer has already invaded into the muscularis propria (Correa, 2004). Histologically, GC is subdivided into diffuse-type adenocarcinoma, characterized by individually infiltrating neoplastic cells without formation of glandular structures, and intestinal-type adenocarcinoma, which is known to develop through multi-step progression (Correa, 1992). Host responses that could be verified to influence progression to gastric carcinogenesis are reduction of acid secretion and gastric inflammation (El-Omar, 2001), thereby representing the basis for severe pathology namely transition of normal mucosa to chronic gastritis. According to the updated Sydney

system, chronic gastritis is classified into mild, moderate and severe forms depending on the amounts of lymphocytes in the tissue during diagnosis (Stolte et al., 2001).

H. pylori-induced inflammation can be accompanied by lack of gastric acid (hypochlorhydria) upon shifting of the bacterial colonization to the corpus resulting in atrophic gastritis and intestinal metaplasia. Epithelial damage facilitates the possible progression towards dysplasia and finally development of intestinal-type adenocarcinoma (Correa, 1996; Sipponen et al., 2000).

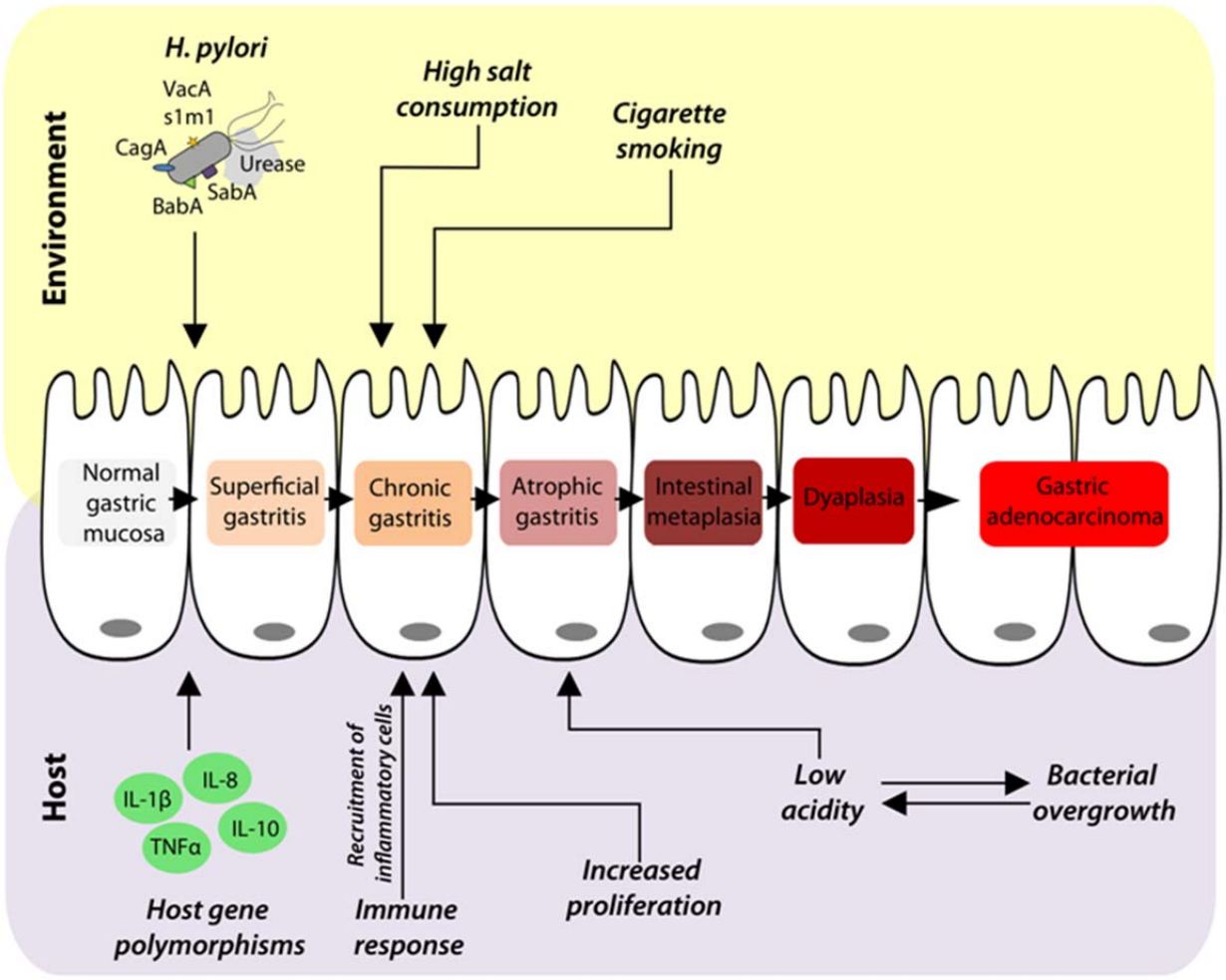

Figure A: **Multifactorial cascade of *H. pylori* induced pathology finalizing in gastric carcinoma.** The interplay of host, bacterial and environmental factors affect the outcome of disease in every person differently (adapted from Wroblewski LE).

The outcome of chronic infection with *H. pylori* varies from patient to patient and is shaped by a diversity of factors, including polymorphisms in the genes for the cytokine IL-1β (El-Omar et al., 2000) and the pro-inflammatory cytokine TNFα (El-Omar et al., 2003) which influence propensity toward gastric carcinogenesis.

3.1.5 Virulence factors of *H. pylori*

3.1.5.1 Adhesins of H. pylori

Around 80% of *H. pylori* remain constantly in the mucus layer, whereas 15-20% adhere directly to the gastric epithelial cells (Noach et al., 1994), thereby facilitating persistence of infection and delivery of virulence factors into the host epithelial cell.

In contrast to other bacterial species, the genome of *H. pylori* encodes for a very high amount of outer membrane proteins (OMPs) (approximately 4%) (Wroblewski et al., 2010), including the Blood group antigen binding adhesin (BabA) (Boren et al., 1993; Gerhard et al., 1999) and the Sialic acid-binding adhesin (Mahdavi et al., 2002; Shet et al.; Yamaoka et al., 2006).

3.1.5.2 The cag pathogenicity island (cagPAI)

The 37 kb-sized cag pathogenicity island (cagPAI) encodes up to 30 different genes whose order on the cagPAI is evolutionary conserved. At least 18 of the genes are components necessary for the functional bacterial type IV secretion system (T4SS) which is capable of translocating bacterial molecules across the bacterial membrane into gastric epithelial cells of the host. Assembly of the secretion apparatus and/or transport of substrates is dependent on energy provided by two ATPases, Caga and CagE (Kutter et al., 2008; Mossey et al., 2010). Intensive research revealed that *H. pylori* strains harboring a cagPAI could augment the risk for more severe clinical pathology (severe and atrophic gastritis or distal gastric cancer) which was observed to be lower in strains lacking cagPAI (Blaser, 1995; Crabtree et al., 1991; Kuipers et al., 1995; Parsonnet et al., 1997; Queiroz et al., 1998).

3.1.5.3 The type 4 secretion system (T4SS) of H. pylori

As pathogen, *H. pylori* uses its T4SS to inject host-cell modulating virulence-associated factors into the epithelial host cell, thereby inducing pro-inflammatory responses as well as multiple cytoskeletal and gene regulatory effects. *H. pylori* displays two different types of T4SS, one for horizontal gene transfer and the *cytotoxin-associated gene* (*cag*) T4SS (Fischer et al., 2010; Kutter et al., 2008) that is encoded on the *cytotoxin-associated gene*-pathogenicity island (cagPAI). So far known, the T4SS of *H. pylori* delivers CagA and components of the bacterial peptidoglycan into gastric epithelial cells which results in activation of the immune response.

3.1.5.4 The CagA protein

The 120-140 kDa CagA protein is the N-terminal gene product of cagPAI. Being expressed in almost 100% of East Asian *H. pylori* strains and around 70% in Western *H. pylori* strains (Alm et al., 1999; Censini et al., 1996; Tomb et al., 1997), CagA is defined as a bacterial oncoprotein capable of inducing malignant transformation of cells (Hatakeyama, 2008). Its expression seems to be directly correlated with gastric pathologies, including chronic active gastritis, peptic ulcer disease, MALT lymphoma and gastric cancer. This was demonstrated in biopsy samples which displayed CagA-positive *H. pylori* strains in more than 90 % of analysed cases (Blaser, 1998). Following attachment of the bacterium to the epithelial cell wall, the CagA protein is translocated into the host cell via the T4SS, where it becomes tyrosine phosphorylated followed by further interacting with various intracellular effectors e.g. an eukaryotic tyrosine phosphatase (SHP-2) which results in epithelial cell elongation (Tsutsumi et al., 2006).

3.1.5.5 The VacA Toxin

In addition to the CagA protein, the vacuolating cytotoxin A plays a major role in the pathogenesis induced by *H. pylori*. The secreted protein affects epithelial cell structure, resulting in barrier function disruption and modulation of the host immune response (Leunk et al., 1988). Binding of VacA to several receptors on the host cell surface e.g. the receptor-type protein

tyrosine phosphatase, affects cell proliferation, differentiation and adhesion (Fujikawa et al., 2003). However it is the antagonizing regulation between CagA and VacA controlled by the bacterium by which *H. pylori* can avoid excessive cellular damage and thereby maintain suitable conditions for persistent colonization of the gastric niche (Oldani et al., 2009; Tegtmeyer et al., 2009).

3.1.6 Chronic inflammation with *H. pylori* and the immune response

Mucosal colonization with *H. pylori* induces a local immune response characterized by recruitment of neutrophils (Crabtree et al., 1994a), macrophages (Suzuki et al., 2002), DCs (Khamri et al., 2010) and lymphocytes (primarily Th1 cells), which produce cytokines and chemokines important to maintain inflammation and to clear the pathogen. During the acute phase of *H. pylori* infection, neutrophils respond to an IL-8 gradient towards the local inflammation (Allen et al., 2000), followed by macrophages which respond to epithelial signals and pathogen-derived products. Macrophages are incapable of killing *H. pylori* (Zheng et al., 2003), yet rather support pathogen survival by formation of megasomes (Schwartz et al., 2006). Furthermore, macrophages amplify the inflammatory reponse by cytokine production, including TNFα and IL-6 which have been both linked to NF-κB signaling activation (Harris et al., 1998; Mai et al., 1991; Pathak et al., 2006). In combination with DCs, macrophages further produce cytokines (including IL-12 or IL-10) (Banchereau et al., 2000; Haeberle et al., 1997; Meyer et al., 2003; Meyer et al., 2000) to stimulate the adaptive immune system, primarily Th1 cells. $CD4^+CD25^-$ effector T cells and NK cells produce interferon γ (IFNγ), which is on the one hand the key molecule in the controlling the *H. pylori*-induced inflammation (Haeberle et al., 1997; Meyer et al., 2003; Meyer et al., 2000), yet also stimulates mucosal pre-neoplastic alterations in the stomach (Sayi et al., 2011). Furthermore, B cells were also shown to contribute to *H. pylori*-driven pathogenesis. In a study of *H. pylori* positive B-cell-deficient mice (μMT, which have a disruption of the membrane exon of the immunoglobulin μ chain gene (Kitamura et al., 1991)),

reduced bacterial colonization was observed which results in increased $CD4^+$ T cell influx and consequently stronger gastric inflammation (Akhiani et al., 2004). In addition this and another study suggested a B-cell mediated counterproductive antibody response to *H. pylori* (Akhiani et al., 2005). However, detailed research on T- and B-cell interaction during *H. pylori*-induced pathology is missing.

3.2 NF-κB signaling

3.2.1 NF-κB, inflammation and cancer

In the year 2000, Doug Hanahan and Robert Weinberg summed up the hallmarks of cancer, a collective of several factors that contribute to a malignant outcome, including cancer: independent control of growth factors and insensitivity to signals crucial for cell growth inhibition, evasion of apoptosis (programmed cell death), indefinite possibility to replicate, continuous angiogenesis and the potential to metastasize tissues (Hanahan et al., 2000). In 2011, inflammation was included on that list and defined as a necessary factor for acquiring the other hallmarks (Hanahan et al., 2011). Cells of the innate immune system (myeloid progenitor cells, mast cells, macrophages and neutrophils) play in general an essential role in the defense against invading organisms. However, accumulating data of the last years have revealed, what was suspected by the Rudolf Virchow already a century ago (Balkwill et al., 2001): persistent inflammation induced by autoimmunity or by infectious agents can not only have a protective effect, but rather leads to a reverse outcome by promoting tumorigenic consequences (Cover et al., 2009; Llovet et al., 2003; Mostafa et al., 1999; Nishiyama et al., 2004; Pohl et al., 2000). The pro-inflammatory NF-κB signaling pathway is a major driver of the immune response. Under physiological conditions, NF-κB initiates the cessation of its own activity by both resynthesis of IκBα and cylandromatosis (CYLD) expression. Furthermore direct ubiquitination followed by nuclear NF-κB degradation is a second possibility for NF-κB downregulation (Yang et al., 2009).

However, activation of NF-κB is accepted to be a double-edged sword. Although it is essential for the activation of the host immune response (innate and adaptive) against pathogens, constitutive pathway activation increases pathogenesis of infectious diseases, chronic inflammation and cancer (Hayden et al., 2008).

3.2.2 The NF-κB transcription factor (TF) family

As one of most intensively studied signaling pathways involved in inflammation, the *nuclear factor 'kappa-light-chain-enhancer' of activated B-cells* (NF-κB) signaling pathway tightly controls cytokine and chemokine responses against either self or infectious agents (e.g. viruses or bacteria), thereby playing a crucial role in immune system regulation. The mammalian family of NF-κB transcription factors comprises p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) and p100 /p52 (NF-κB2), which share an amino-terminal Rel homology domain (RHD) essential for dimerization, interaction with IκBs and DNA binding (Ghosh et al., 2012). With support of their transactivation domain (TAD) NF-κB transcription factors can stimulate recruitment of transcriptional co-activators to the DNA. The tight regulation of the transcription factors is provided by seven IκB proteins (including the precursors p100 and p105 (Hinz et al., 2012)), whose main function is to prevent the binding of the NF-κB dimer to the DNA. Transcription requires proteasomal degradation of the precursor forms p100 to p52 and p105 to p50 and association with the TAD-containing transcription factors RelB or RelA, respectively.

3.2.3 The canonical (classical) NF-κB signaling pathway

Activation of the canonical NF-κB signaling pathway can be stimulated by extracellular and intracellular signals. Among many others, tumor necrosis factor (TNF) α, a pro-inflammatory cytokine which is a member of the TNF superfamily plays a major role. Binding of TNF family

members to the specific receptor triggers a rapid transcription of genes controlling inflammatory responses, cell survival, proliferation and differentiation.

In addition, also toll-like receptors (TLR) induce canonical NF-κB signaling following recognition of bacterial components, since the invading microbes are commonly recognized by the innate immune system of the host organism.

The canonical NF-κB signaling pathway depends on degradation of IκB proteins. Following TNFR triggering, the receptor interacting kinase 1 (RIP1) and TRAF2 are recruited to the receptor via the adaptor molecule like TNFR-1 associated DEATH domain protein (TRADD). Rapid RIP1 ubiquitination, probably induced by TRAF2 via K63-linked polyubiquitin chains leads to stabilization of the TNFR complex with the ubiquitin-binding domain of NEMO, which in turn recruits the IkB kinase complex (IKK) (Ea et al., 2006; Hacker et al., 2006; Wu et al., 2006). The release of NF-κB dimers from IκB proteins is reliant on phosphorylation by the IκB kinase (IKK) complex, a trimeric complex consisting of two catalytic (IKKα and IKKβ) and one regulatory (IKKγ/NEMO (NF-κB essential modulator)) subunit. Phosphorylation of IκB leads to recognition and further polyubiquitination of IκB proteins by the ubiquitin ligase machinery (SCF family) and finally to degradation or – in case of p100 and p105 – further processing by the proteasome. Eventually the heterotrimeric NF-κB p50-RelA molecule gets translocated into the nucleus and initiates target gene expression (Hayden & Ghosh, 2008; Vallabhapurapu et al., 2009) (Figure B).

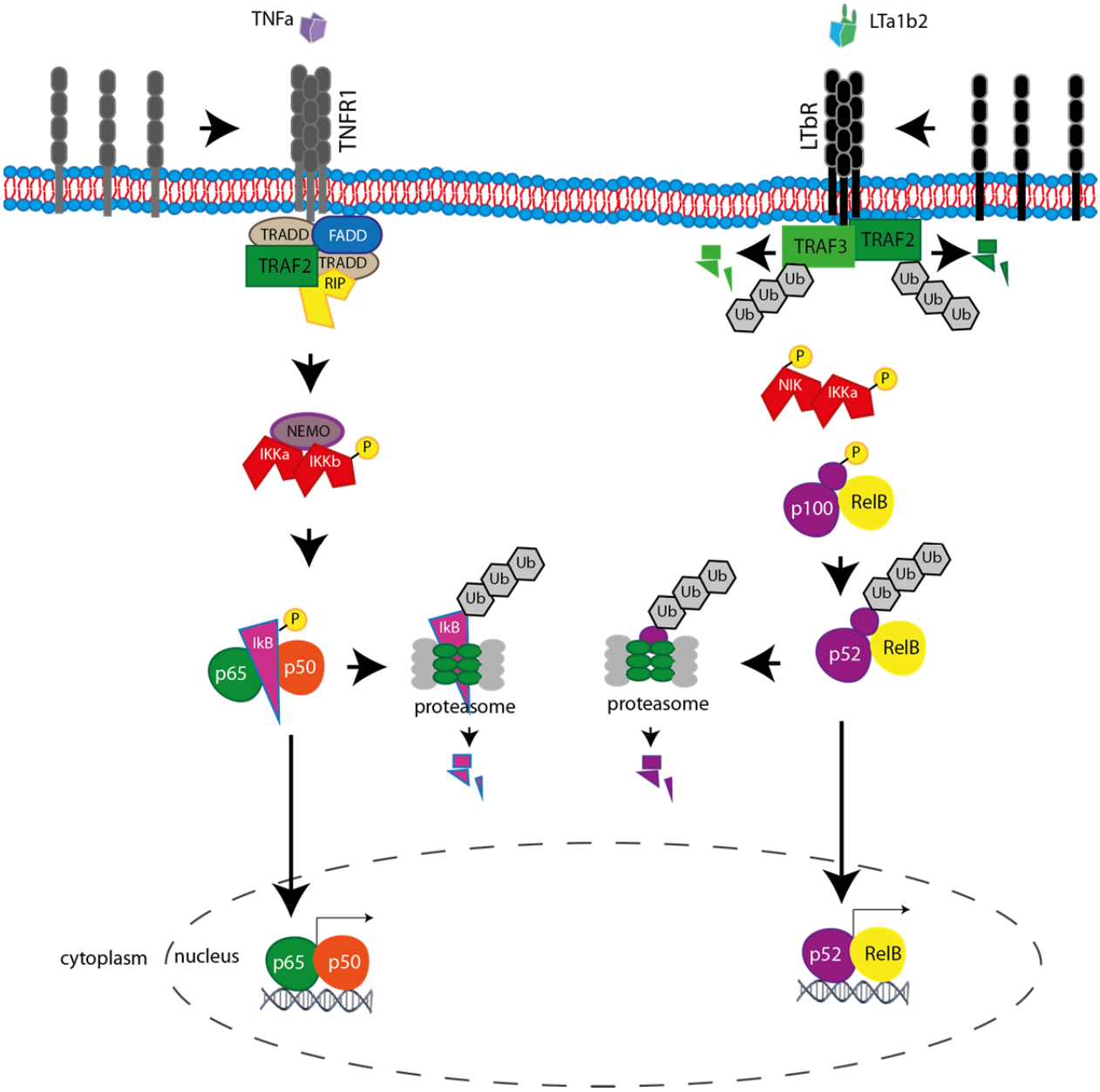

Figure B: Canonical and alternative pathways of NF-κB signaling.
In general - under unstimulated conditions - inhibitory IκB proteins bind inactive NF-κB complexes in the cytoplasm. Following stimulation, IκB protein degradation is initiated through phosphorylation by the IκB kinase (IKK) complex, consisting of two catalytically (IKKα and IKKβ) and one regulatory subunit (IKKγ/NEMO). Phosphorylated IκB proteins are targeted for ubiquitination and degradation by the proteasome. The released NF-κB dimers can translocate to the nucleus and initiated target gene expression.
Stimulation of the canonical NF-κB signaling pathway (left), here represented by TNFα/TNFR1 signaling, induces binding of TRADD, which provides an assembly platform for recruitment of FADD and TRAF2 molecules. TRAF2 associates with RIP1 for IKK activation and final phosphorylation of IκBα in an IKKβ- and NEMO-dependent manner, resulting in the nuclear translocation of p65-containing (typically p50-p65). In contrast, the alternative pathway (right), here represented by LTα1β2/ LTβR signaling, involves IKKα-mediated phosphorylation of p100 associated with RelB. IKKα activation and phosphorylation of p100 depends on NIK, which is subject to complex regulation by TRAF3, TRAF2 and additional ubiquitin ligases. Partial processing of p100 to p52 generates transcriptionally active p52-RelB complexes that translocate into the nucleus and initiated target gene expression.
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3.2.4 The non-canonical (alternative) NF-κB signaling pathway

3.2.4.1 Receptors and ligands

Alternative NF-κB signaling can be elicited by five receptors (Sun, 2011), in contrast to canonical NF-κB signaling, which can be activated by diverse receptors. Alternative NF-κB receptors include CD40 and its corresponding ligand CD40L, RANK and ligand RANKL, BAFFR and ligand BAFF, Fn14 and ligand TNF-like weak inducer of apoptosis (TWEAK) and the lymphotoxin β receptor (LTβR) with the corresponding ligands lymphotoxin (LT) and LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes) (Sun, 2011).

3.2.4.2 The LT system

3.2.4.2.1 The ligands LT and LIGHT

LT system signaling through the LTβR is a signaling pathway involved in various inflammatory and cancer-associated pathologies (Drayton et al., 2006; Haybaeck et al., 2009; Kratz et al., 1996; Luther et al., 2000; Picarella et al., 1992; Seleznik et al., 2012). LT was first described around 1968 as a soluble lymphocytes-derived secreted protein with cytotoxic activity (Granger et al., 1968; Ruddle et al., 1968). The structural similar cytokines LTα and LTβ (Browning et al., 1993) are members of the TNF superfamily (TNFSF) (Hehlgans et al., 2005) and were described to be expressed by T, B, natural killer (NK) and lymphoid tissue-inducer cells in mice and men under physiological conditions (Aggarwal, 2003). Whereas mRNA expression of LTα is inducible, LTβ mRNA is, in most cases, constitutively produced (e.g. by B cells) (Browning et al., 1993; Worm et al., 1994). Under pathological conditions such as tissue injury/damage or chronic infectious diseases, both LTs can also be expressed by stromal or parenchymal cells as was shown for hepatocytes in Hepatitis B and C viruses-caused chronic hepatitis and subsequent hepatocellular carcinoma or acinar cells in the context of autoimmune pancreatitis (Haybaeck et

al., 2009; Seleznik et al., 2012). After induction, LTα is secreted as soluble $LT\alpha_3$ homotrimers that can act locally or on distal cells by binding and signaling (like TNFα) through the TNF receptors TNFR1 or TNFR2. LTβ in turn is a type II transmembrane-protein, anchored in the cell membrane, thereby primarily acting locally via cell-cell contact, which can bind LTα to form either $LT\alpha_2\beta_1$ or $LT\alpha_1\beta_2$ heterotrimers on the cell surface of activated lymphocytes (Browning et al., 1995; Browning et al., 1993) (Figure C). Cleavage of cell surface-associated LT by various enzymatic active proteins (e.g. matrix metalloproteases) elicits responses on distal cells (Young et al., 2010).

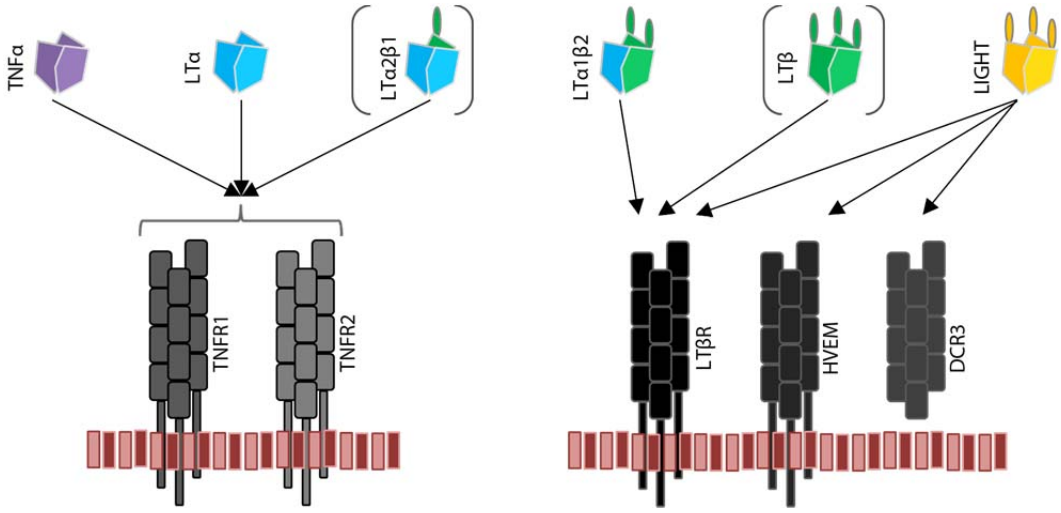

Figure C: Ligands and receptors of the LT system
Soluble and membrane-bound trimeric ligands are shown in the upper row. Arrows indicate binding to the corresponding receptors in the lower row. Low affinity ligands (LTα2β1) or ligands without known biological function/ physiological existence are marked in brackets (Adapted from Browning, JL)

Although $LT\alpha_2\beta_1$ triggers both TNFR1 and TNFR2, this ligand was reported to be a diminutive form of LT, solely expressed in very low amounts (<2%) by T cells (Ware, 2005) with its biological role yet to be defined. The second and most prevalent/abundant LT heterotrimer, $LT\alpha_1\beta_2$, binds and signals specifically through the LTβR, a TNFR superfamily member, expressed on lymphoid stromal and epithelial cells, but was also shown to be expressed on myeloid cells (Norris et al., 2007). In addition to $LT\alpha_1\beta_2$, triggering of LTβR can happen through

the alternative ligand LIGHT that is also capable of interacting with herpesvirus entry mediator (Schneider et al., 2004) or the decoy receptor 3 (DCR3) (Ware, 2005). Furthermore it was recently shown that single-chain LTβββ is able to bind to and activate LTβR on its own with similar specificity as $LT\alpha_1\beta_2$ (Schuepbach-Mallepell et al., 2015).

The interplay of ligands (LTα, LTβ and LIGHT) and receptors (TNFR1, TNFR2 and LTβR) – in general summarized as LT system – ensures an effective signaling network between lymphocytes and tissue cells both under physiological as well as pathological conditions.

3.2.4.2.2 The biological functions of the LT system

Similar to the biological functions of TNF, LT is crucial for the regulation of various cellular processes including proliferation, survival / apoptosis and differentiation (Locksley et al., 2001). Studies in LT system component knockout mice revealed an essential role of LTβR signaling in the organogenesis of secondary lymphoid tissues, the maintenance of lymphoid tissue microarchitecture but also in the regulation of gastrointestinal immune function (Banks et al., 1995; De Togni et al., 1994; Futterer et al., 1998; Koni et al., 1997; Matsumoto et al., 1996a; Matsumoto et al., 1996b; Ngo et al., 1999; Pfeffer et al., 1993). For instance, LTα and LTβ-deficient mice failed to form any peripheral and mesenteric lymph nodes or Peyer's Patches and showed a disrupted microarchitecture of the spleen due to lack of follicular dendritic cells (FDC) and germinal centers displaying the importance of LT under physiological conditions (De Togni et al., 1994; Koni et al., 1997). Signaling through LTβR is necessary for the expression of several chemokines (CXCL13, CCL21, CCL19, and SFD-1a) which are essential for both organization of lymphoid organs and homing of lymphocyte to peripheral non-lymphoid tissues (Dejardin et al., 2002).

Development of tertiary (ectopic) lymphoid organs (TLO) could be observed in inflammation-driven malignancies, like chronic inflammation triggered by persistent infection (Drayton et al., 2006) or cancer (Bergomas et al., 2011; Cipponi et al., 2012; Martinet et al., 2011). TLOs are lymphoid cell accumulations which resemble lymph nodes with regard to their cellular content (T and B cells, DCs, FDCs and stromal cells) and micro-anatomical organization e.g. compartmentalization of T and B cells, high endothelial venules, and lymphatic vessels (LVs) (Link et al., 2011; Penaranda et al., 2010; Stranford et al., 2012). These structures are defined by the expression of lymphoid chemokines (e.g. CXCL13), which already regulate lymphocyte and DC movement during development (Drayton et al., 2006; Hjelmstrom et al., 2000). It is generally accepted that TLOs provide local sites of antigen presentation, lymphocyte activation and also class switching in B cells (Schroder et al., 1996), thereby facilitating local immune responses induced by e.g. microorganisms.

3.2.5 NF-κB and *H. pylori* infection

Invading microbes are commonly recognized by the innate immune system of the host organism via pathogen-associated molecular patterns (PAMPs), which include LPS or bacterial peptides (e.g. peptidoglycan). Detection is possible via germ-line encoded pattern-recognition receptors (PRRs) that are primarily expressed on innate immune cells but also on a variety of non-hematopoietic cells, e.g. epithelial cells. PRRs comprise the family of Toll-like receptors (TLRs) (Beutler et al., 2006) and NOD-like receptors (NLRs) (Ting et al., 2005). Recent data suggest that at least three different bacterial products contribute to *H. pylori*-associated activation of NF-κB signaling: 1) *H. pylori* LPS which is recognized by TLR2 or TLR4 (Ishihara et al., 2004; Kawahara et al., 2001; Ting & Williams, 2005), 2) bacterial wall component peptidogylcan via NOD1 (Allison et al., 2009) and 3) CagA which activates NF-κB signaling via translocation into the epithelial cell (Suzuki et al., 2009). Sokolova and colleagues could show that *H. pylori*

transiently activates the IKK complex in a cagPAI-dependent manner but independently of CagA translocation (Sokolova et al., 2014). Assembly of the IKK complex and therefore its integrity results in stabilization of the regulatory subunit NEMO which is necessary for phosphorylation of IκBa and RelA. In the case of an *H. pylori* infection the IKK complex is controlled by MEKK3 and TAK1, leading to transient recruitment of TRAF6 to TAK1. The resulting autophosphorylation and K63-linked ubiquitinylation of the TAK1 protein induces canonical NF-κB downstream signaling including transient TAK1 interaction with the IKK complex (Sokolova et al., 2014).

Although the canonical NF-κB signaling pathway was well characterized in the context of an *H. pylori infection,* the molecular effects on pathology provided by the alternative NF-κB arm induced by the microbe remain unknown. Studies in *H. pylori* infected patients revealed high expression levels of CXCL13, implying active alternative NF-κB signaling (Galamb et al., 2008; Nakashima et al., 2011). Also, cell culture models displayed *H. pylori*-induced NF-κB2 activation in gastric epithelial cells via the downstream key molecule of LTβR signaling NIK (Maeda et al., 2000; Neumann et al., 2006). Thus far, specific activation of alternative NF-κB signaling by downstream p100/p52 processing during *H. pylori* infection has only be observed in B cells (Ohmae et al., 2005). However, the mechanisms of H. pylori-induced pathology in epithelial cells as well as of alternative NF-κB signaling in this process are still poorly understood.

3.3 Aim of study

A pro-inflammatory environment triggered by viral or bacterial infection initiates rapid activation of distinct signaling cascades such as the intensively studied NF-κB signaling pathway.

Chronic active inflammation is the major hallmark of an *H. pylori* infection. Various studies confirmed that canonical NF-κB plays a crucial role in the pathology of an *H. pylori* infection and can eventually contribute to the development of gastric cancer and progression. Accumulating data suggest an essential role of the alternative NF-κB signaling pathway in chronic inflammatory diseases induced by e.g. infectious agents, such as hepatitis B and C viruses or autoimmune diseases such as rheumatoid arthritis. However, the importance of the alternative NF-κB signaling pathway in the context of a persistent *H. pylori* infection remains elusive.

The aim of this thesis was to study the role of alternative NF-κB signaling during an *H. pylori* infection with a primary focus on its contribution to gastric inflammation/pathology induced by the bacterium.

In the first part of this study human gastric biopsy samples should be characterized with regard to alternative NF-κB ligand/receptor expression, the resulting activation of the signaling pathway and downstream target gene expression. To specifically address the underlying molecular effects of alternative NF-κB signaling induced by the pathogen, an *in vitro* cell culture model should be established, in which the canonical arm of NF-κB signaling was selectively blocked in different gastric cell lines by specific antibody treatment. The alternative NF-κB signaling pathway separated from the canonical arm in several gastric cancer cell lines and the interplay with the bacterium should reveal the specific role of the alternative NF-κB signaling in *H. pylori* driven gastric pathology.

In the second part of this study, subsequent analyses should be undertaken to investigate the secondary effects of activated signaling in terms of inflammatory cell influx into the gastric tissue

with either protective or aggravating capacity in several stages of gastric pathology. Furthermore alternative NF-κB signaling should be analyzed in *H. pylori*-mediated gastric pathology of an intact living organism (mouse model), where signaling can either be blocked or stimulated by specific antibodies thereby supporting data gained from human tissue analyses.

4 Materials and Methods

4.1 Materials

4.1.1 Antibodies

Target	Clone	Company	Host	Reactivity	Dilution WB/FACS/in vivo	Conc.IHC	ARC
B220	RA3-6B2	BD Biosciences	rat	M		1:3000	20 min EDTA, 100°C
b-actin	13 E5	Cell Signaling	rabbit	M, H	1:10000		
CD20	MS4A1	R&D systems	mouse	H		1:100	30 min EDTA, 95°C
CD21/35	7G6	BD Biosciences	rat	M		1:2000	Aceton 10 min, + Formalin 30 min
CD3	SP7	NeoMarkers/ Lab Vision Corporation	rabbit	M, H		1:300	20 min EDTA, 95°C
CD4P	4SM95	eBioscience	rat	M		1:1000	30 min EDTA, 95°C
CD68	FA-11	Serotec	rat	H		1:200	30 min EDTA, 100°C
F4/80	BM8	BMA Biomarkers	rat	M		1:120	10 min enzyme, 37°C
FDC-M1	mono	BD Biosciences	rat	M		1:60	Aceton 10 min, + Formalin 30 min
goat anti-rat IgG Alexa488	poly	BD Biosciences		H	1:1000		
HRP	secondary	Promega	rabbit	M	1:10000		

IL-6	mono	R&D	goat	H	1:1000		
IL-6, recomb.	*E. coli*	Pepprotech		H			
LTb	B27	Biogen Idec	mouse	H		1:1000	60 min EDTA, 100°C
LTbR	ACH6	Biogen Idec		M	1 µg/mL		
LTbR	BS-1	Biogen Idec		H	1 µg/mL		
LTbR	6D66	Santa Cruz		H	1 µg/mL		
LTbR-mIgG (fusion protein)		Biogen Idec		M	1 µg/mL		
LTbR-hIgG (fusion protein)		Biogen Idec		H	1 µg/mL		
p100/p52	poly	Cell Signaling		M	1:1000		
NF-kappa b p52	mono	Merck Millipore	mouse	H		1:100	20 min EDTA, 95°C
NF-kappa b p65 (Rel-A)	poly	NeoMarkers	rabbit	M, H		1:500	20 min EDTA, 95°C
NF-kB (p105/p50)	poly	Cell Signaling	rabbit	H	1:1000		
p-NF-kB p65 (Ser536)	93H1	Cell Signaling	rabbit	H	1:1000		
RelB	poly (C-19)	Santa Cruz	rabbit	M, H	1:1000	1:400	20 min EDTA, 100°C
p-RelB (ser 552)	poly	Santa Cruz	rabbit	M, H	1:1000		
TPCA-1		Sigma-Aldrich		H	1:1000		

Tab. Antibodies used for Western Blot analyses or IHC staining in alphabetical order.

4.1.2 Chemicals

Compound	Company	Country
Accutase	Sigma-Aldrich	Taufkirchen, Germany
Acetic Acid	Roth	Karlsruhe, Germany
Albumine bovine fraction V	Biomol GmbH	Hamburg Germany
Amphotericin B	Oxoid	Wesel, Germany
Brain heart infusion (BHI)	MP Biomedical	Eschwege, Germany
Cefsulodin	Oxoid	Wesel, Germany
Citrate	Sigma Aldrich	Taufkirchen, Germany
Disodium hydrogen phosphate	Sigma Aldrich	Taufkirchen, Germany
DTT (DL-Dithiothreitol)	Promega	Mannheim, Germany
Eosin	Waldeck GmbH & Co. KG	Münster, Germany
Ethanol absolute	Merck KGaA	Darmstadt, Germany
Ethylene glycol tetraacetic acid	Sigma Aldrich	Taufkirchen, Germany
Ethylenediaminetetraacetic acid	Sigma Aldrich	Taufkirchen, Germany
Fetal Bovine Calf Serum (FCS) Gold	PAA	Freiburg, Germany
Glycerol	Sigma Aldrich	Taufkirchen, Germany
Horse serum	Invitrogen	Darmstadt, Germany
Igepal-CA630	Sigma Aldrich	Taufkirchen, Germany
Kanamycin (50µg/ml)	Sigma-Aldrich	Taufkirchen, Germany
Lennox-L-Broth	Invitrogen GIBCO	Darmstadt, Germany
L-Glutamine	Sigma-Aldrich	Taufkirchen, Germany
LPS, ultrapure	Sigma-Aldrich	Taufkirchen, Germany
Milk powder	Roth	Karlsruhe, Germany
Monopotassium phosphate	Sigma Aldrich	Taufkirchen, Germany
PBS	Invitrogen GIBCO	Darmstadt, Germany
Penicillin (Pen)	Invitrogen GIBCO	Darmstadt, Germany
Phloxin	Merck KGaA	Darmstadt, Germany
Pierce ECL solution	Thermo-Scientific	Dreieich, Germany
Potassiumchlorid	Sigma Aldrich	Taufkirchen, Germany
Potassium dihydrogen phosphate	Sigma Aldrich	Taufkirchen, Germany
Prestained Low range Protein Molecular Weight Marker	MBI Fermentas	St.Leon-Rot, Germany
Protran Nitrocellulose membrane	Sigma-Aldrich	Taufkirchen, Germany
Sodium	Oxoid	Wesel, Germany
Sodium bicarbonate	Sigma Aldrich	Taufkirchen, Germany
Sodium carbonate	Sigma Aldrich	Taufkirchen, Germany
Sodium dodecyl sulfate solution 20%	Sigma Aldrich	Taufkirchen, Germany
Sodium hydrogen phosphate	Sigma Aldrich	Taufkirchen, Germany
Sodiumchlorid	Sigma Aldrich	Taufkirchen, Germany

Sodiumdeoxycholate	Sigma Aldrich	Taufkirchen, Germany
Streptomycin (Ishikawa, M. et al.)	Invitrogen GIBCO	Darmstadt, Germany
Tris(hydroxymethyl)aminomethane	VWR International GmbH	Erlangen, Germany
Triton X-100	Sigma Aldrich	Taufkirchen, Germany
Tween-20	BioRad	München, Germany
Vancomycin	Oxoid	Wesel, Germany
X-VIVO 15	Lonza	Köln, Germany

Tab. Chemicals in alphabetical order.

4.1.3 Buffers / solutions / media

Buffers/Solutions/Media	Recipe / Company
1x PBS	135 mM NaCl 2.5 mM KCl 10 mM Na2HPO4 1.8 mM KH2PO4
1x SDS sample buffer	62.5 mM Tris-HCl pH 6.8 50 mM DTT 2% (w/v) SDS 10% (v/v) Glycerol 0.01% (w/v) bromphenol blue
1x TBST Washing buffer)	0.1 M Tris-Cl (pH 8,0) 1,5 M NaCl 0.5% Tween-20
Acrylamide solution	40% (w/v) acrylamide / Bis 19:1 (Ambion)
BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution	BD Bioscience, San Jose, USA
BD Perm/Wash™ Buffer	BD Bioscience, San Jose, USA
Blocking buffer	TBST + 5% BSA or TBST + 5% milk powder
Brain heart infusion (BHI) medium	36 g BHI 0.25% (w/v) yeast extract 5% (v/v) FCS 1 l aqua
Dent-Supplement	Vancomycin Amphotericin B Cefsulodin Sodium Trimethoprimlactat
Electrophoresis buffer	25 mM Tris-HCl 250 mM Glycin

	0.1% (w/v) SDS pH 8.3
Eosin Stock Solution	4 g Eosin 0,4 g Phloxin 440 mL ddH2O
Eosin Staining Solution	60 mL Eosin Stock solution 430 mL 96% ethanol 2,2 mL glacial acidic acid
Flow cytometry buffer	1x PBS 20 mM EDTA 2% FBS
Freezing medium for *H. pylori*	BHI-medium 20% Glycerol 20% FCS
Infection medium	RPMI + Gln 10% heat-inactivated FCS Gold
Luria-Bertani (LB)-Medium	20 g/l Lennox-L-Broth, autoclaved
Ponceau S Solution	0.1% PONCEAU-S (w/v) in 5% acetic acid solution
Stacking gel	125 mM Tris-HCl pH 6.8 0.1% SDS 0.1% APS 0.1% TEMED
Separating gel	375 mM Tris-HCl pH 8.8 0.1% SDS 0.1% APS 0.04-0.06% TEMED
Transfer buffer	25 mM Tris-HCl 190 mM Glycin 0.01% (w/v) SDS 20% Ethanol
Wilkins Chalgren (WC)-dent	21.5 g WC-Agar 500 ml aqua 50 ml inactivated horse serum 0.4 g potassium nitrate Dent-Supplement

Tab. Buffers in alphabetical order with recipe or if commercially available company listed.

4.1.4 Bacterial strains

bacterial strain	isogenic strains	plating	provider / source
Helicobacter pylori G27	-	Wilkins-Chalgren blood agar	(Xiang et al., 1995)
Helicobacter pylori G27	G27ΔCagA	20 µg/ml of kanamycin or 15 µg/ml of chloramphenicol under the same conditions	KanR, (Figura et al., 2004)
Helicobacter pylori G27	G27ΔCagE		(Censini et al., 1996; Figura et al., 2004)
Helicobacter pylori G27	G27ΔCagF		(Pattis et al., 2007)
Helicobacter pylori G27	G27ΔCagI		(Segal et al., 1997)
Helicobacter pylori G27	G27ΔBabA		(Boren et al., 1993)
Helicobacter pylori G27	G27ΔSabA		(Talarico et al., 2012)
Helicobacter pylori G27	G27ΔVacA		(Oertli et al., 2013)
Helicobacter pylori G27	G27ΔgGT		(Schmees et al., 2007)
Helicobacter pylori G27	G27ΔUreA/B		transformation from 2808 ΔUreA/B
PMSS-1	human gastric cancer cell line	Wilkins-Chalgren blood agar	(Arnold et al., 2011; Lee et al., 1997)
SS-1	human gastric cancer cell line	Wilkins-Chalgren blood agar	(Jeong et al., 2000; Lee et al., 1997)
Tx30	human gastric cancer cell line	Wilkins-Chalgren blood agar	(Aras et al., 2003)
clinical isolates I-VII	-	Wilkins-Chalgren blood agar	Städtisches Klinikum München

Tab. *H. pylori* strains and isogenic mutants with growing conditions and publication.

4.1.5 Cell lines

Cell line	Origin	Medium	source
HEK293	human embryonic kidney cells	DMEM 10% FCS 1x Pen/Strep	kindly provided by Martin Sprinzl
HepG2	human hepatoma cell line	DMEM 10% FCS 1x Pen/Strep	kindly provided by Martin Sprinzl

AGS	human gastric cancer cell line	DMEM 10% FCS 1x Pen/Strep	ATCC
AZ521	human gastric cancer cell line	MEM + Glutamax 10% FCS 1x Pen/Strep	Japanese Collection of Research Biosources, Osaka, Japan
MKN45	human gastric cancer cell line	DMEM 10% FCS 1x Pen/Strep	(Motoyama et al., 1986)
KATO III	human gastric cancer cell line	DMEM 10% FCS 1x Pen/Strep	ATCC
ST3051	human gastric cancer cell line	DMEM 10% FCS 1x Pen/Strep	(Vollmers et al., 1993)
NUGC-4	human gastric cancer cell line	DMEM 10% FCS 1x Pen/Strep	(Akiyama et al., 1988)
SNU1	human gastric cancer cell line	DMEM 10% FCS 1x Pen/Strep	ATCC
St2957	human gastric cancer cell line	DMEM 10% FCS 1x Pen/Strep	(Vollmers et al., 1993)

Tab. Cell lines used in described experiments in alphabetical order with information on tissue background and respective culture media composition.

4.1.6 Oligonucleotides

	human		**murine**	
Primer	**forward**	**reverse**	**foward**	**reverse**
A20	TCCTCAGGCTTT GTATTTGAGC	TCTCCCGTATCT TCACAGCTT	TGGTTCCAATTTT GCTCCTT	CGTTGATCAGAG TCGTG
CCL17 (TARC)	ACCGTTGGTGTT CACCGCCC	GGCCCTTTGTGC CCATGGCT	TACCATGAGGTC ACTTCAGATGC	GCACTCTCGGCC TACATTGG
CCL2	CTTCGGAGTTTG GGTTTGCTT	CATTGTGGCCAA GGAGATCTG	TTAAAAAACCTG GATCGGAACCAA	GCATTAGCTTCA GATTTACGGGT
CCL20 (LARC)	GCTACTCCACCT CTGCGGCG	CAGCTGCCGTGT GAAGCCCA	ACTGTTGCCTCT CGTACATACA	ACCCACAATAGC TCTGGAAGG
CXCL10 (IP-10)	TATTCTGCAAGC CAATTTTGTC	TCTTGATGGCCT TCGATTCTG	AAGTGCTGCCGT CATTTTCT	CCTATGGCCCTC ATTCTCAC
CXCL13 (BLC)	TGCCCCGTGGGA ATGGTTGTC	TCAGCTTGAGGG TCCACACACACA	ATATGTGTGAATC CTCGTGCCA	GGGAGTTGAAGA CAGACTTTTGC

GAPDH	CCTGGTCACCAG GGCTGC	CCGTTCTCAGCC TTGACGG	CCACCCCAGCAA GGAGAC	GAAATTGTGAGG GAGATGCT
KC			TGCACCCAAACC GAAGTCAT	TTGTCAGAAGCC AGCGTTCAC
IL-6	TCGAGCCCACCG GGAACGAA	GCAACTGGACC GAAGGCGCT		
LIGHT	CTGGCGTCTAGG AGAGATGG	CTGGGTTGACCT CGTGAGAC	TCCGCGTGCCTG GAAA	AAGCTCCGAAAT AGGACCTGG
LTa	CCACCCTACACC TCCTCCTT	AGTCTGGGCAG CTGAAGGT	TCCACTCCCTCA GAAGCACT	AGAGAAGCCATG TCGGAGAA
LTb	GAGGACTGGTAA CGGAGACG	GGGCTGAGATCT GTTTCTGG	TACACCAGATCC AGGGGTTC	ACTCATCCAAGC GCCTATGA
LTbR	GAGAACCAAGGT CTGGTGGA	GAGCAGAAGAA GGCCAGTG	GCCGAGGTCACA GATGAAAT	CAGGACACTGGT GAAGAGCA
TNFa	GGCGCTCCCCAA GAAGACAGG	CCAGGCACTCAC CTCTTCCCT	CGATGGGTTGTA CCTTGTC	CGGACTCCGCAA AGTCTAAG
TNFR1	CTGCCTCAGCTG CTCCAAA	CGGTCCACTGTG CAAGAAGAG		

Tab. Oligonucleotides used for quantitative PCR analysis in alphabetical order.

4.1.7 Kits

Kit	Company	Country
BCA protein Assay	Thermo Scientific	Ulm, Germany
BondMax	Leica Novocastra	Wetzlar, Germany
Immun-Star™WesternC™Chemiluminescent Kit	BioRad	München, Germany
PeqGold HP Kit	VWR International	Erlangen, Germany
QuantiTec Reverse Transcription Kit	Qiagen, Hilden, Germany	Hilden, Germany
RNAscope 2.0 brown FFPE Assay kit	Advanced cell diagnostic	Hayward CA, USA

Tab. Kits in alphabetical order

4.1.8 Mice

Female C57BL/6 mice of 7-9 weeks were obtained from Harlan Laboratories (Rossdorf, Germany). All animal studies were conducted in compliance with European guidelines for the care and use of laboratory animals (licenses 63/2008 and 24/2013t to Anne Müller) and were approved by the Zurich Cantonal Veterinary Office as well as the Institutional Animal Care and Use Committees (IACUC) of Technische Universität München, Regierung von Oberbayern.

4.1.9 Software

Software	Company
Adobe Photoshop CS5	Adobe
BD FACS Diva™	BD Biosciences
Digital Image Hub (DIH)	Leica Biosystems
DIH-TissueIA	Leica Biosystems
Endnote	Microsoft Corporation
Tree Star	FlowJo, LLC
GraphPad Prism	GraphPad Software Inc.
Image Lab™	BioRad
LabImage 1D	INTAS
Magellan™ - Data Analysis Software	Tecan
Microsoft Office	Microsoft Corporation
NanoDrop2000	Thermo Scientific
PrimerBlast	Open Source (NCBI)
Quantity One™	BioRad
SCN400 viewer	Leica Biosystems
SDS 2.4 ™	Applied Biosystems

Tab. Software applications used in alphabetical order.

4.1.10 Consumables

Product	Company	Country
384 well qPCR plates colourless, ABI type	Biozym Scientific GmbH	Oldendorf, Germany
96-,24-,12-,6-well plates polystyrene cell culture	Corning Life Sciences	Tewksbury, USA
Adhesive Microplate Seal, 135 x 80 mm, Sheets	Biozym Scientific GmbH	Oldendorf, Germany
Cassettes	Medite	Burgdorf, Germany
Cell-Star Serological Pipets 5mL, 10mL, 25mL	Greiner Bio-one	Frickenhausen, Germany
Conical Sterile Polypropylene Centrifuge Tubes 15mL; 50 mL	Thermo Scientific™ Nunc™	Ulm, Germany
Cryotubes 1.8mL	Thermo Scientific	Ulm, Germany
Disposal Bags, autoclavable	Roth	Karlsruhe, Germany
Drigalski spatula, polypropylene	A. Hartenstein	Marburg, Germany
GentleMACS M-tubes	Miltenyi	Bergisch-Gladbach , Germany
Kimtech Science Professional tissues	Kimberly Clarke	Reigate Surrey, UK
Nitrex Nextgen examination gloves	Rösner-Mautby Meditrade	Kiefersfelden, Germany

NuPAGE® Novex® Bis-Tris Protein Gels	Life Technologies (Invitrogen)	Frankfurt, Germany
Omnican single use fine dosage syringe	Braun-Melsungen	Melsungen, Germany
Omnifix syringes 5 mL /10 mL	Braun-Melsungen	Melsungen, Germany
PCR tube stripes and lid stripes	Biozym Scientific GmbH	Oldendorf, Germany
Pipette filter tips (10µl; 20µl; 200µl; 1000µl)	VWR International GmbH	Erlangen, Germany
Pipette tips (20-200µl; 1000µl)	VWR International GmbH	Erlangen, Germany
Preparation Sets (scissors, foreceps…)	Omnilab	München, Germany
Reaction tubes (0.5 mL; 1.5mL; 2mL)	Eppendorf	Hamburg, Germany
Rotilab Syringefilters 0.45 µm, 0.22µm	Roth	Karlsruhe, Germany
Superfrost Plus Microscope Slides	Thermo Scientific	Ulm, Germany
Surgical blades	Schreiber	Fridingen, Germany
Surgical disposable scalpels	Braun-Melsungen	Melsungen, Germany
Tissue culture flasks with filter lid (T15;T75)	Greiner Bio-one	Frickenhausen, Germany

Tab. Consumables listed in alphabetical order

4.1.11 Lab equipment

Device	Source	Country
-20°C fridge	Liebherr	Switzerland
4°C fridge	Bauknecht	Stuttgart, Germany
7900HT Fast Real Time PCR System	Applied Biosystems	Darmstadt, Germany
-80°C fridge Herafreeze	Heraeus	Hanau, Germany
AG Protect Cryostat	Leica Microsystems	Wetzlar, Germany
Automatic Ice Machine AF10	Scotsman	USA
Biofuge Fresco Microcentrifuge	Heraeus	Hanau, Germany
BondMaxII	Leica Microsystems	Wetzlar, Germany
Eppendorf Research 10µl; 20µl; 200µl; 1000µl	Eppendorf	Hamburg, Germany
gentleMACS^{TM} Dissociator	Miltenyi	Bergisch Gladbach, Germany
Hybaid Shake'n Stack Hybridization oven	Thermo Scientific	Ulm, Germany
HybEZ oven	Advanced cell diagnostic	Hayward CA, USA
Hyrax M25 Microtome	Zeiss	Jena, Germany
IKAMAG RCT magnetic stirrer	IKA Werke	Staufen, Germany
Megafuge 1.0	Heraeus	Hanau, Germany

Micro centrifuge MiniStar Silverline	VWR	Darmstadt, Germany
Microscope Axiovert 25	Zeiss	Jena, Germany
Microscope BX53	Olympus	Hamburg, Germany
Milli-Q® Advantage A10 Ultrapure Water Purification System	Merck Millipore	Darmstadt , Germany
Molecular Imager® ChemiDoc™ XRS	Bio Rad	München, Germany
Nanodrop 2000 Spectrophotometer	Peqlab	Erlangen, Germany
Novex® NuPAGE® SDS-PAGE Gel System	Life Technologies (Invitrogen)	Frankfurt, Germany
PipetBoy	Integra Bioscience	Zizers, Switzerland
Power 300 Power Supply	Peqlab	Erlangen, Germany
SCN400 slide scanncer	Leica Microsystems	Wetzlar, Germany
Subcell Agarose Electrophoresis System	Bio Rad	München, Germany
Tetra Blotting Module	Bio Rad	München, Germany
Thermomixer comfort	Eppendorf	Hamburg, Germany
Tissue drying oven TDO66	Medite	Burgdorf, Germany
TransBlot Turbo	Bio Rad	München, Germany
UNITWIST RT shaker	UniEquip	Planegg, Germany
Varioklav autoclave	HP-Medizintechnik	Oberschleißheim, Germany
Vortexer Reax 200	Heidolpf	Kelheim, Germany

Tab. Equipment listed in alphabetical order.

4.2 Methods

4.2.1 Cultivation of *H. pylori*

H. pylori wild type strain G27 is used as laboratory strain that expresses the Cag pathogenicity island and produces VacA. Frozen cryo aliquots were thawed at once in a 37°C water bath and centrifuged for 2 min, 1.000 x g to remove the glycerol-containing freezing medium. The pellet was resuspended in warm BHI-dent + 10% FCS, plated out on a WC-dent plate and cultivated for 2 - 3 days at 37°C under microaerophilic conditions (10% CO_2, 5% O_2, 85% N_2).

G27 isogenic mutant strains G27ΔCagA, G27ΔBabA, G27ΔVacA, G27ΔgGT, and G27ΔUreA/B were grown on WC-dent plates with 50µg/ml of kanamycin for selection under identical

conditions. *H. pylori* was transferred on a new plate for another day to increase its vitality. Bacteria were harvested, resuspended in warm BHI medium and directly used for experiments. The multiplicity of infection (MOI) was determined by measurement of the optical density (OD_{600}). For that reason dilutions of the main culture were incubated on WC-dent plates. Colonies were counted after 2 days and the amount of vital bacteria/ml was calculated. An amount of ~2x 10^8 *H. pylori* / ml were determined at OD_{600} = 1.0.

4.2.2 Freezing of *H. pylori*

For preparation of aliquots, bacteria were harvested from a WC-dent plate and resuspended in BHI-dent + 20% FCS + 20% glycerol. Aliquots were immediately frozen in liquid nitrogen and stored at -80°C until further use.

4.2.3 Cell line cultivation

Cells (see 2.1.5) were maintained at 37°C in a CO_2 atmosphere, *Mycoplasma* contamination was routinely tested. The respective media for each cell line is listed in table 2.1.5.

4.2.4 Cell culture experiments

Cultured cells were infected with either *H. pylori* strains, *H. pylori* mutant strains or clinical isolates for the indicated time points and multiplicities of infection (MOI). The human LTβR agonist monoclonal antibody BS-1 provided by Jeff Browning, (Biogen Idec, Cambridge, Massachusetts) was used as a positive control at a 0.5 µg/ml. LTβR was blocked by incubating cells with a LTβR-Ig fusion protein also provided by Jeff Browning, (Biogen Idec, Cambridge, Massachusetts) at the indicated concentrations.

Inhibition of the canonical NF-kB signaling pathway was performed by treating cells with an inhibitor of IkB Kinase-2 (IKK2), TPCA-1 (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide) (Sigma-Aldrich, Steinheim), in a concentration of 1μM or 5μM.

4.2.5 Western blot analysis

Cells were lysed using SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue). Equal amounts of lysate were loaded on 8 % SDS-PAGE gels and separated proteins were transferred to nitrocellulose membrane (Protran, Germany). After blocking, anti-p100/p52, p-p65 and p65 (Cell Signaling, USA) or anti-β-actin (Sigma-Aldrich, USA) antibodies were used according to manufacturer's protocol. Quantification of band density was performed using LabImage 1D software (INTAS).

4.2.6 Histology

4.2.6.1 Human gastric tissue samples

Diagnosis	*Case*	*H. pylori*	*Age*	*Gender*
Normal stomach	NS1	negative	17	M
Normal stomach	NS2	negative	81	F
Normal stomach	NS3	negative	46	M
Normal stomach	NS4	negative	58	M
Normal stomach	NS5	negative	40	F
Normal stomach	NS6	negative	71	F
Normal stomach	NS7	negative	30	F
Normal stomach	NS8	negative	29	F
Normal stomach	NS9	negative	63	M

Diagnosis	*Case*	*H. pylori*	*Age*	*Gender*
Severe gastritis	SG1	positive	69	F
Severe gastritis	SG2	positive	81	F
Severe gastritis	SG3	positive	81	M
Severe gastritis	SG4	positive	63	F
Severe gastritis	SG5	positive	60	F
Severe gastritis	SG6	positive	78	M
Severe gastritis	SG7	positive	39	M
Severe gastritis	SG8	positive	76	M
Severe gastritis	SG9	positive	53	F

Normal stomach	NS10	negative	45	F
Normal stomach	NS11	negative	21	F
Normal stomach	NS12	negative	56	M
Normal stomach	NS13	negative	50	M
Mild gastritis	MiG1	positive	37	M
Mild gastritis	MiG2	positive	65	F
Mild gastritis	MiG3	positive	42	F
Mild gastritis	MiG1	positive	43	M
Mild gastritis	MiG5	positive	71	F
Mild gastritis	MiG6	positive	52	M
Mild gastritis	MiG7	positive	50	M
Mild gastritis	MiG8	positive	60	M
Mild gastritis	MiG9	positive	51	F
Mild gastritis	MiG10	positive	42	M
Mild gastritis	MiG11	positive	59	M
Mild gastritis	MiG12	positive	49	M
Mild gastritis	MiG13	positive	85	M
Moderate gastritis	MoG1	positive	70	M
Moderate gastritis	MoG2	positive	40	F
Moderate gastritis	MoG3	positive	50	M
Moderate gastritis	MoG4	positive	41	F
Moderate gastritis	MoG5	positive	73	F
Moderate gastritis	MoG6	positive	72	F
Moderate gastritis	MoG7	positive	37	M
Moderate gastritis	MoG8	positive	64	F
Moderate gastritis	MoG9	positive	52	M

Severe gastritis	SG10	positive	38	M
Severe gastritis	SG11	positive	73	F
Severe gastritis	SG12	positive	78	M
Severe gastritis	SG13	positive	71	M
Intestinal-type early gastric adenocarcinoma	EGA1	positive	62	M
Intestinal-type early gastric adenocarcinoma	EGA2	positive	79	M
Intestinal-type early gastric adenocarcinoma	EGA3	positive	74	M
Diffuse-type early gastric adenocarcinoma	EGA4	Ex-*H. pylori*	57	F
Intestinal-type early gastric adenocarcinoma	EGA5	Ex-*H. pylori*	81	F
Diffuse-type early gastric adenocarcinoma	EGA6	Ex-*H. pylori*	79	M
Intestinal-type early gastric adenocarcinoma	EGA7	Ex-*H. pylori*	63	M
Diffuse-type early gastric	EGA8	positive	57	M

Moderate gastritis	MoG10	positive	51	M
Moderate gastritis	MoG11	positive	48	M
Moderate gastritis	MoG12	positive	71	F
Moderate gastritis	MoG13	positive	79	F
adenocarcinoma				
Diffuse-type early gastric adenocarcinoma	EGA9	positive	63	F
Intestinal-type early gastric adenocarcinoma	EGA10	Ex-*H. pylori*	76	F
Intestinal-type early gastric adenocarcinoma	EGA11	positive	74	F

Table 1: Clinicopathological characteristics of human tissue samples used for immunohistochemistry (IHC) analyses. Different stages of *H. pylori*-induced chronic gastritis are classified according to the updated Sydney system by the amount of inflammatory cells present in the analyzed tissue.

4.2.6.2 Dissection, tissue fixation and embedding

Euthanization of all experimental animals was performed by using CO_2 as determined by the German animal protection law. Prior to dissection, fur was moisturized with 80 % ethanol, following by opening of the abdomen via central cut. Organs were taken out dissected as needed and directly put into 4% PFA for paraffin embedding or deep frozen in TissueTek® cryosection medium via liquid nitrogen for cryo cutting. Samples for RNA and protein analyses were shock-frozen in liquid nitrogen and stored on -80°C until further use. Stomach tissue for paraffin blocks was fixated in stripes between 2 filter papers in a embedding cassette and fixated in 4% PFA for 5 days, followed by dewatering and paraffin embedding in a 90° angle to guarantee the intersection of the cut. Blocks were cut on a microtome into consecutive 2 µm ultrathin sections on Superfrost glass slides and stored at room temperature for further processing.

Tissue cuts were incubated at 37°C overnight to allow dehydration before staining.

4.2.6.3 Histochemistry

4.2.6.3.1 Hematoxylin-Eosin (HE) staining

Paraffin sections

Paraffin slides were baked for 15 minutes at 70°C. De-paraffinization of slides was performed by three times 5 minute-incubation in xylene solution and three times incubation for 1 minute in absolute ethanol, followed by a shortly washing in ddH_2O. Staining was proceeded by immersing slides for 5 minutes in Harris hematoxyline solution. Following three ddH_2O washing steps, slides were blued by dipping several times into ammoniac water (40 drops, 25% ammonium solution per 500 mL ddH_2O). Slides were washed with ddH_2O followed by a differentiation step with HCL-water (2,5mL 37%HCL solution per 500 ml ddH2O) via short dipping. Differentiation is stopped by putting slides under running tap water followed again by ammonia water-bluing, washed again two times with ddH_2O and one short 70% ethanol step. Eosin staining was performed for 2 minutes and slides were subsequently immersed in absolute ethanol for three times washing out eosin solution. Slides were incubated in clear xylene solution for three times 2 minutes, shortly dehydrated and were finally mounted in Eukitt mounting medium.

Cryosections

Sections were dried at room temperature for 1-2 h. Fixation of tissue was performed by 10 min immersing the slide in absolute ethanol. Afterwards slides were shortly washed twice in ddH_2O and immersed in freshly filtered Harris hematoxyline solution for 1 minute and 30 seconds, followed by three times washing with ddH2O and bluing under running tap water for 5 minutes. Afterwards, slides were again shortly washed in ddH_20 and immersed in eosin staining solution for 30 seconds. Dehydration was performed by three times immersing slides in 100 % ethanol and followed by three times dipping into fresh xylene solution. Slides were mounted in Eukitt mounting medium.

4.2.6.3.2 Immunohistochemistry (IHC)

Sections were incubated in Bond Primary antibody diluent (Leica) and staining was performed on a BOND-MAX immunohistochemistry robot (Leica Biosystems) using BOND polymer refine detection solution for DAB or AP. Depending on primary antibody, citrate (H1)- or EDTA (H2)-based antigen retrieval was performed upon staining. Antibodies, used concentrations and antigen retrieval conditions (ARC) can be found in sections 2.1.1.

4.2.6.3.3 Quantification of positive cells

Whole gastric tissue area sections (paraffin embedded) were quantified by using SlidePath TissueIA image analysis software (Leica).

The number of cells in stomach tissue positively stained for the different markers (CD3 for T cells or B220 for B cells) was determined by counting whole tissue by hand normalized per mm^2 area. Nuclear stain of RelB indicating activation of non-canonical NF-κB, was quantified by analyzing 5 independent desktop screen shots. Representative pictures were taken by scanning in whole tissue sections using a Leica SCN400 slide scanner.

4.2.7 *In situ* hybridization

RNA *in situ* hybridization was performed by using RNAscope 2.0 brown FFPE Assay from Advanced Cell Diagnostics according to protocol. Summarized, 2 µm paraffin-embedded tissue sections were baked in a dry oven for 1 h, 60°C, followed by de-paraffinization and blocking of endogenous peroxidases. Slides were cooked for 15 min in pre-treatment solution 2 and protease digestion (human gastric biopsy samples 30 min pretreatment solution 3). Tissues treated with a *in situ* probe specifically for human LTb receptor (Advanced Cell Diagnostic) were incubated for 2 hrs at 40°C in a hybridization oven, followed by signal amplification and detection according to manufactures protocol. Counterstaining was done following protocol using Gill'

Hematoxylin I (American Master Tech Scientific) and slides were finalized by using EcoMount mounting medium (Biocare). Pictures were taken with LEICA SCN400 Slides Scanner.

4.2.8 RNA Isolation and reverse transcription

4.2.8.1 RNA Isolation from tissue samples

Total RNA from human biopsy samples was extracted from formalin-fixed paraffin-embedded (FFPE) tissue sections, using the RNeasy FFPE kit (QIAGEN, Hilden, Germany) according to protocol “Total RNA from microdissected FFPE tissue sections” including an extended digestion step of 3h instead of 15 min at 56°C.

Total RNA from human gastric cancer cell lines and gastric mouse tissue was purified using the peqGOLD HP Total RNA kit from Peqlab, Erlangen, Germany. Quantity and quality of the RNA was determined spectroscopically by using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific).

4.2.8.2 Reverse Transcription

Generation of cDNA (1µg of total RNA (mouse tissue) or 400ng (biopsy tissue)) was performed using the Qiagen QuantiTec Reverse Transcription Kit© (QIAGEN, Hilden). In brief 1µg/400ng of RNA in a total volume of 12 µl (RNase free water) has been incubated at 42°C mixed with 2 µl of genomic DNA wipeout buffer. After 7 min of genomic DNA digestion, 6 µl of master mix – 4 µl reaction buffer, 1 µl primer mix and 1 µl reverse transcriptase - was added and incubated again for 20 minutes at 42°C. Reverse transcription was stopped by incubating samples for 3 minutes on 95°C. CDNA Samples were diluted in a 1:40 ratio with ddH_2O water and - until further use - frozen at -20°C.

4.2.8.3 Quantitative PCR analysis

Gene expression levels were quantified by qRT-PCR performed on a 7900 HT qRT-PCR system (Applied Biosystems, Life Technologies) using the ΔΔCT method and analyzed via the second derivative maximum method that includes both normalization to the reference gene and primer efficiency. In brief, per sample 7 µl of a master mix (6 µl of 2x FastStart Universal SybrGreen ROX Mix 2x from Roche, 0.06 µl of each forward and reverse primer (100 µM stock concentration) and 0.88 µl ddH_2O water) together with 5 µl of cDNA were placed in a 384-well plate following by centrifuging the plate 2 min at 1800 rpm. Standard cycling procedure contained a pre-heating step for 5 min on 95°C to activate hot start polymerase, 15 s double strand dissociation at 95°C, 15 s annealing and elongation at 60°C. In total, 40 cycles were run. After the last cycle a melting curve ramping from 65°C to 95°C was added to control for single specific amplification products. Data analysis was performed using the SD2.4 software. Relative mRNA levels of all target genes were normalized to average ΔCT GAPDH RNA levels.

4.2.9 Flow cytometry: human LTβR staining

1 million cells of gastric cancer cell lines MKN45, KATO III and ST3051, HepG2 as positive control and HEK293 cells as negative control for LTβ receptor expression were seeded. Cells were washed in FACS buffer (PBS 2% FCS), fixed and permeabilised for 20 min in the dark on ice using Cytofix/Cytoperm reagent kit (BD Pharmingen). Afterwards cells were washed three times with 1x Perm/Wash Buffer (R&D Systems) and incubated for 30 min in 2% goat serum diluted in 1x Perm/Wash Buffer. Three washing steps in 1x Perm/Wash buffer were followed by 45 min intracellularly staining on ice in rat anti-human LTβR (1 µg/mL, clone 6D66, Santa Cruz) diluted in 1x Perm/Wash Buffer. Primary antibody was washed off by three washing steps with 1x Perm/Wash Buffer buffer. Afterwards, cells have been incubated for another hour in anti-rat IgG Alexa 488 (BD Biosciences, 1 µg/ml) antibody solution diluted in 1x Perm/Wash Buffer for

signal detection. Final washing steps were performed using FACS buffer and cells were resuspended in 350 µl FACS buffer for analysis on a BD LSR II Flow Cytometer. FlowJo sofware TreeStar was used for acquisition and data analysis.

4.2.10 Animal experiments

Antibodies used for animal experiments include a systemic antagonist of the LTβR, mLTBR-mIgG1 (LTβR-Ig) and a control murine monoclonal IgG1 antibody, MOPC21 which were prepared at Biogen/Idec and stored at -80°C until use. 1 week prior to experimental start, mice were injected intraperitoneal with 100 µg/mouse of either antibody on day -7 and day -2. On day 0, 2 and 5, mice were infected orogastrically with 1 dose of approximately $2x10^7$ c.f.u. human *Helicobacter pylori* strain *PMSS-1* and further injected once per week with 100 µg/mouse of either LTβR-Ig or MOPC-21. *H. pylori* infection was confirmed by c.f.u. of stomach tissue and antibodies efficacy was confirmed by loss of splenic follicular dendritic cell networks, visualized by anti-FDC-M1, PNA and CD21/35 staining of LTβR-Ig-/MOPC-21-treated mice.

Agonization of the alternative NF-κB signaling pathway was performed by treating the mice twice/week with 50 µg/mouse ACH6 antibody in parallel to the above described *in vivo* experiments.

4.2.11 Statistics

Data were analyzed by using GraphPad Prism software version 5.0 (GraphPad Software). Statistics were performed by analysis of variance (ANOVA) with either the post hoc Bonferroni multiple comparison test (cell culture) or the One-way ANOVA Kruskal-Wallis with Dunn's multiple comparison test (biopsy samples, mouse tissue analyses). Analyses of two sample groups were performed with Student's t test, and statistics for scoring of inflammation have been

calculated using Lauren test. Statistical significance is indicated as follows: $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$; “n.s.” indicates not significant.

5 Results

5.1 *H. pylori*-induced gastritis and early gastric tumors display signs of alternative NF-κB signaling pathway activation

Various studies revealed an essential role of the alternative NF-κB signaling pathway in chronic inflammatory diseases e.g. induced by infectious agents (hepatitis B or hepatitis C virus) or as autoimmune diseases (rheumatoid arthritis) (Fava et al., 2003).

Activation of alternative NF-κB signaling requires at least one of five specific surface receptors (CD40, RANK, BAFFR, Fn14 or LTβR) which are known to mediate downstream p100 processing (Sun, 2011). To interrogate the role of alternative NF-κB signaling during chronic *H. pylori* infection, ligand expression was tested by real-time PCR in gastric tissue samples of patients suffering from *H. pylori*-induced chronic moderate gastritis and compared to uninfected normal control biopsies.

RANKL, which is mainly involved in bone resorption and formation, could not be detected in gastric tissue samples. As for the Fn14 ligand TWEAK, BAFFR ligand BAFF and the CD40L, expression levels were detectable but only slightly affected by the pathogen (Figure 1A). Notably, the ligand showing highest upregulation in response to chronic *H. pylori* infection compared to uninfected controls was LT - in particular LTβ – a ligand that is necessary to form LT heterodimers to activate LTβR. Further testing of gastric biopsy samples ranging from normal mucosa, *H. pylori*-induced mild, moderate and severe gastritis and early gastric tumor samples displayed LTβ-positive inflammatory cells (Figure 1B), corroborating studies in other inflamed tissues e.g. autoimmune pancreatitis (Seleznik et al., 2012) or chronic hepatitis (Haybaeck et al., 2009). Furthermore, in all stages of gastritis and early gastric tumors, epithelial cells expressed LTβ, thereby creating the prerequisites for interaction (either direct or indirect) between

pathogen and gastric epithelial cells in an inflammation-independent autocrine and

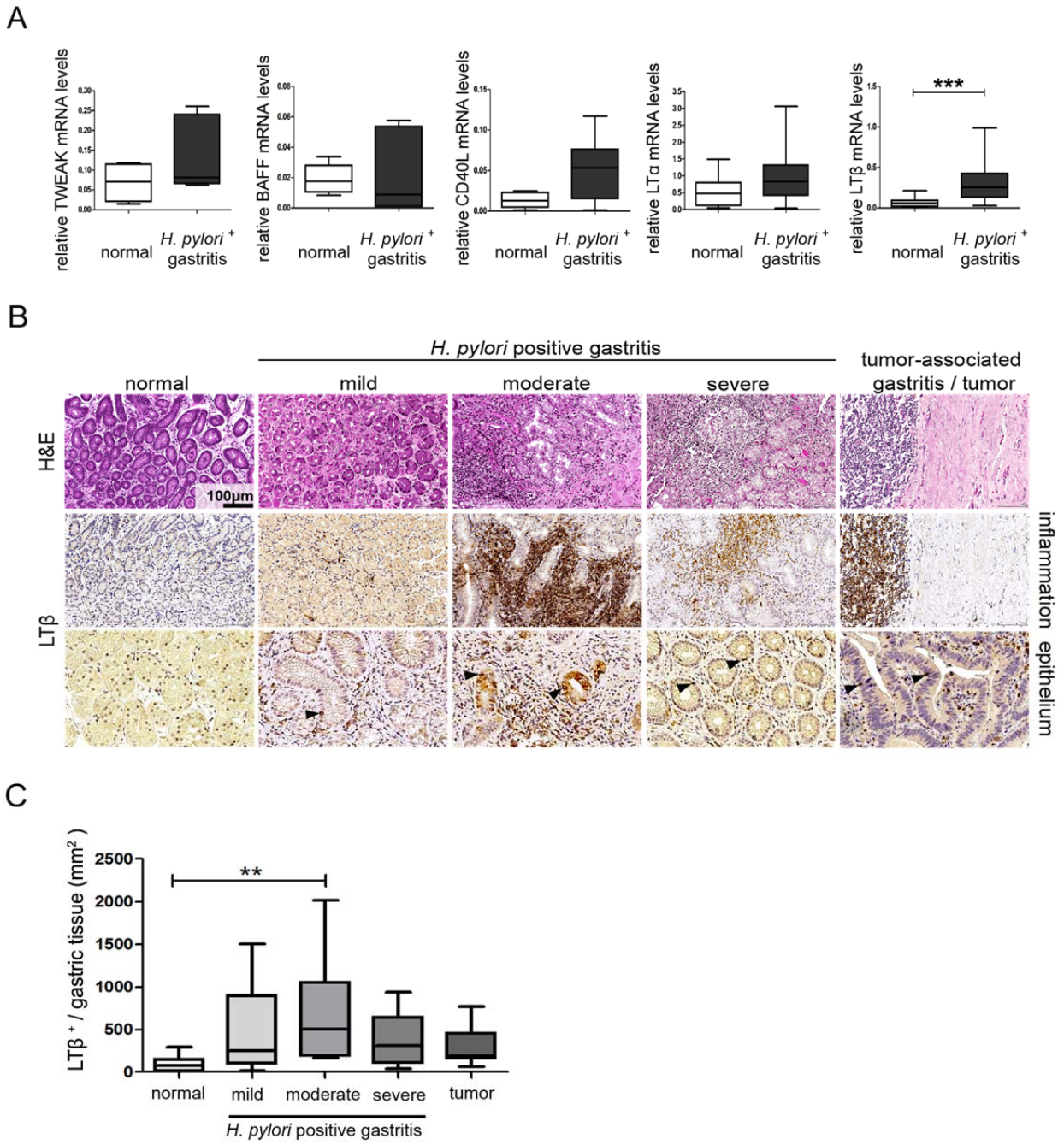

Figure 1: *H. pylori*-induced chronic gastritis displays high levels of the alternative NF-κB signaling LTβ.
(A) Whiskers (median and minimum to maximum) box plots show relative mRNA levels of alternative NF-κB ligands TWEAK, BAFF, CD40L, LTα and LTβ, detected in human normal gastric mucosa (n=11) and *H. pylori*-induced gastritis (n=6). ***p≤0.001. (Student's t test). (B) Representative images of LTβ expression in inflammatory and epithelial cells of human gastric tissue samples detected in human normal gastric mucosa *H. pylori*-induced mild, moderate and severe gastritis and early gastric adenocarcinomas by immunohistochemistry (IHC). (Scale bar: 100µm). (C) Quantification of LTβ^{+} cells (median and minimum to maximum values) in human normal gastric mucosa (n=13), mild (n=13), moderate (n=13) and severe gastritis (n=13), as well as in early gastric adenocarcinoma (n=11) tissue samples. **p≤0.01 (ANOVA Kruskal-Wallis, Dunn's multiple comparison test).

probably paracrine manner. When quantifying both inflammatory and epithelial LTβ positive cells in gastric mucosa and submucosa, LTβ expression was shown to be increased in all *H. pylori*-induced pathological stages compared to unaffected gastric tissue (Figure 1C). Furthermore, highly inflamed gastric tissue such as moderate gastritis samples showed highest levels of LTβ expression. Unalike to moderate gastritis, levels of LTβ positive cells again decreased in severe gastritis. These reduced levels are most likely related to altered morphology of the gastric tissue including more necrotic area. Similar to severe gastritis samples also early gastric tumor biopsies displayed decreased levels of LTβ protein when compared to other gastritis samples correlating with loss of tissue structure and increased cell death and also smaller total number of inflammatory infiltrates. Consecutive cut analyses of the inflammatory cells showed high levels of T and B cells as main LTβ producers. Furthermore, macrophages could be detected (Figure 2).

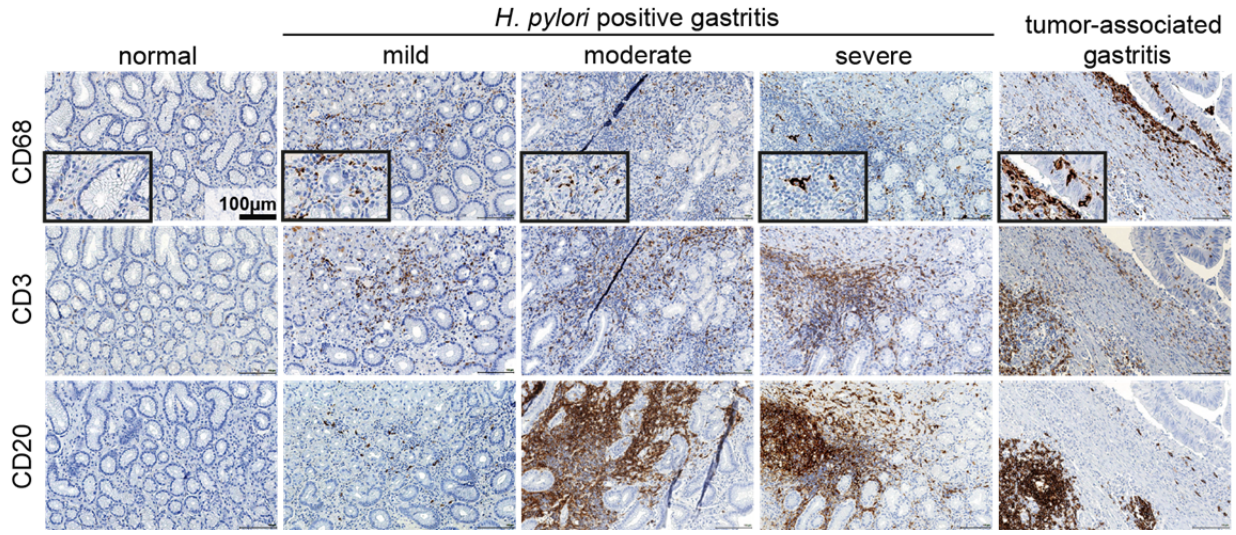

Figure 2: LTβ secreting inflammatory cells.
Macrophages (CD68) T cells (CD3) and B cells (CD20) detected by IHC in normal gastric mucosa and progressing stages of gastritis as well as in tumor-associated gastritis. Insets in the pictures of macrophage staining show a higher magnification. Representative images are shown. (Scale bar: 100µm).

LTβR signaling has already been stated to be a crucial pathway in inflammatory or autoimmune disease disorders (Browning, 2008; Drayton et al., 2006; Gommerman et al., 2003; Ware, 2005). Chronic infection with *H. pylori* induces pathology that progresses through several steps to

gastric cancer (Correa, 1992). To assess whether receptor expression is affected by *H. pylori* and/or severity of pathology, LTβR mRNA levels were analyzed via *in situ* hybridization in gastric tissue samples of healthy donors and patient samples with pathological progressing forms of *H. pylori*-induced mild, moderate and severe gastritis and early gastric tumors (Figure 3).

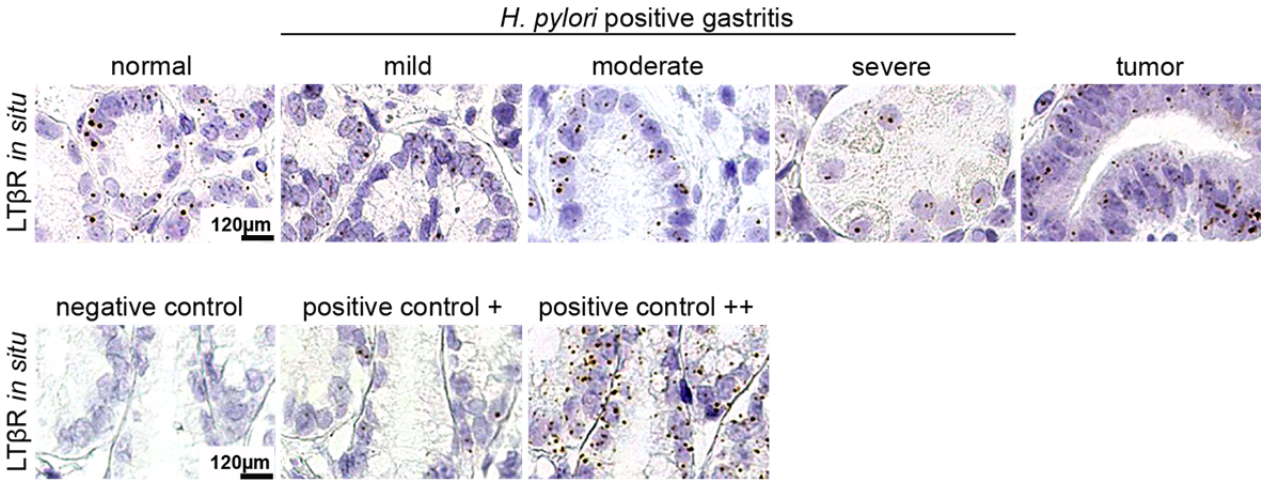

Figure 3: LTβR expression is not influenced by H. pylori infection in gastric pathology.
LTβR mRNA expression detected in human normal gastric mucosa *H. pylori*-induced mild, moderate and severe gastritis and early gastric adenocarcinomas detected by *in situ* hybridization. Negative (DapB) and positive controls (Pol2A + and UBC++) used for LTβR *in situ* hybridization. Scale bar represents 120µm.

Notably, mRNA expression of LTβR was not influenced by the severity of pathology, thereby giving the possibility to activate alternative NF-κB signaling. LT-mediated activation of canonical or alternative NF-κB signaling results in nuclear translocation of RelA and RelB, respectively. Whereas the induction of the canonical pathway is triggered by LTα, LTβR signaling is mediated by both LTα and LTβ ligands which predominantly form the $LT\alpha_1\beta_2$ heterodimers (Ware et al., 1995).

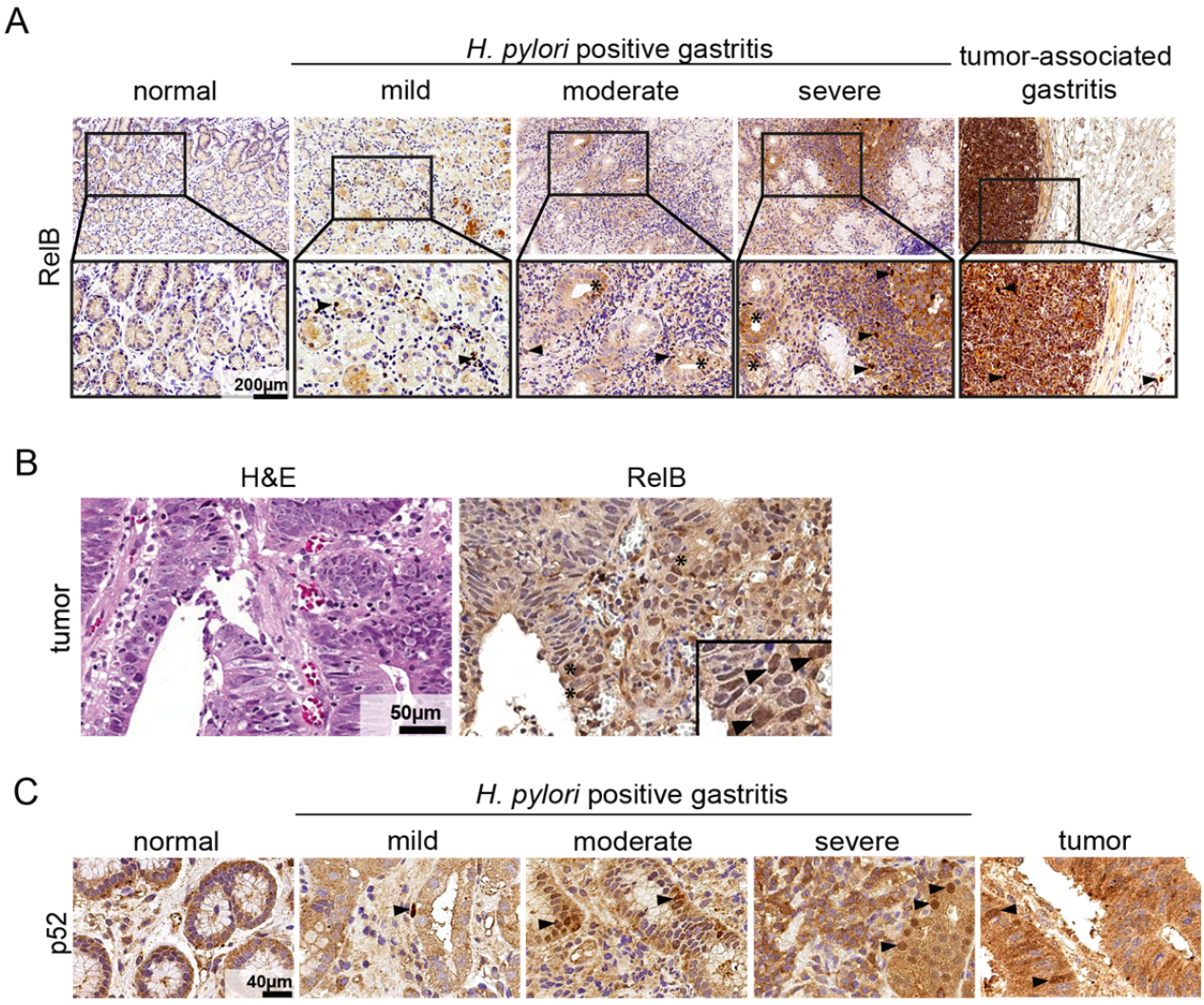

Figure 4: Inflammatory cells and epithelial cells show active alternative NF-κB signaling in gastric pathological tissues
(A) Representative images of RelB expression by inflammatory cells in chronic forms of human gastritis samples detected by immunohistochemistry (IHC). Arrows (inflammatory cells) and asterisks (epithelial cells) indicate $RelB^+$ nuclei. (B) Representative image of RelB expression in gastric tumors detected by IHC. Arrows indicate $RelB^+$ positive nuclei. (Scale bar: 50µm). (C) Representative images of active NF-κB signaling activation shown by p100 processed p52 in different stages of chronic gastritis and early tumors. Arrows indicate $p52^+$ nuclei. (Scale bar: 40µm).

In addition to ligand and receptor expression, activation of the alternative NF-κB signaling pathway in response to *H. pylori* could be demonstrated through nuclear translocation of RelB. Different stages of chronic gastritis displayed RelB translocation in both inflammatory and epithelial cells (Figure 4A) as well as in early gastric tumor cells (Figure 4B). Active alternative NF-κB signaling in different stages of chronic gastritis was further confirmed by analysis of the p100 processed subunit p52 in the nuclei (Figure 4C). Interestingly, p52 positive nuclei were

earliest detected in epithelial cells of moderate gastritis although the signaling was already active in mild gastritis, as demonstrated by p52 positive inflammatory cells.

Processing of p100 to p52 followed by nuclear translocation of RelB results in the expression of a specific alternative NF-κB gene profile. This includes the homeostatic (constitutively expressed in non-inflamed tissue) chemokine CXCL13, a B lymphocyte chemoattractant (BLC), whose main producer are FDCs (Cyster, 2010).

To confirm *H. pylori*-induced activation of the alternative NF-κB, *H. pylori*-positive moderate gastritis samples were analyzed for NF-κB target gene expression. Indeed, *H. pylori* positive patient samples displayed significantly increased expression levels of

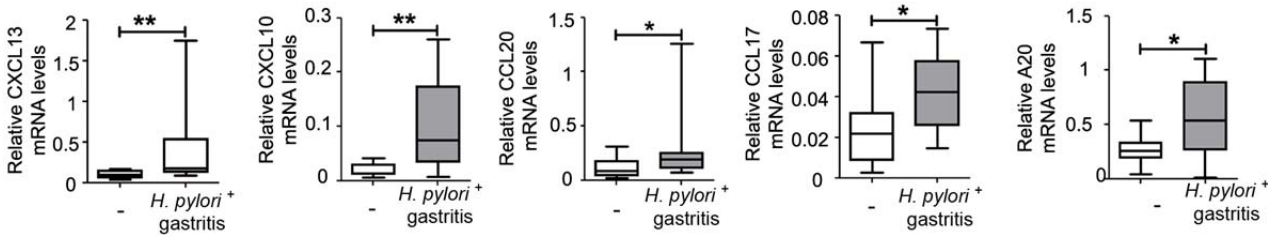

Figure 5: LTβR signaling downstream target genes are increased in *H. pylori* colonized gastritis patient samples
Whiskers (median and minimum to maximum) box plots show relative mRNA levels of CXCL13, CCL17, CXCL10, CCL20 and A20 detected in human normal gastric mucosa (n=11) and *H. pylori*-induced gastritis (n=11). *p≤0.05, **p≤0.01, ***p≤0.001. (ANOVA Kruskal-Wallis, Dunn's multiple comparison test).

CXCL13, confirming studies highlighting the importance of CXCL13 during a chronic *H. pylori* infection (Figure 5) (Galamb et al., 2008; Nakashima et al., 2011). Further NF-κB-regulated cytokines and chemokines that were increased in *H. pylori*-induced chronic gastritis when compared to healthy donors include CXCL10, CCL17 and CCL20 which could be shown to play a role in both canonical and alternative NF-κB signaling in various forms of pathological diseases (Haybaeck et al., 2009; Sirard et al., 2009). Notably some of these genes have already been mentioned in the context of an *H. pylori* infection, e.g. CCL20 or CXCL10. However, CCL20 was described in the literature to be induced by canonical NF-κB signaling (Allison et al., 2013) and

CXCL10 was mentioned specifically in the context of *H. pylori* induced inflammation after antibiotic treatment without going into detail about the signaling pathway (Zhang et al., 2014). Active classical NF-κB signaling in the presence of *H. pylori* is also supported by enhanced expression of an endogenous inhibitor of canonical NF-κB, known as A20, in *H. pylori*-associated gastritis compared to healthy controls (Figure 5). The second ligand required for heterodimer formation, LTα, as well as the CCL2 chemokine which recruits monocytes and granulocytes showed slightly higher expression levels.

Together, these results obtained from *H. pylori*-infected patients indicate a parallel activation of NF-κB signaling via both the classical and alternative pathway. LTβR-triggered activation of alternative NF-κB signaling could be observed both in inflammatory and epithelial cells in several progressive forms of chronic gastritis and early gastric tumors.

5.2 *H. pylori* activates alternative NF-κB in human gastric cancer cell lines

Data obtained from human biopsy samples supported the hypothesis of active alternative NF-κB signaling as response to persistent colonization of *H. pylori* in the stomach. To explore the mechanism by which *H. pylori* activates the alternative NF-κB signaling pathway during *H. pylori*-induced chronic inflammation studies on human gastric cancer (GC) cell lines were performed.

As a first step, a set of different gastric cancer cell lines was characterized for their basal expression levels of both ligands and receptors of the classical (TNFα/TNFR1) and alternative (LTα, LTβ, LIGHT / LTβR) NF-κB signaling pathways. The HepG2 hepatic cell line described to express both TNFR1 (Zhu et al., 1998) and LTβR (Wu et al., 1997) was therefore used as positive control to compare GC cell lines for their receptor expression. As a positive control for stimulated LT signaling a GC cell line treated with the humanized tetravalent LTβR agonistic antibody BS-1 (Bista et al., 2010) was used. Eight GC cell lines displayed different mRNA levels

of the LTβR, ranging - compared to the positive control cell line HepG2 - from high expression (cell lines St3051, KATO III, AGS or St2957) to similar (MKN45 or NUGC-4) or low expression levels (AZ521), whereas the ligands LTα, LTβ and LIGHT were hardly detectable (Figure 6A). Furthermore, three of these GC cell lines were tested in addition via FACS analysis for LTβR surface expression compared to both HepG2 cells and a negative control (HEK 293 cells), which does not express the receptor. In all three cell lines (MKN45, KATOII and St3051) LTβR surface expression was observed to be higher expressed than in HepG2 cells (Figure 6B). Notably, all cell lines also expressed TNFR1 with expression levels comparable or increased to HepG2 cells, yet under physiological conditions also very low basal levels of TNFα (Figure 6C).

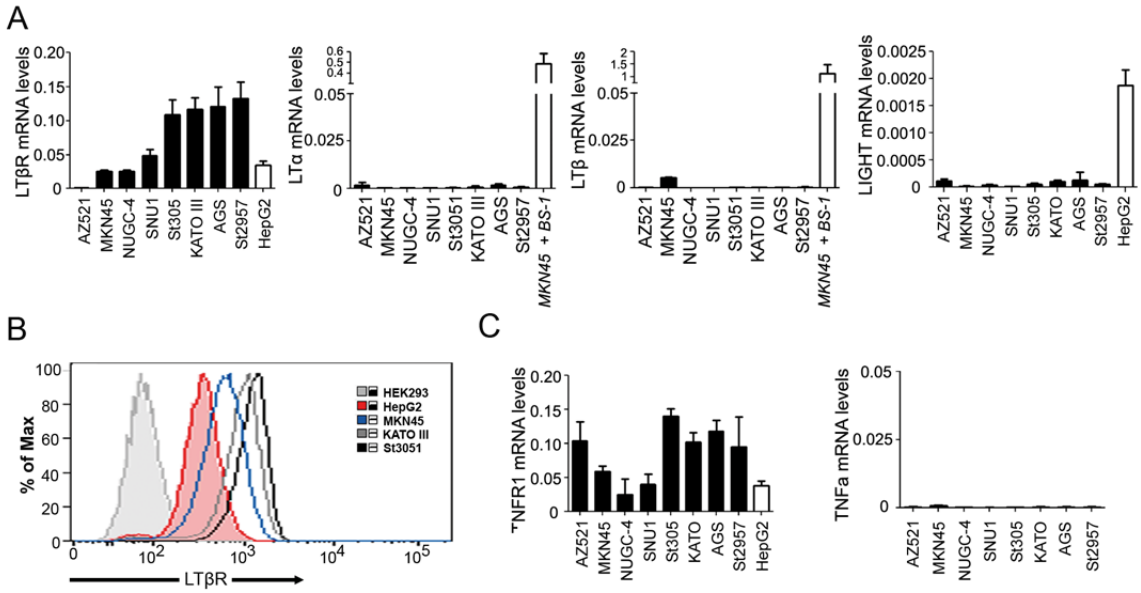

Figure 6: Gastric cancer cell lines express NF-κB receptors but lack ligand expression without further stimulation
(A) LT system component mRNA basal expression levels in gastric cancer cell lines. HepG2 cells or MKN45 cells treated with LTβR agonist BS-1 (0.5 µg/ml) were used as positive controls. Results (mean ± S.D.) of two independent experiments performed in duplicates are shown. (B) LTβR expression on the surface of MKN45, KATOIII and St3051 GC cells detected by flow cytometry. HepG2 and HEK293 cells were used as positive and negative control, respectively. One representative experiment is shown. (C) TNFR1/TNFα mRNA basal expression levels in gastric cancer cell lines. HepG2 cells were used as positive control. Results (mean ± S.D.) of two independent experiments performed in duplicates are shown.

NF-κB is a major regulator of innate immunity and inflammation and is therefore frequently observed to be aberrantly regulated in many cancers, which can result in cell-autonomous

genetic alterations including constitutive activation, amplification, mutations or deletions in cancer cells (Karin, 2006). To further assess whether the GC cell lines show alternative NF-κB pathway signaling activation under physiological conditions, basal activation of alternative NF-κB signaling was analyzed by p100/p52 processing status. Via Western Blot analysis processing of p100 to p52 could be detected in all cell lines (Figure 7A). Notably, several of the cell lines displayed constitutive p100 processing such as AZ521 and NUGC-4. Constitutive activation of LTβR signaling due to production of high amounts of the specific ligands LTα, LTβ or LIGHT by the respective cell lines without additional triggering could already be excluded (Figure 6A) which leaves mutations in the NF-κB promoter region as a second possibility for constitutive p100 processing. In addition, canonical NF-κB signaling was analyzed showing varying levels of - in parallel ongoing - TNFR1 stimulation in all tested gastric cell lines (Figure 7B).

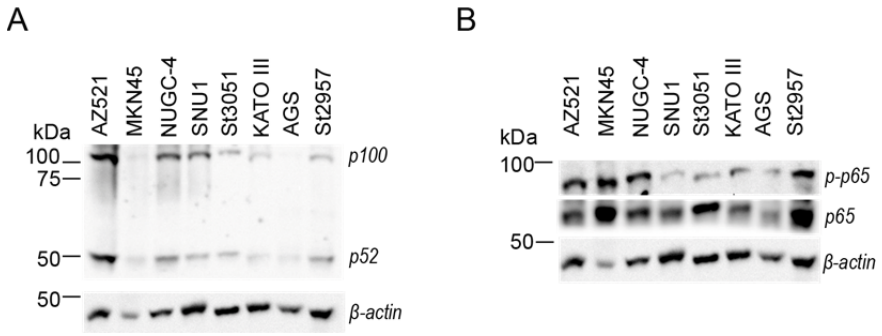

Figure 7: Gastric cancer cell lines display active alternative NF-κB signaling
(A) p100/p52 basal protein expression levels in different GC cell lines analyzed by western blot. β-actin served as loading control. (B) p-p65 and p65 basal protein expression levels in different gastric cancer cell lines analyzed by western blot. β-actin served as loading control.

Activation of canonical NF-κB shown by phosphorylation of p65 could be detected in most cell lines independently of the respective basal TNFα expression (Figure 6C). The obtained results qualified MKN45 and St3051 as suitable cell lines for further investigation of *H. pylori*-induced alternative NF-κB signaling due to 1.) different LTβR expression levels and 2.) lack of constitutive activation of alternative NF-κB signaling, which can be shown by a lack of p100 processing.

To test the hypothesis whether *H. pylori* is capable of inducing LTβR signaling the two GC cell lines were infected with *H. pylori* strain G27 (Xiang et al., 1995). To see, whether activation of alternative NF-κB by *H. pylori* is dose-dependent, MKN45 and St3051 cells were infected with *H. pylori* strain G27 at varying multiplicities of infection (MOI) (MOI 2 to MOI 50) for 12h.

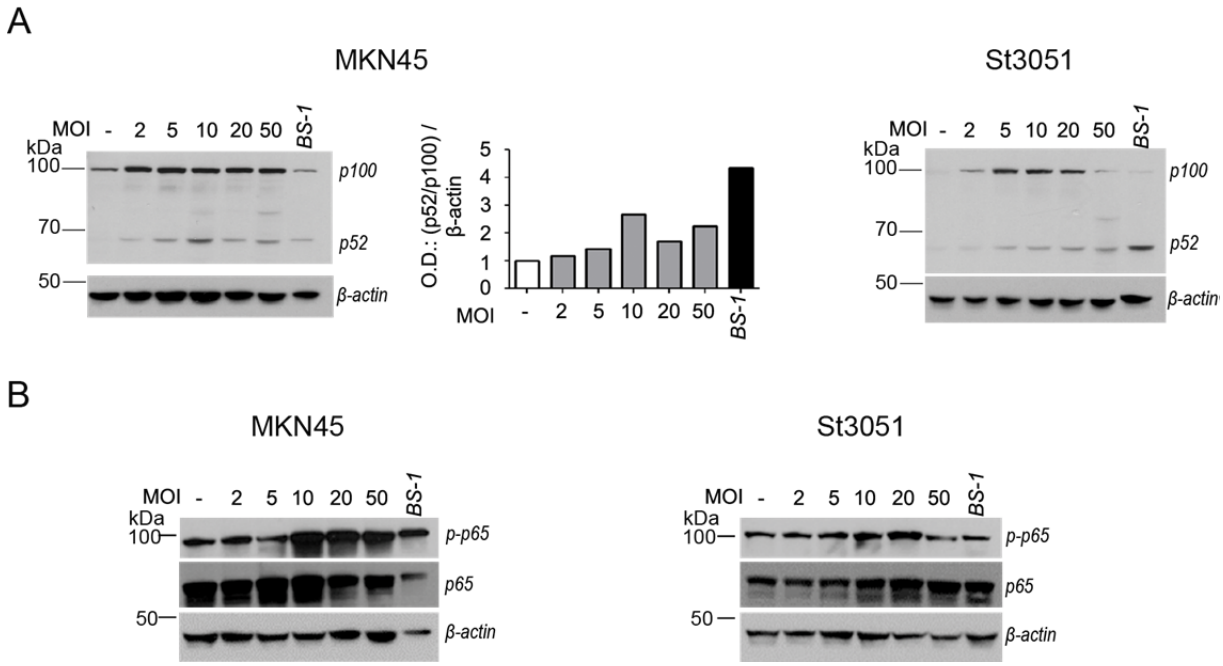

Figure 8: *H. pylori* infection with a MOI of 10 displays stable activation of alternative NF-κB
(A) MKN45 and St3051 cells were infected with *H. pylori* G27 strain at the indicated MOI and p100/p52 processing was analyzed by western blot. The agonist of LTβR signaling BS-1 (0.5µg/ml) was used as positive and β-actin as loading control. One representative blot and band quantification of MKN45 cells showing the ratio between p100 and p52 levels from 3 independent experiments is shown. O.D. optical density (arbitrary units). (B) p-p65 and p65 basal protein expression levels in gastric cancer cell lines analyzed by western blot. β-actin served as loading control.

H. pylori MOI dependency on p100/p52 processing was analyzed by Western Blot analyses of MKN45 cells. A low MOI of 2 or 5, an average MOI of 10 and higher MOI of 20 and 50 confirmed a dose-dependent induction of p100 expression and cleavage to p52 in both cell lines, showing highest processing with an MOI of 10 in MKN45 cells (Figure 8A). Results were confirmed by treating cells in addition with BS-1 as positive control for p100/p52 processing. Also at higher infectious doses of MOI 20 to 50, p100/p52 processing was observed to be stable but p100 expression decreased again exclusively in St3051 cells. Overall, p100/p52 processing was *H.*

pylori dose dependent and signal intensity quantification (O.D.:p52/p100) showed highest processing at an MOI10, with levels comparable to the BS-1 control. Results of the second cell line St3051 were similar, although p100/p52 processing was best for an MOI between 5 and 20 (Figure 8A). As expected, infection with the G27 *H. pylori* strain also resulted in a dose-dependent activation of canonical NF-κB, shown by p65 phosphorylation, which was also most effective with a MOI of 10 or 20 for both cell lines (Figure 8B).

Since gastric cell lines infected with *H. pylori* undergo a morphological change formerly described as hummingbird phenotype with elongated cell protrusions (Segal et al., 1999). Therefore, MOIs that showed stable p100/p52 processing were tested for morphological appearance (Figure 9). MKN45 cells showed obvious signs of cell death with MOI of 50 12h post infection. Thus, all further cell culture experiments were performed with an MOI of 10.

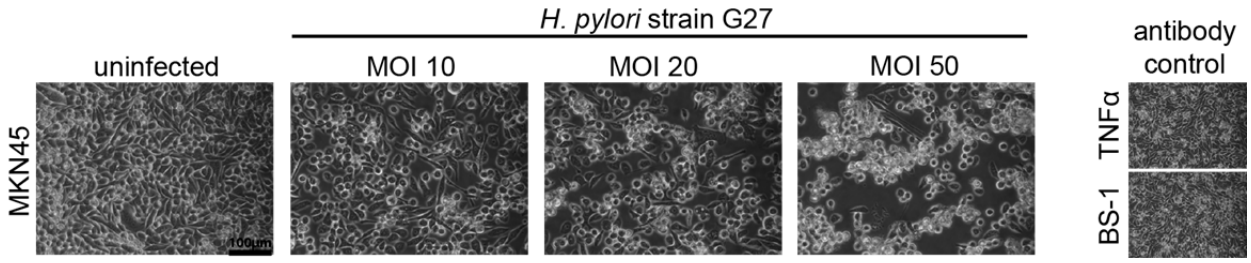

Figure 9: Gastric cancer cell line MKN45 shows increase cell death with an MOI higher than 10
Gastric cancer cell line MKN45 was infected with different multiplicity of infection (MOI). TNFα treatment served as control of morphology, BS-1 administration showed no effect on MKN45 cells.

Furthermore, the effect of the LTβR-agonist BS-1 treatment (which will be used in further experiments as positive control for active p100/p52 processing) was tested and compared to TNFα administration, to exclude effects on the cell survival due to the antibody. In addition, administration of BS-1 was tested since antibody administration stimulates LTβR signaling. However, administration of 0.5ng/µl BS-1 did not cause any abnormalities e.g. extensive cell death in neither MKN45 nor St3051 GC cells and will be therefore used in further experiments as positive control for cells with active LTβR signaling.

Canonical NF-κB was described to be a rapid signaling pathway. Among others, Keates and colleagues could demonstrate by using the gastric cancer cell lines AGS and KATO III, detection of nuclear translocation of p50/p65 heterodimers and p50 homodimers 30 min after exposure to IL-1β (25ng/ml) (Keates et al., 1997). Furthermore, they observed increased amounts of IL-8 mRNA after 1 hour and protein levels 4 hours post treatment, initiating the pro-inflammatory cascade in response to the invading bacterium. To assess the question, whether *H. pylori*-induced kinetics of LTβR signaling follows the same timeframe as canonical NF-κB, a time course study was performed starting from 15 min on with the endpoint of 24h. Notably, p100 expression increased earliest 6h post-infection obtaining a plateau at 8h, which was steady even 24 hours post-*H. pylori* infection (Figure 10). Processing of p100 to p52 could be detected earliest 2 hours after p100 upregulation at 8 hours post-infection with stable processing to the endpoint of the experiment after 24 hours. Notably, p100 expression remained at high levels in *H. pylori*-infected GC cell lines, whereas p100 appeared to be almost completely processed to p52 in BS1-treated, *H. pylori*-negative cells 12h post treatment.

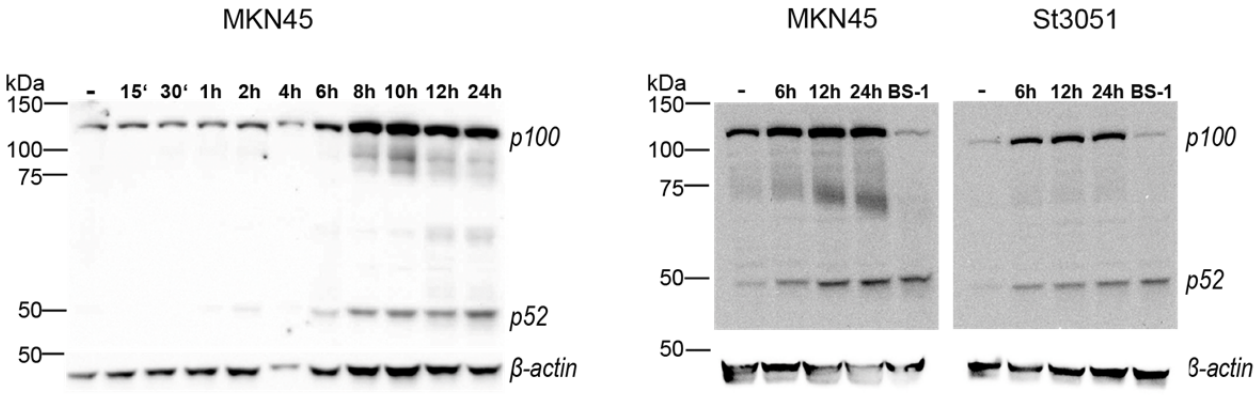

Figure 10: *H. pylori* induced 100/p52 processing is initiated 6h post infection
MKN45 and St3051 GC cells were infected with *H. pylori* G27 strain at MOI 10 for different time points and processing of p100 to p52 was analyzed by western blot. The agonist BS-1 (0.5µg/ml for 12h) was used as positive control. β-actin was used as loading control. One representative blot is shown.

Note that all cell culture experiments described above were performed using the *H. pylori* G27 strain. In order to exclude that the observed effect on p100 processing was not a specific feature

of the infection with this particular laboratory strain of *H. pylori* and to model the human situation more closely, additional ex vivo experiments were performed using seven different clinical H. pylori isolates (kindly provided by the *Städtisches Krankenhaus München*). These isolates were tested for their capacity to induce p100 expression and its subsequent processing to p52 in MKN45 cells. P100 expression was highly increased in cells infected with clinical isolates and was similar when compared to cells infected with the common experimental *H. pylori* G27 strain (Figure 11A). Enhanced processing of p100 to p52 on the other hand could be observed in 3 (II, V and VII) out of 7 clinical isolates which is shown by quantification of one representative western blot (Figure 11B). These data demonstrate that distinct *H. pylori* isolates are capable of inducing alternative NF-κB signaling in gastric cancer cells.

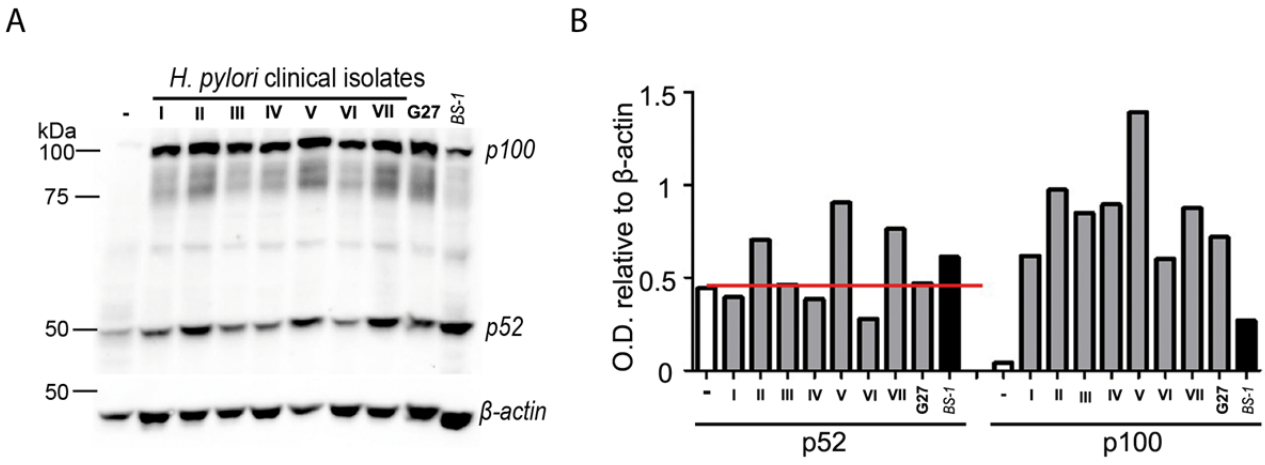

Figure 11: Active alternative NF-κB signaling can be shown with different *H. pylori* strains
(A) p100 processing to p52 detected by Western Blot in MKN45 cells infected with different clinical isolates (I-VII) at MOI 10 for 12 hours. One representative blot of 3 independent infections is shown. (B) Signal intensity is quantified for p52 and p100 relative to loading control. O.D. optical density (arbitrary units).

Activation of alternative NF-κB signaling was verified in MKN45 and St3051 cells. To link *H. pylori* infection to active LTβR downstream signaling, correlation between expression of LTβR ligands and *H. pylori* infection was analyzed. Remarkably, an upregulation of both LTα and LTβ mRNA was already detectable at 3 hours post infection in both cell lines (Figure 12A). In fact, increased LTα and LTβ mRNA levels were already evident 4-5 hours prior to first signs of p100/p52processing (Figure 11), suggesting that another signaling pathway may participate in

the induction of these ligands. Notably, both cell lines, MKN45 and St3051 displayed significantly elevated levels of LTβ in response to the *H. pylori* infection, whereas expression levels of LTα appeared less affected. Also, LTβR expression remained completely unaltered in response to infection (Figure 12B).

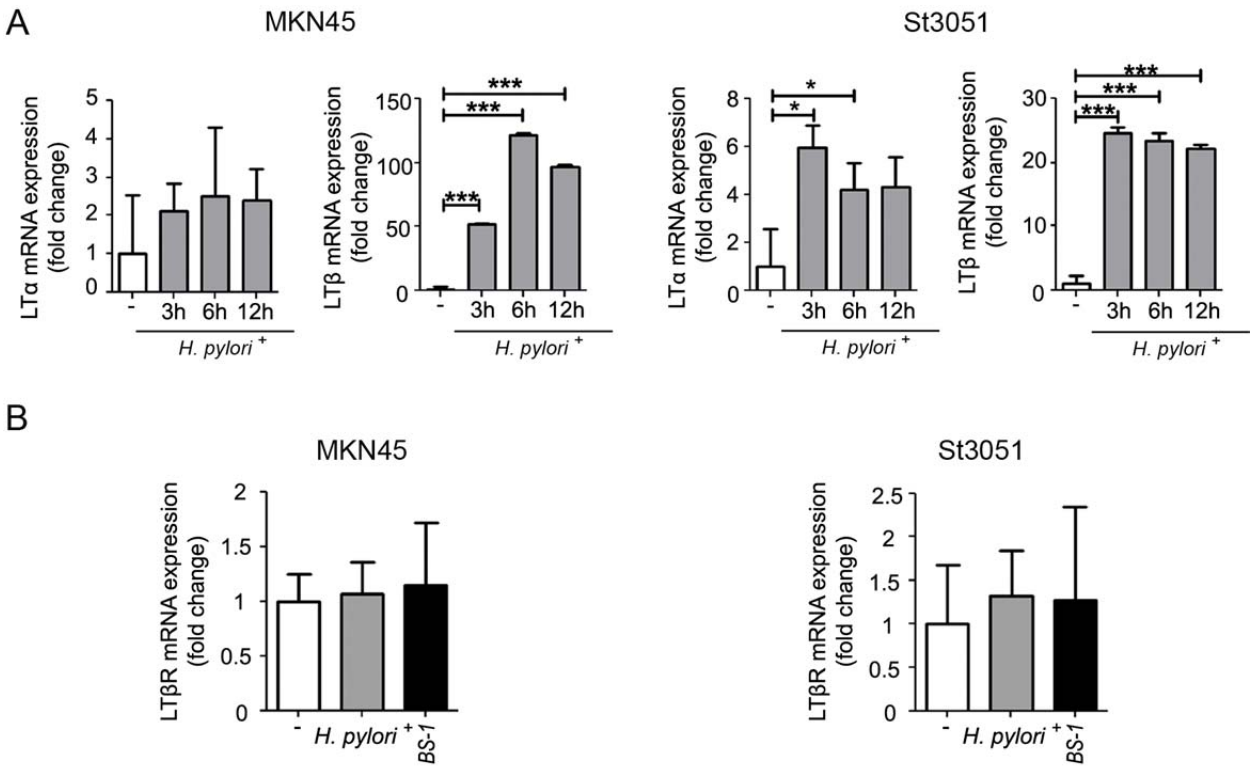

Figure 12: *H. pylori*-induced upregulation of LT expression can be observed 3 h post infection
(A) mRNA expression levels (mean ± S.D.) of LTα and LTβ upon *H. pylori* G27 strain infection at MOI 10 for 3 (n=3), 6 (n=4) and 12 hours (n=4) in MKN45 and St3051 cells. (ANOVA, Dunnett's multiple comparison test). (B) Gastric cancer cells MKN45 and St3051 were infected with *H. pylori* G27 at MOI 10 for 12 hours. The mRNA expression levels of LTβR were analyzed by real time PCR. Ct values were normalized to GADPH. Results (mean ± S.D.) of three independent experiments are presented as fold induction. Significances were calculated using One-way ANOVA with Bonferroni's multiple comparison test.

To further confirm activation of LTβR signaling in the human GC cell lines MKN45 and St3051, expression of the identical panel of NF-κB target genes already used for analyses in human biopsies and murine *in vivo* experiments was analyzed after 12 hours post *H. pylori* infection. LTβR-agonist BS-1 treatment was again used as positive control for target gene expression after specific alternative NF-κB signaling activation. Indeed, both gastric cancer cell lines tested

showed upregulation of the cytokine A20 and chemokines CCL20, CXCL10 and CXCL13 in response to *H. pylori* infection. CXCL13, the specific target gene of the LTβR signaling pathway showed a two-fold (yet not significant) (MKN45) (Figure 13A) or significant 3.5-fold (St3051) (Figure 13B) up-regulation compared to the respective non-treated uninfected cells. A similar upregulation of CXCL13 was also noted in uninfected cells, receiving the LTβR-agonist BS-1. CXCL10 displayed a significant five-fold increase in expression level after *H. pylori* challenge or BS-1 treatment in the MKN45 cell line. CXCL10 displayed a significant five-fold increase of expression level after *H. pylori* challenge or BS-1 treatment in the MKN45 cell line. The increase of CXCL10 detected in *H. pylori* infected St3051 cells exceeded the already significantly up-regulated expression levels of CXCL10 after BS-1 treatment compared to uninfected cells, corroborating activation of alternative NF-κB signaling by *H. pylori* colonization in the human stomach. On the other hand, A20 and CCL20 levels were also significantly increased after infection with *H. pylori.* Notably, BS-1 treated cells showed lower levels in comparison, suggesting that another signaling pathway, most likely canonical NF-κB, drives A20 and CCL20 upregulation upon infection with *H. pylori*.

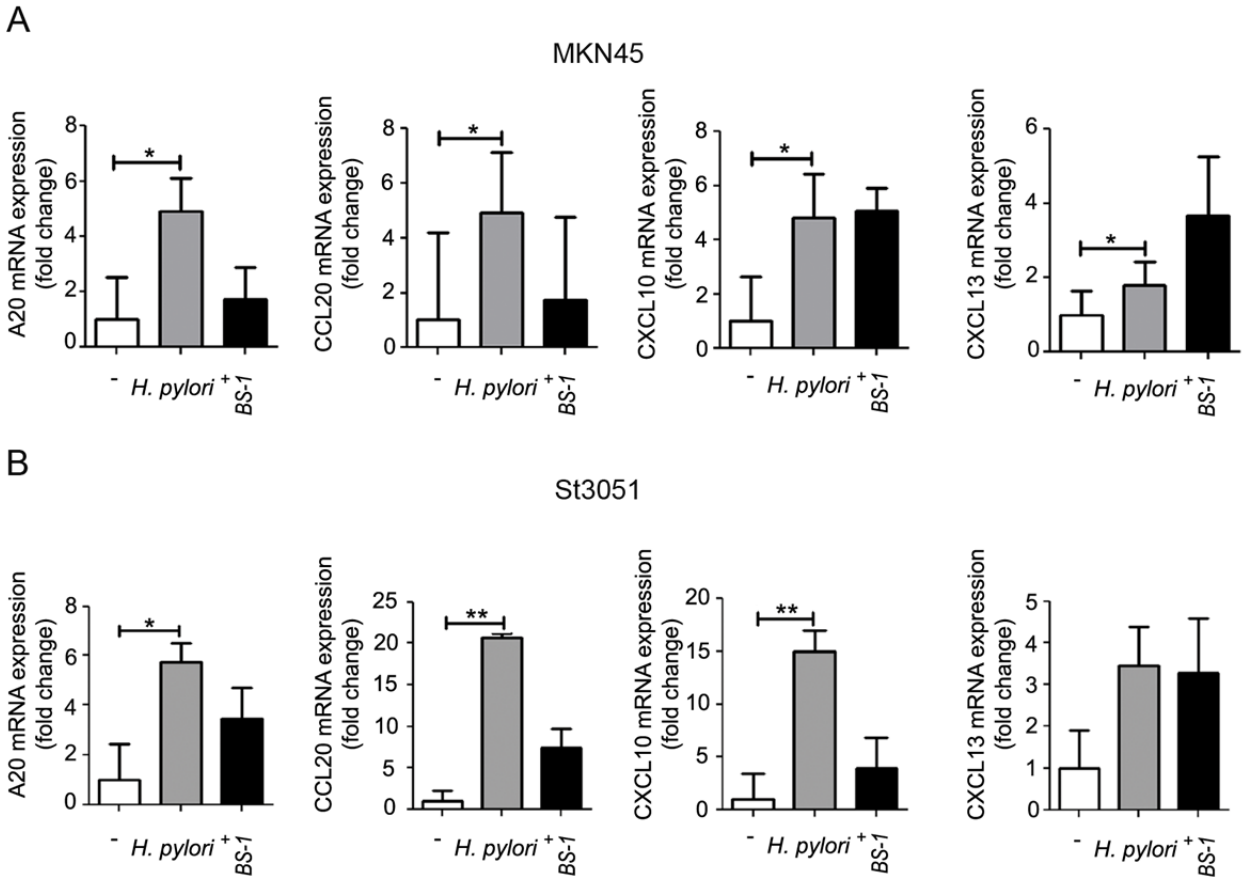

Figure 13: *H. pylori* infection results in upregulation of NF-κB target genes.
(A) A20, CCL20, CXCL10 and CXCL13 upon *H. pylori* G27 strain infection at MOI 10 for 12 hours in MKN45 cells. The agonist BS-1 (0.5µg/ml) was used as positive control. Ct values were normalized to GADPH. Results (mean ± S.D.) from 3 independent experiments are presented as fold induction. (Control and *H. pylori*-infected cells were compared using Student's t-test). *ü≤0.05. (B) Analysis of mRNA expression of target genes with MOI 10 after 12 hours in ST3051 cells. The agonist BS-1 (0.5µg/ml) was used as positive control. Ct values were normalized to GADPH. Results (mean ± S.D.) from 3 independent experiments are presented as fold induction. (Control and *H. pylori*-infected cells were compared using Student's t-test). *p≤0.05, **p≤0.01.

Together, these data indicate a very rapid *H. pylori*-induced increase in LTα and LTβ expression, which results in p100 processing several hours later, thereby promoting the expression of downstream chemokines in gastric epithelial cells.

5.3 LT secreted in response to *H. pylori* induces alternative NF-κB signaling

Several studies have provided proof for a direct interaction of *H. pylori* with host receptors such as MUC5AC (Van de Bovenkamp et al., 2003) or TNFR1 (Ansari et al., 2014), raising the question as to whether *H. pylori* might also directly interact with the LTβR. One example of an infectious agent directly binding to LTβR is hepatitis C virus which mediates activation of the signaling pathway via direct interaction of viral particles with the LTβR (Chen et al., 1997; Matsumoto et al., 1997). To test the possibility of a direct engagement between the pathogen and LTβR, immunoprecipitation studies were performed. Cell lysates, collected 3h post-infection, were incubated with antibodies against IgG (negative control), LTβR and *H. pylori* CagA (positive control). However, the analysis indicated no direct interaction of *H. pylori* with functional human recombinant LTβR (fusionprotein LTβR-Ig) as shown by the absence of *H. pylori* protein in the immunoprecipitate (Figure 14), implying other mechanisms might induce alternative NF-κB signaling via LTβR during *H. pylori* infection.

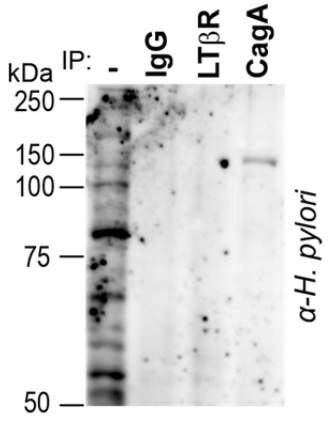

Figure 14: No direct interaction of *H. pylori* and LTβR to induce alternative NF-κ signaling.
MKN45 cells were infected with G27 *H. pylori* strain for 3 hours and lysates were immunoprecipitated with anti-LTβR or anti-CagA antibodies. Irrelevant rabbit IgG were used as control. Immunoprecipitates were subjected to western blot for detection of *H. pylori* proteins.

Signaling activation proceeds via receptor-binding of the trimeric complexes $LT\alpha_1\beta_2$ or LIGHT. Based on the previous finding that LT is upregulated early post infection (Figure 12), but p100/p52 processing occurred rather late, it was hypothesized that LT might feed into a secondary signaling loop to activate LTβR signaling after *H. pylori* infection. To test this hypothesis MKN45 and St3051 cells were infected with *H. pylori* G27 strain prior to administration of an LTβR-Ig fusion protein which scavenges LTβR ligands $LT\alpha_1\beta_2$ and LIGHT.

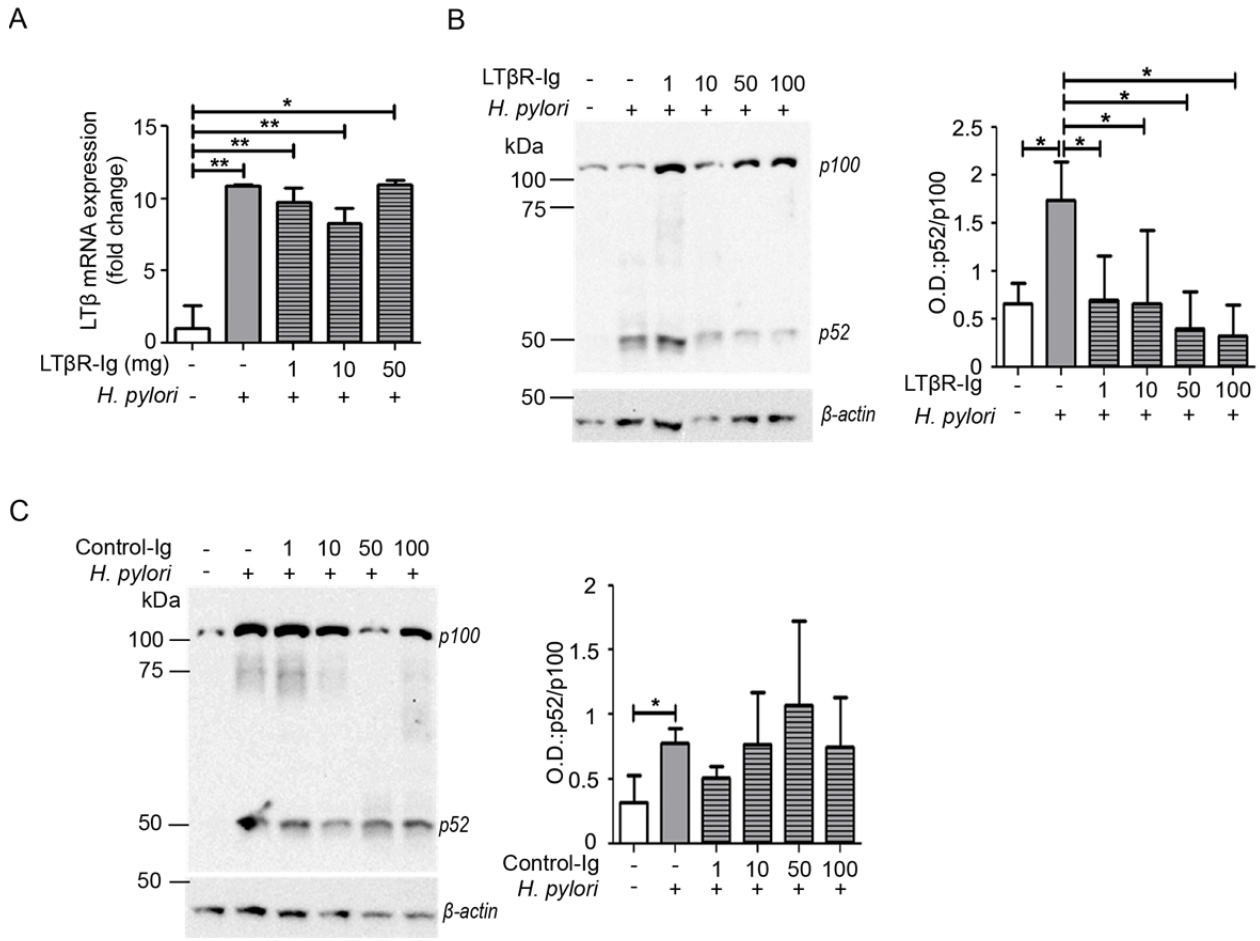

Figure 15: *H. pylori* infection induced p100/p52 processing via the LTβR signaling pathway.
(A) MKN45 cells were incubated with LTβR-Ig one hour prior to *H. pylori* infection. LTβ mRNA levels were measured 12 hours post infection. Values were normalized to GADPH. Results (mean ± S.D.) of three independent experiments are shown. p values were calculated using One-way ANOVA with Bonferroni's multiple comparison test. *p≤0.05, **p≤0.01. (B) MKN45 cells were incubated with LTβR-Ig for 1h before *H. pylori* infection at MOI 10. p100 processing to p52 was evaluated by western blot after 12h infection. β-actin served as loading control. One representative blot and quantification of 3 independent experiments (mean ± S.D.) are shown. (C) MKN45 cells were incubated with the isotype control MOPC-21 (Control-Ig) for 1h before *H. pylori* infection at MOI 10. p100 processing to p52 was evaluated by western blot after 12h infection. β-actin was used as a loading control. One representative blot and quantification of three independent experiments are shown. *p≤0.05.

Notably, LTβ mRNA levels which were strongly enhanced during *H. pylori* infection remained unaffected throughout the entire incubation period with LTβR-Ig in the GC cell line MKN45 (Figure 15A). Also, p100 protein expression was stable under antibody treatment. In contrast, p100 cleavage to p52 was significantly suppressed in *H. pylori* infected MKN45 cells treated with LTβR-Ig (Figure 15B), p100 processing was unaltered in infected cells treated with the isotype control (MOPC-21) (Figure 15C). Taken together, these results suggest an LTα- and LTβ-mediated LTβR-triggered secondary loop of alternative NF-κB signaling activation as a response to *H. pylori* colonization.

5.4 *H. pylori*-induced increase of LT via canonical NF-κB signaling induces feed-forward activation of alternative NF-κB and upregulation of LIGHT

H. pylori stimulates various signaling pathways that might play a role in the upregulation of LT during inflammation. IL6 and glycoprotein (gP) 130-dependent signaling of Signal Transducer and Activator of Transcription (STAT3) have been identified as key elements of cancer-related inflammation (Karin, 2006; Langowski et al., 2006; Voronov et al., 2003; Yu et al., 2007). *H. pylori*-stimulated chronic gastritis and gastric carcinogenesis in humans have been associated with activated STAT3 signaling (Jackson et al., 2007; Kanda et al., 2004). Downstream signaling of NF-κB transcription factor induces expression of primary inflammatory cytokines including TNF-α, IL-1β and IL-6. Furthermore, Subrata and colleagues could show canonical NF-κB-induced enhancement of both LTα and LTβ levels in hepatic cells by identifying IL-6 as a cytokine capable of activating LTβ expression (Subrata et al., 2012). These observations prompted the question, whether the observed LT up-regulation regulation was mediated via either IL-6-STAT3 signaling or the canonical NF-κB pathway.

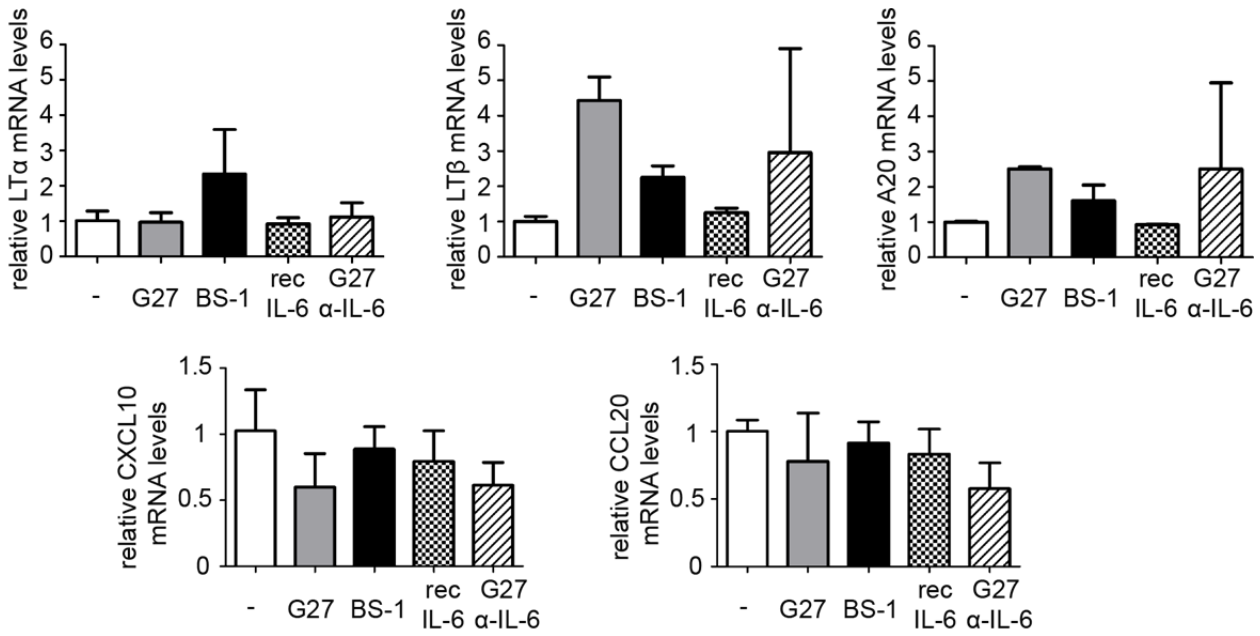

Figure 16: LT gene expression is not dependent IL-6 mediated STAT3 signaling in MKN45 cells
mRNA levels of LTα LTβ A20, CXCL10 and CCL20 were analyzed by real time PCR in MKN45 cells 12h post-infection with *H. pylori* strain G27 (MOI 10). MKN45 cells where either infected with G27, BS-1, recombinant IL-6 or *H. pylori* infected MKN45 cells treated with IL-6 antibody.

Stimulation of uninfected MKN45 cells with recombinant IL-6 did not lead to an increase in LTα or LTβ expression. Expression of LTα and LTβ which was induced in *H. pylori* infected cells remained unchanged upon blockade of IL-6 signaling using anti-IL-6 antibodies. Additional downstream NF-κB target genes were also not affected, which suggested an IL-6-STAT3 independent signaling pathway of LT expression upon *H. pylori* infection (Figure 16).

To address the question whether the increased LT mRNA expression observed in infected gastric cancer cells was resulting from canonical NF-κB pathway activation, *H. pylori*-infected MKN45 cells were compared to TNFα stimulated cells. The target gene A20 served as positive control for activated canonical NF-κB signaling. Notably, expression of LIGHT was not affected by TNFα, confirming that LIGHT expression is regulated independently of canonical NF-κB signaling. However, expression levels of LTα were slightly increased upon TNFα stimulation and LTβ expression was significantly upregulated (Figure 17). Furthermore, the effects observed for

LTβ were mirrored in the significant induction of A20 mRNA expression under TNFα treatment, identifying canonical NF-κB signaling as responsible signaling pathway for LT upregulation.

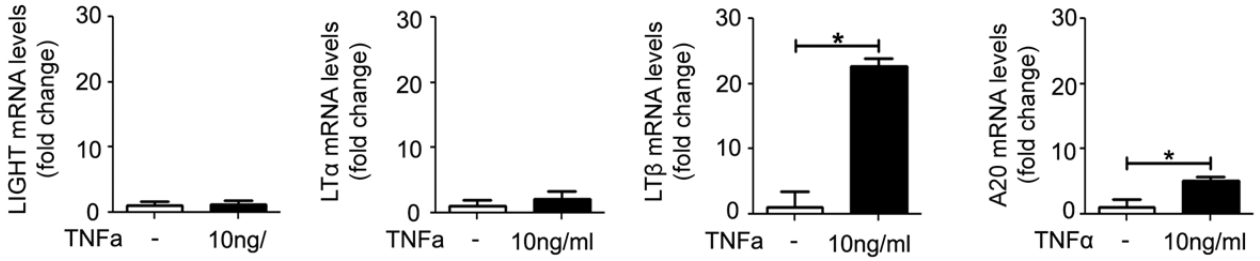

Figure 17: The canonical NF-κB signaling pathway induced upregulation of LT in MKN45 cells.
mRNA levels of LIGHT, LTα, LTβ and A20 in TNFα (10ng/ml)-treated MKN45 cells 12h post-infection with *H. pylori* strain G27 (MOI 10) were analyzed by real time PCR. Comparison was performed using Student's t-test. (*p≤0.05).

To further confirm that canonical NF-κB signaling accounted for the increase in LT expression during pathogen infection, canonical NF-κB signaling pathway was blocked by addition of the IKK2 inhibitor [5-(p-fluorophenyl)-2-ureido]thiophene-3-carboxamide (TPCA-1). As a control for effective blockage of the signaling pathway, A20 was analyzed. As expected, A20 expression levels significantly dropped in TCPA-1-treated infected cells when compared to untreated infected cells (Figure 18A). Interestingly, although LTα expression was only slightly changed in cells treated with *H. pylori* or TPCA-1, expression levels of LTβ were significantly increased after *H. pylori* infection and could be reduced again by TPCA-1 treatment. This suggests that LTβ expression is primarily controlled by the canonical NF-κB signaling pathway in GC cells in response to *H. pylori* infection. Furthermore these results imply that either LTα is regulated by a third signaling pathway or that LTβ as a homotrimer might be capable of stimulating LTβR signaling during chronic *H. pylori* infection. As expected, inhibition of canonical NF-κB through TPCA-1 reduced levels of p100 in both MKN45 and St3051 cells (Figure 18B), since expression of p100 depends on canonical NF-κB. Remarkably, cleavage of p100 to p52 could still be observed, implying an involvement of a canonical NF-κB-independent ligand in the stimulation of LTβR signaling. As control for specific activation of the canonical NF-κB signaling pathway,

BS-1, TPCA-1 and TNFα were tested in both cell lines by assessing the phosphorylation of p65 via Western blot. BS-1 showed no effect on p-p65, but - as expected - TPCA-1 administration to *H. pylori*-infected or TNFα-treated cells led to p-p65/p65 signal intensity downregulation (Figure 18C).

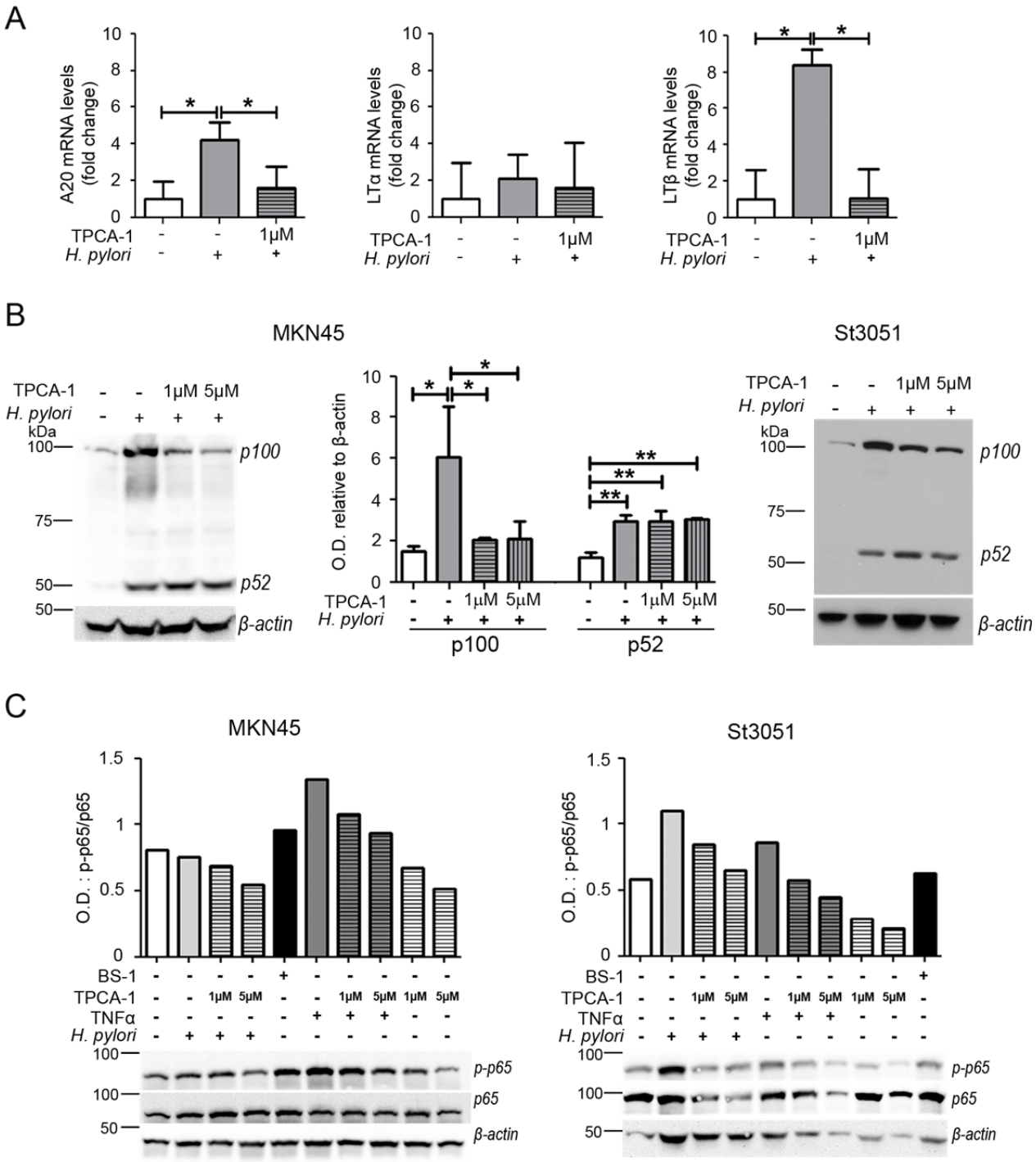

Figure 18: LT gene expression is regulated by the canonical NF-κB signaling pathway in gastric cancer cells
(A) mRNA levels of A20, LTα and LTβ were analyzed by real time PCR in MKN45 cells after 12h infection with *H. pylori* strain G27 (MOI 10). Cells were incubated with the inhibitor of human IκB kinase 2 (IKK2) TPCA-1 to block canonical NF-κB signaling 1h prior to infection. Ct values were normalized to GADPH. Results expressed as mean ± S.D. from 3 independent experiments are shown. (B) MKN45 ans St3051 cells were incubated with TPCA-1 for 1h before infection with the *H. pylori* strain G27 (MOI 10). Cell lysates were obtained after 12h infection and levels of p100/p52 were assessed by western blot. β-actin was used as loading control. One representative blot (mean ± S.D.) of 3 independent experiments is shown as well as quantification of the representative MKN45 western blot. p values were calculated using One-way ANOVA with Bonferroni's multiple comparison test. *p≤0.05, **p≤0.01. (C) MKN45 and St3051 cells were incubated with TPCA-1 and cells were stimulated with 50ng/ml of TNFα or the LTβR agonist BS-1 (0.5µg/ml) where indicated. Cell lysates were obtained after 12h infection with the *H. pylori* strain G27 and levels of p-p65 and p65 were assessed by western blot. β-actin was used as a loading control. One representative blot and quantification from three independent experiments is shown.

So far, the only other known ligand for LTβR signaling activation is LIGHT. Indeed, upon *H. pylori* infection of MKN45 and St3051 cancer cells, LIGHT expression levels were increased if canonical NF-κB was blocked (Figure 19), implying that upon cessation of canonical NF-κB signaling LIGHT may serve as the main ligand to maintain active LTβR signaling upon *H. pylori* infection, to compensate for the decrease in LT levels. Furthermore, CXCL13 was also upregulated when canonical NF-κB was blocked. On the other hand, expression of CCL22 (an NF-κB target gene, which was observed to be exclusively regulated by canonical signaling during *H. pylori* infection) was completely abrogated after treatment with TPCA-1 (Figure 19).

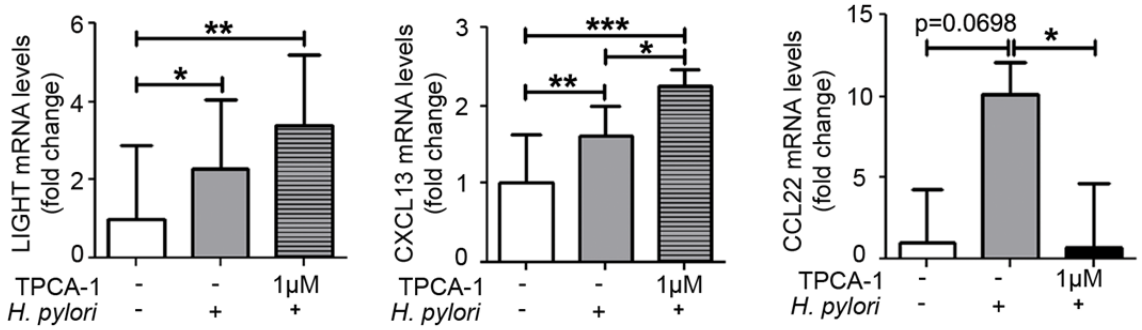

Figure 19: Blocking of canonical NF-κB signaling during *H. pylori* infection leads to LIGHT upregulation.
LIGHT mRNA expression levels after infection of MKN45 cells with the *H. pylori* strain G27 at MOI 10 for 12 hours. Cells were incubated with TPCA-1 for 1h before infection. Results (mean ± S.D) from 3 independent experiments are shown. (E) mRNA expression levels of CXCL13 and CCL22 in MKN45 cells infected with *H. pylori* G27 (MOI 10, 12h). Where indicated, cells were incubated with TPCA-1 1h before infection. Ct values were normalized to GADPH. Results (mean ± S.D) from 3 independent experiments are shown. Significances were calculated using One-way ANOVA with Bonferroni's multiple comparison test. *p≤0.05**p≤0.01, ***p≤0.001.

As was already described by many studies, chronic inflammation is driven by both arms of the NF-κB signaling pathway and LT plays a significant role in the process. To confirm the role of the alternative LTβR ligand LIGHT *in vivo*, human biopsy samples were tested for LIGHT expression under pathological conditions. IHC staining for LIGHT in normal unaffected gastric tissue, mild, moderate and severe gastritis induced by *H. pylori*, and early gastric tumor samples revealed LIGHT positivity in inflammatory cells in all pathological stages. However, also gastric epithelial cells expressed LIGHT, which was detected earliest in pathological stages of moderate gastritis (Figure 20A). Furthermore, when comparing LIGHT expression between tissue samples of patients suffering from chemical reactive *H. pylori* negative gastritis (*H. pylori* negative gastritis samples were confirmed by histology and after analyzing expression of *H. pylori* antigens by ELISA and Lineblot (Formichella et al., 2013)) or *H. pylori*-induced gastritis, only tissue samples of *H. pylori* positive gastritis displayed a significant increase of LIGHT (Figure 20B), suggesting a direct correlation between LIGHT expression and *H. pylori*-induced pathology.

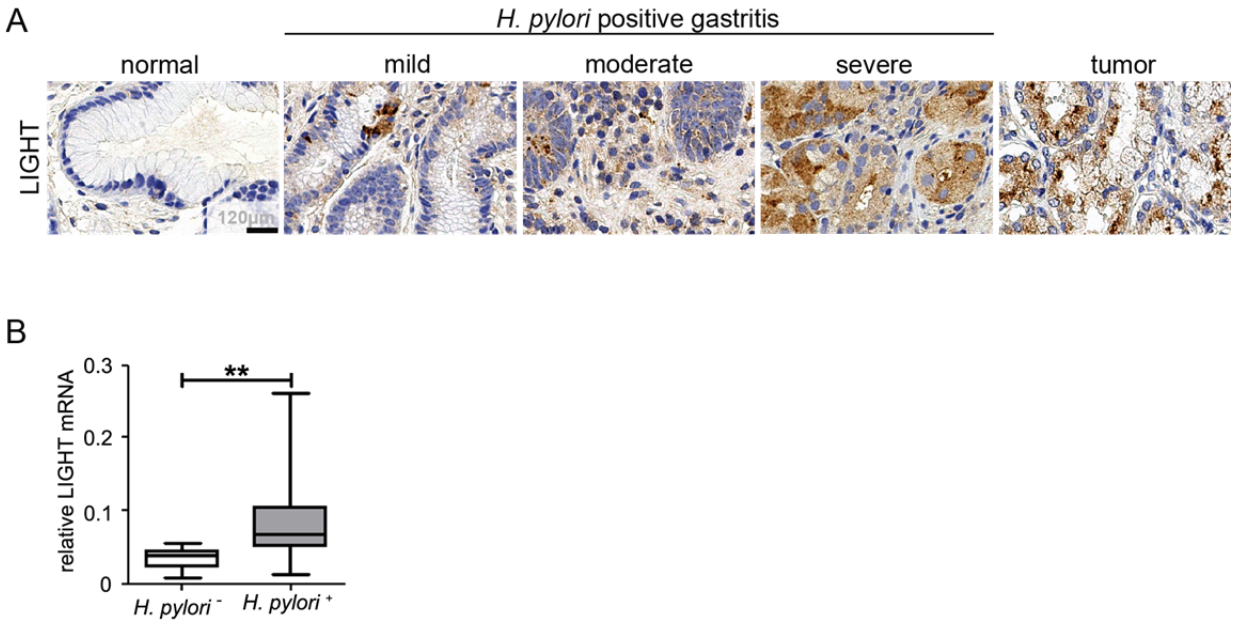

Figure 20: LIGHT is highly upregulated in *H. pylori* induced gastric pathology.
(A) Representative images of LIGHT expression in human normal gastric mucosa, mild, moderate and severe gastritis as well as in early gastric adenocarcinoma biopsy samples, detected by immunohistochemistry. (B) Whiskers (median and minimum to maximum) box plot showing relative mRNA levels of LIGHT in gastric *H. pylori* positive and *H. pylori* negative gastritis (n=11). Statistics were calculated with ANOVA Kruskal-Wallis, Dunn's multiple comparison test (**p≤0.01).

Taken together, the *in vitro* results provide proof for *H. pylori*-induced activation of LTβR signaling, first by canonical NF-κB-mediated increase of LTs, followed by activation of alternative NF-κB signaling and compensatory upregulation of LIGHT in the absence of LT. The *in vivo* situation implies a direct *H. pylori*-mediated control of LIGHT expression probably to amplify alternative NF-κB signaling,

5.5 The cagPAI of *H. pylori* is essential for LTβR-mediated activation of alternative NF-κB, however independent of CagA

H. pylori strains display a variety of virulence factors which shape the pathological response. The outcome can differ from patient to patient depending on the pathogen strain and the influence of environmental and host factors on the signaling pathway induction in gastric epithelial cells. By using a wide panel of virulence factors which are important for bacterial attachment, T4SS assembly or cytotoxicity, *H. pylori* is capable of inducing pathology in gastric epithelial host cells. For example, as was demonstrated by several groups the canonical NF-κB signaling pathway in combination with the virulence factor CagA plays a significant role in the inflammatory response towards bacterial colonization (Backert et al., 2010; Lamb et al., 2009). To assess the role of virulence factors on activation of alternative NF-κB MKN45 and St3051 cells were infected with different virulence factor mutant strains to see effects on p100 processing.

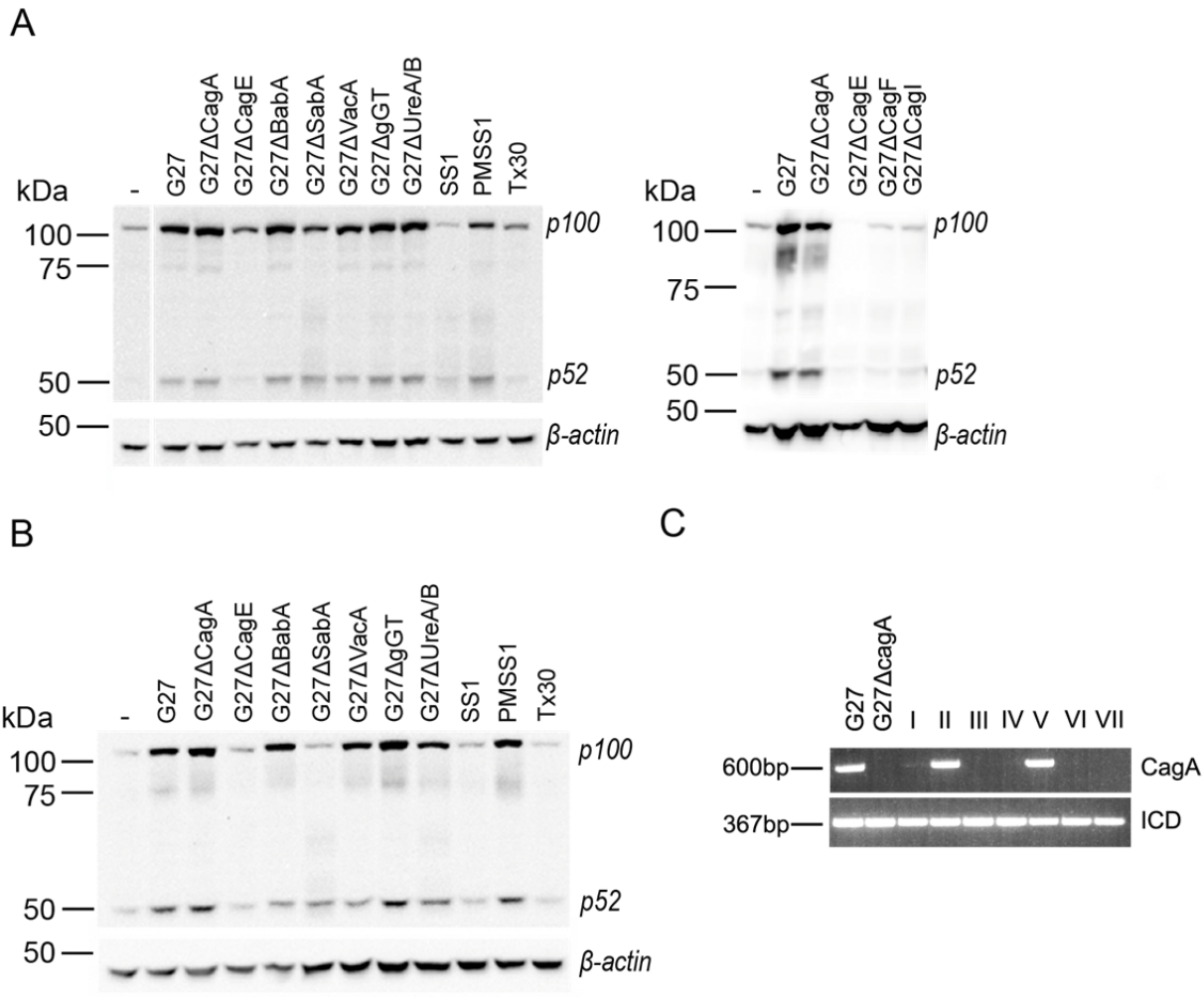

Figure 21: Alternative NF-κB signaling is independent of CagA but dependent of the T4SS
(A) MKN45 cells were infected with the *H. pylori* wild type strains G27, PMSS1, SS1, and Tx30 and the G27 isogenic mutant strains G27ΔCagA, G27ΔCagE, G27ΔBabA, G27ΔSabA, G27ΔVacA, G27ΔgGT and G27ΔUreA/B as well as CagF and CagI at MOI 10 for 12 hours. BS-1 served as positive control. p100 processing to p52 was detected by western blot. β-actin was used as loading control. One representative blot from 3 independent experiments is shown. (B) St3051 cells infected with identical G27 isogenic virulence strains than MKN45 cells at MOI10 for 12hours. p100 processing to p52 was detected by western blot. β-actin was used as loading control. One representative blot from 3 independent experiments is shown. (C) Expression of CagA and isocitrate dehydrogenase (ICD), used as a housekeeping gene, detected by PCR in different *H. pylori* clinical isolates.

With regard to LTβR signaling, the CagA-deficient mutant strain did neither change expression levels of p100 nor p100 cleavage to p52 in both MKN45 (Figure 21A) and St3051 cells (Figure 21B). Furthermore, no changes in p100/p52 processing was observed in virulence factor mutant strains of toxin VacA, adhesins BabA and SabA, γ-Glutamyltranspeptidase (gGT) or Urease. However, virulence factor mutant stains without essential components of the cagPAI, namely the

mutant strains G27ΔCagE, G27ΔCagF, G27ΔCagI as well as wildtype strains SS1 and Tx30, which also lack a functional T4SS, could not induce p100 processing to p52. This implies a crucial role of the T4SS in the activation of the alternative NF-κB signaling pathway, although processing is independent of CagA.

To further confirm the hypothesis of that CagA is dispensable for *H. pylori*-mediated activation of the LTβR signaling pathway in gastric epithelial cells, MKN45 cells infected with clinical isolates - positively tested for p100/p52 processing (Figure 11) - were analyzed for CagA protein expression. Notably, out of 7 clinical isolates tested, 3 isolates displayed p100/p52 processing in MKN45 cells. Two of these 3 isolates tested positive for CagA (Figure 21C), but nevertheless exhibited active alternative NF-κB signaling, supporting the theory of *H. pylori* CagA-independent triggering of LTβR signaling in gastric cancer cell lines.

Overall, the data collected so far indicated that *H. pylori,* which induced secretion of LTα, LTβ and in the beginning low amounts of LIGHT activates LTβR signaling. Therefore, expression of ligands of the LT system followed by p100 processing was analyzed in the absence of a functional type IV secretion system.

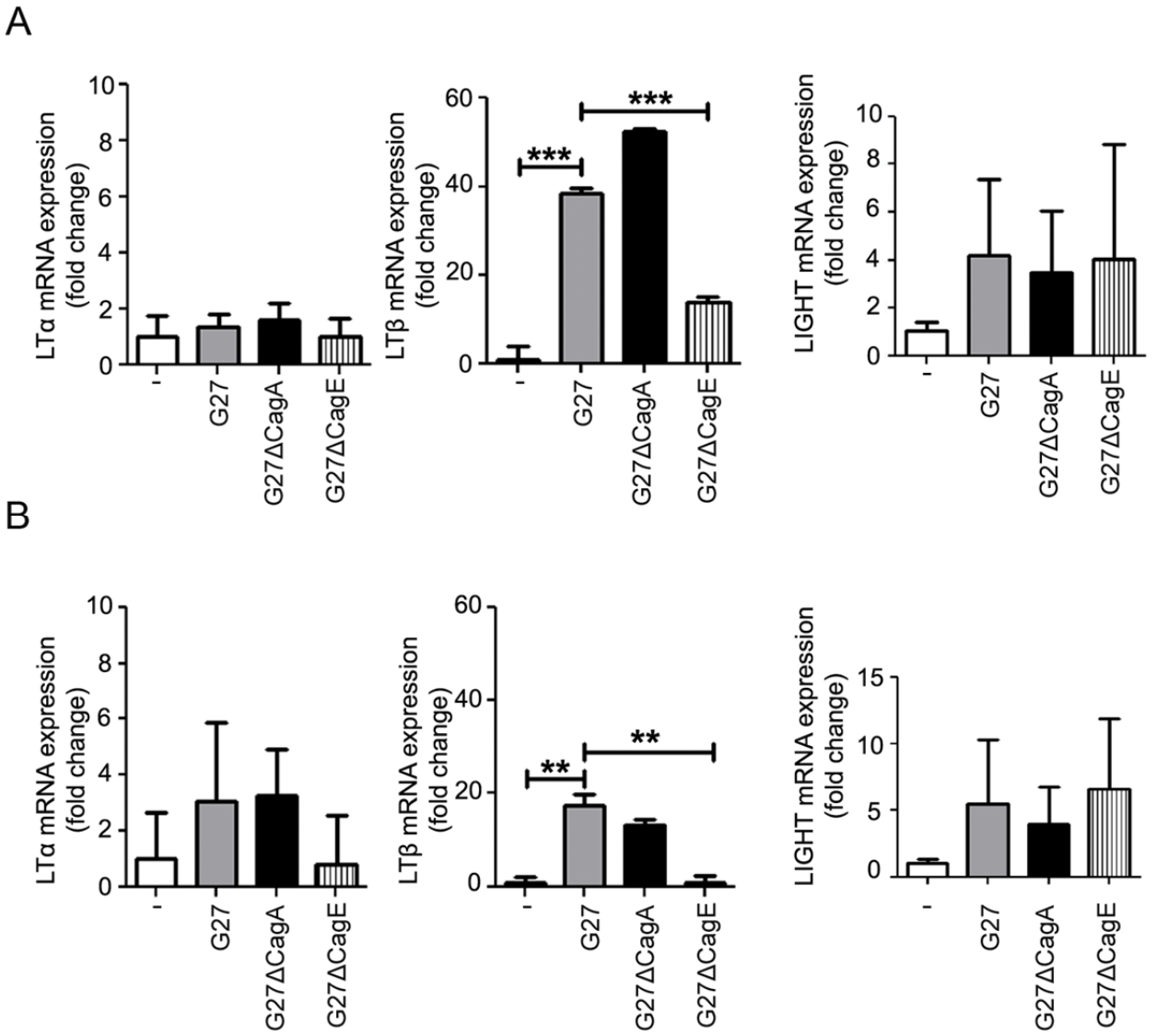

Figure 22: Expression of LT system ligands is regulated by the T4SS but independent of CagA
(A) LTβR ligand mRNA expression levels in MKN45 cells after infection with wild type *H. pylori* G27 strain and the isogenic mutant strains G27ΔCagA and G27ΔCagE. Ct values were normalized to GADPH. Results (mean ± S.D) from 4 independent experiments are shown. ***p≤0.001. (ANOVA, Bonferroni's multiple comparison test). (B) LT system ligand mRNA expression levels in St3051 cells after infection with wild type *H. pylori* G27 strain and the isogenic mutant strains G27ΔCagA and G27ΔCagE. Ct values were normalized to GADPH. Results (mean ± S.D) from 4 independent experiments are shown. **p≤0.01. (ANOVA, Bonferroni's multiple comparison test).

Notably, human gastric cancer cells infected with the mutant strain G27ΔCagE expressed significantly lower levels of LTβ in both cell lines compared to the wildtype strain G27 (Figure 22), whereas LTα was only slightly reduced. On the other hand LIGHT expression remained

entirely unaffected. Combined, these results suggest that the presence of a functional type IV secretion system influences LT expression upon *H. pylori* infection.

5.6 *In vivo* blocking of LTβR signaling reduces *H. pylori*-induced gastric inflammation

Data obtained from *H. pylori*-infected human gastric tissue samples and *in vitro* cell culture experiments show a tight co-operation of the canonical and alternative NF-κB signaling pathways in response to *H. pylori* infection. In order to assess what specific role LTβR signaling plays in gastric pathology during *H. pylori* infection a mouse model was established. Infecting mice with *H. pylori* for one month highly increased LTα, LTβ as well as LIGHT expression levels in gastric tissue samples (Figure 23).

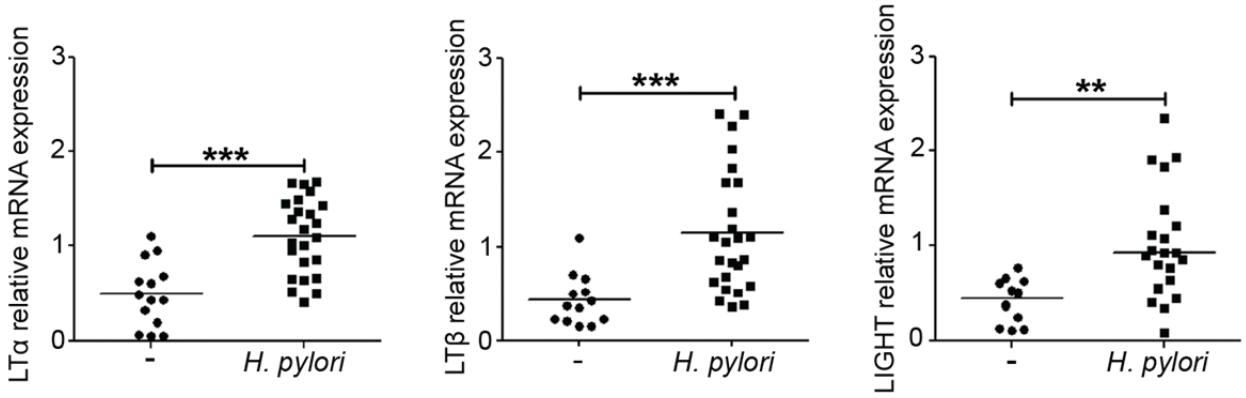

Figure 23: Mice suffering from *H. pylori* infection have high levels of LT and LIGHT.
LTα, LTβ and LIGHT mRNA levels in mice infected with the *H. pylori* strain PMSS1 for one month. Ct values were normalized to GADPH. (Mann-Whitney U test).

Female C57BL/6 mice at the age of 6-8 weeks were injected one week prior to infection with a soluble LTβR immunoglobulin fusion protein (mLTβR-mIgG1, in the following named LTβR-Ig) (Browning et al., 2005) (Figure 24A). This fusion protein was described to intercept ligands ($LT\alpha_1\beta_2$ and LIGHT) capable of inducing LTβR signaling, thereby blocking the alternative NF-κB

signaling pathway. Following infection with the pathogenic *H. pylori* PMSS1 strain, mice were injected once per week over 30 days with either LTβR-Ig antibody, or its isogenic control MOPC-21, to study the effect of LTβR signaling during an active chronic inflammation.

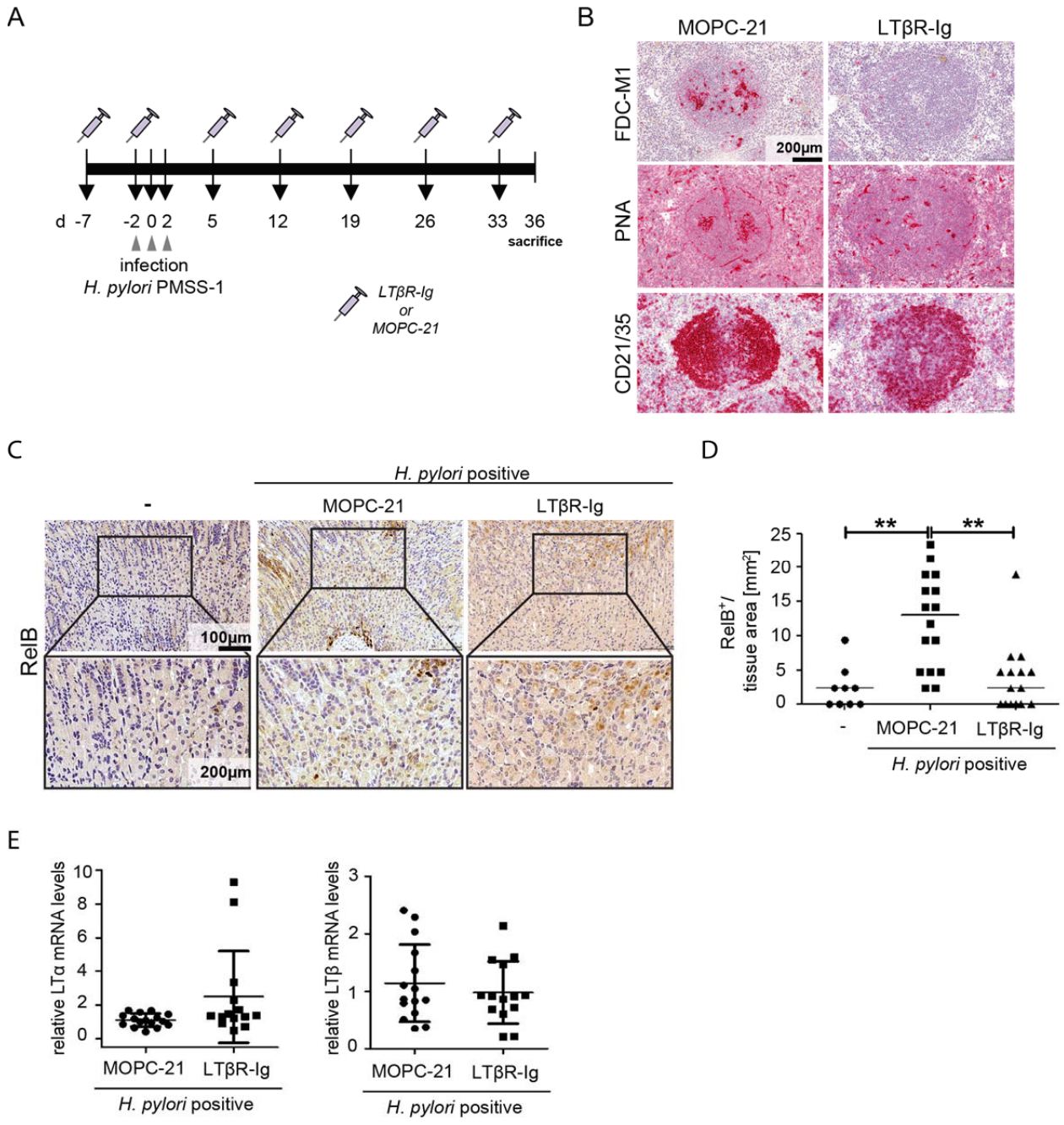

Figure 24: LTβR signaling in *H. pylori* infected mice is blocked via LTβR-Ig treatment
(A) Time schedule of LTβR-Ig treatment. C57BL/6 mice were injected with 100µg of LTβR-Ig or the isotype control MOPC-21 at the indicated time points ( ). At day 0, 2 and 5, mice were infected with a 10^9 orogastric dose of *H. pylori* strain PMSS1. Mice were sacrificed at day 36 for analyses. (B) IHC staining of FDC-M1 or CD21/35 in cryosections of spleens to detect FDCs from MOPC-21 or LTβR-Ig-treated mice, infected with *H. pylori* PMSS1.

(Scale bar: 200µm). (C) Murine stomach samples were stained for $RelB^+$ cells by IHC. Representative pictures are shown. (scale bar represents 100µm and 200µm for magnifications). (D) $RelB^+$ nuclei were calculated per area of tissue (mm^2). **p≤0.01, ***p≤0.001 (ANOVA Kruskal-Wallis, Dunn's multiple comparison test). (E) Relative mRNA expression levels of LTα and LTβ in the stomach of *H. pylori*-infected mice treated either with MOPC-21 or LTβR-Ig. Ct values were normalized to MOPC-21. (Student's t test).

Extensive studies on the LT system revealed its unique role in both development and functional organization of lymphoid tissues (Beutler et al., 1994; Ware et al., 1995). It is crucial for the maintenance of several aspects of the lymphoid microenvironment e.g. development of follicular dendritic cell (FDC) (Ettinger et al., 1998) and also their localization and accumulation within lymphoid follicles, which is essential for successful B cell maturation in lymphoid tissue following antigen stimulation (Clark et al., 1994; Rajewsky et al., 1987; Szakal et al., 1989). Logically, the dependency of FDC networks on LTβR signaling in splenic germinal centers was used to confirm the efficacy of LTβR-Ig treatment, since mice injected with the fusion protein lack FDC networks (Haybaeck et al., 2009). Histochemically analyzed spleens of *H. pylori* infected mice that received the control antibody MOPC-21 displayed normal FDC networks in the germinal centers of the spleen shown by staining with the monoclonal antibody FDC-M1 (Figure 24B). Additionally, germinal centers were analyzed with regard to germinal center B cells, which can be identified as proliferating and peanut agglutinin (PNA)-positive centrocytes (Grabner et al., 2009). Whereas MOPC-21-treated mice displayed an ordered appearance of germinal center B cells the structure in LTβR-Ig treated mice was disrupted. As a third confirmative approach complement receptors CR1 (CD35) and CR2 (CD21) were stained with anti-CD21/35 antibody whose high expression on FDCs were described to be important for maintenance of B cell memory (Fischer et al., 1998). LTβR-Ig treated mice exhibited a rather diffuse distribution of complement receptors within germinal centers, in contrast to the ordered spatial organization of complement receptors within the germinal centers of control treated animals. Furthermore, the missing activation of alternative NF-κB signaling in response to the pathogen in LTβR-Ig treated mice was also reflected in a reduced amount of $RelB^+$ nuclei when compared to control mice, since less RelB was translocated (Figure 24C/D). Notably, *H. pylori* positive mice that received

the LTβR-Ig antibody also showed no changes on LTα and LTβ expression when compared to *H. pylori* infected mice (Figure 24E). This confirms the observations obtained from *in vitro* experiments showing that regulation of LT expression is regulated by canonical NF-κB signaling (Figure 18). Altogether, *H. pylori*-infected and LTβR-Ig treated mice lack a functional LTβR-Ig signaling and therefore provide a good model to study *H. pylori*-induced pathology in the absence of LTβR signaling.

First, *H. pylori* colonization of the gastric mucosa was analyzed. *H. pylori* infected mice which received LTβR-Ig presented a significantly higher gastric colonization compared to MOPC-21-injected mice, suggesting that LTβR-mediated signaling is important for the establishment of infection within the gastric mucosa (Figure 25A). In contrast to the significant increase in c.f.u. in LTβR-Ig treated mice, the inflammatory score, as determined by the amount of infiltrating inflammatory cells, in LTβR-Ig-treated mice did not reach the same level as in in control mice (Figure 25B) demonstrating that the regulation of the *H. pylori*-induced inflammation was partly dependent on LTβR signaling.

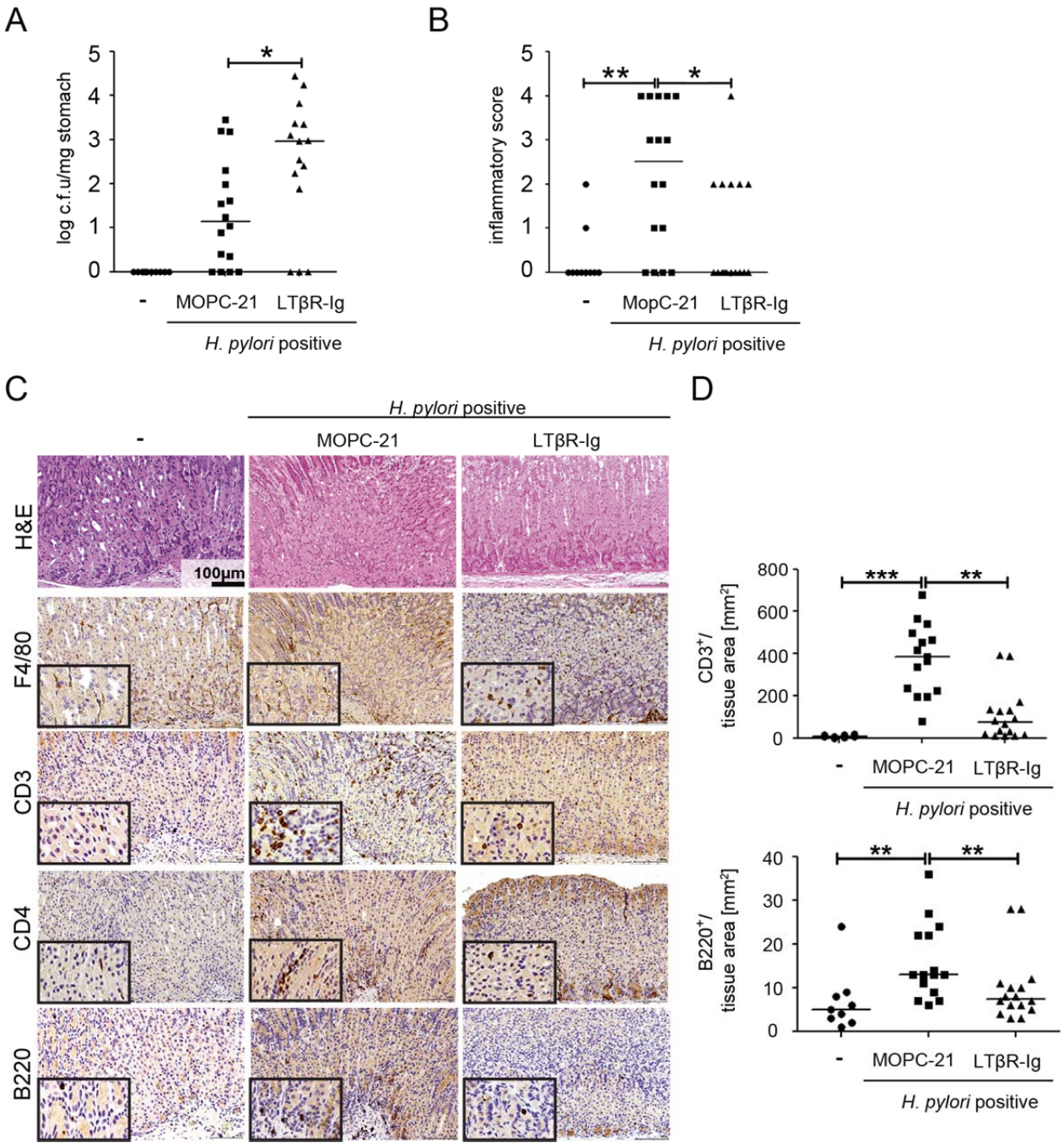

Figure 25: Blocking of LTβR signaling during *H. pylori* infection reduces gastric inflammation
(A) Colony-forming units (c.f.u) per mg of stomach. (B) Inflammatory score. Activity and chronicity of infection was evaluated in antrum and corpus of murine stomach samples according to the updated Sydney system. (C) Murine stomach samples were stained for F4/80$^+$, CD3, CD4 and B220 by IHC. Representative pictures are shown. (scale bar represents 100µm and 200µm for magnifications (D) CD3$^+$ and B220$^+$ cells and RelB$^+$ nuclei were calculated per area of tissue (mm^2). **p≤0.01, ***p≤0.001 (ANOVA Kruskal-Wallis, Dunn's multiple comparison test).

Acute infection with *H. pylori* initiates the first line of defense of the host, the innate immune system. However, the host innate immune system is insufficient to clear *H. pylori* infection, as

the bacterium has evolved effective strategies to avoid elimination by neutrophilic granulocytes (Evans et al., 1995) macrophages (Schwartz & Allen, 2006) or dendritic cells (Khamri et al., 2010). Nevertheless, upon infection with the pathogen strong influx of $CD4^+$ T cells into the gastric lamina propria was described (Eaton et al., 2001). In the study at hand, analyses of the inflammatory cells was done according to the *in vivo* experiments of gastric biopsy samples (Figure 2).

Notably, the higher bacterial load in LTβR-Ig treated mice was accompanied by less F4/80 positive macrophages and less T cell (mostly $CD4^+$) influx (Figure 25C/D). In addition, also slightly fewer amounts of $B220^+$ B cells could be observed after one month of LTβR-Ig treatment in the gastric mucosa of infected mice, supporting the hypothesis that the LTβR signaling pathway as important driver of gastric pathology induced by *H. pylori*.

Furthermore, expression levels of cytokines and chemokines, which are important for inflammatory cell recruitment to the tissue, were analyzed in the different groups. Remarkably, several NF-κB pathway downstream target genes that were so far described to be canonically regulated showed also reduced levels *H. pylori* positive mice with blocked LTβR signaling. For example, one of the fastest NF-κB target genes upregulated in response to *H. pylori* infection is IL-8, also known as neutrophil chemotactic factor (Crabtree et al., 1994a; Crabtree et al., 1994b; Sharma et al., 1998), since it is a key chemokine for neutrophil recruitment (Baggiolini et al., 1989).

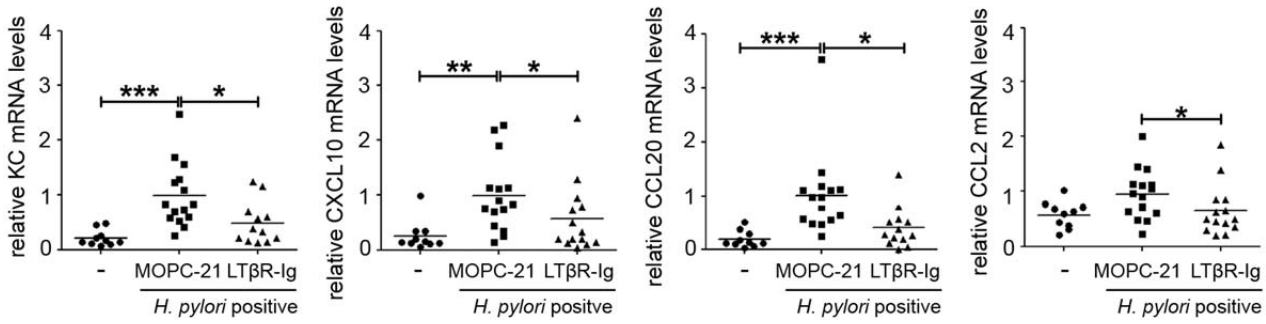

Figure 26: Blocking of LTβR signaling during *H. pylori* infection reduces gastric inflammation
Relative mRNA expression levels of KC, CXCL10, CCL20 and CCL2 in the stomach of uninfected mice or *H. pylori*-infected mice treated either with MOPC-21 or LTβR-Ig. Ct values were normalized to MOPC21. *p≤0.05, **p≤0.01, ***p≤0.001. (ANOVA Kruskal-Wallis, Dunn's multiple comparison test).

Notably, mice infected with *H. pylori* PMSS-1 showed significantly increased levels of the murine IL-8 homolog keratinocyte-derived protein chemokine (KC) (Rovai et al., 1998) compared to aged-matched non-infected mice. On the other hand, mice that had inhibited LTβR signaling displayed also increased levels of KC, however, KC expression was significantly less compared to non-blocked *H. pylori* positive mice (Figure 26). IL-8 is mainly produced by macrophages but also epithelial cells (Cromwell et al., 1992; Ribeiro et al., 1991). The - under physical conditions - high influx of macrophages into the gastric mucosa as response to the bacterial colonization was inhibited due to the absence of LTβR signaling. Consistent with this reduced recruitment of macrophages, LTβR-Ig treated mice showed reduced levels of CXCL10, a chemokine produced by macrophages after IFN-γ stimulation. CXCL10 was demonstrated to play a crucial role in T cell trafficking (Dufour et al., 2002). The reduced amounts of macrophages resulting in lower levels of CXCL10 expression therefore seemed to be regulated by LTβR signaling in response to *H. pylori* which supports the results obtained in *in vitro* experiments. In line with this, significantly lower levels of the NF-κB downstream target genes CCL20 (lymphocyte chemoattractor (Hieshima, 1997 #5385)) and CCL2, also known as monocytes chemoattractant protein-1, which is a T cell chemoattractant (Carr et al., 1994) were detected in LTβR-Ig treated *H. pylori*-infected mice (Figure 26).

Together, these *in vivo* data reveal a significant role of *H. pylori*-induced activation of alternative – LTβR-mediated – NF-κB signaling in both inflammatory but also epithelial cells during chronic gastric pathology. The absence of an inflammatory milieu in cell culture experiments with gastric cancer cell lines underlines the role of epithelial cells in the process of *H. pylori* induced pathology which might be further amplified by ongoing LTβR signaling and increased levels of chemokine-secreting immune cells.

5.7 Activation of LTβR signaling aggravates gastric pathology induced by *H. pylori* colonization

Gastric pathology induced by *H. pylori* is a step-wise process culminating in the development of gastric adenocarcinoma. To get further insight into the role of LTβR signaling in the context of *H. pylori*-induced exacerbation of local pathology, infected mice received the LTβR-agonizing antibody ACH6. Mice were injected with ACH6 twice/week and sacrificed after 36 days (Figure 27A).

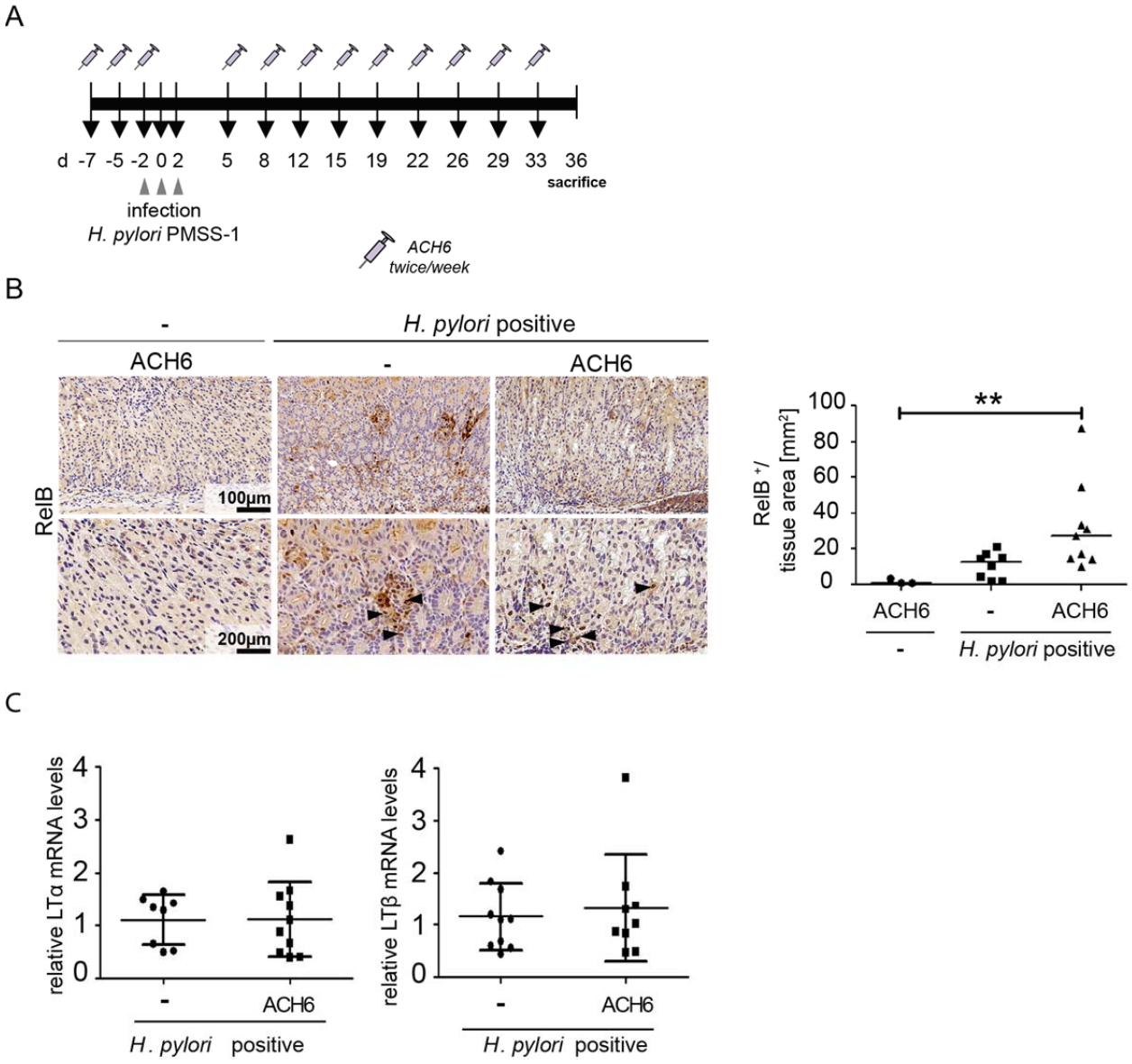

Figure 27: Agonization of the LTβR signaling pathway in *H. pylori* infected mice.
(A) Time schedule of ACH6 treatment. C57BL/6 mice were injected with 50µg of ACH6 at the indicated time points ( ). At day 0, 2 and 5, mice were infected with a 10^9 orogastric dose of *H. pylori* strain PMSS1. Mice were sacrificed at day 36 for analyses. (B) Murine stomach samples were stained for $RelB^+$ cells by IHC. Representative pictures are shown. (scale bar represents 100µm and 200µm for magnifications). (D) $RelB^+$ nuclei were calculated per area of tissue (mm^2). **p≤0.01 (ANOVA Kruskal-Wallis, Dunn's multiple comparison test). (C) Relative mRNA expression levels of LTα and LTβ in the stomach of *H. pylori*-infected mice untreated or treated with ACH6. Ct values were normalized to untreated *H. pylori* mice. (Student's t test).

Successful treatment was confirmed by analysis of RelB translocation (Figure 27B). LT expression was also not affected by ACH6 treatment in *H. pylori* positive mice (Figure 27C).

Upon confirmation of successful treatment, colony forming unit (c.f.u.) was determined in gastric tissue. Notably, intensified activation of LTβR signaling via ACH6 reversed the effects observed in the experimental approach of blocked alternative NF-κB signaling: whereas a reduced bacterial load in the stomach of infected mice was noted (Figure 28A) the inflammatory score was significantly higher (Figure 28B). Analysis of stomach-infiltrating inflammatory cells showed increased levels of macrophages in *H. pylori*-infected mice comparable to the human situation. Furthermore, slightly lower levels of total $CD3^+$ cells were detected in ACH6-treated mice when compared to *H. pylori* infected animals, yet no apparent differences were seen number of infilitrating the $CD4^+$ T cells (Figure 28C and 28D). Apart from T cells, *H. pylori* infection induced an influx of B cells which varied depending on the severity of inflammation. This finding was consistent with the data obtained from human *H. pylori*$^+$ biopsy samples, where B cell amounts in samples of moderate, severe or tumor-associated gastritis were significantly higher than in mild gastritis and normal mucosa samples (Figure 2). Significantly increased levels of B cells compared to *H. pylori* infected-only mice got recruited into the inflamed tissue, indicating that LTβR signaling promotes recruitment of B cells as response to the pathogen colonization (Figure 28E). However, it has to be noted that ACH6 treatment by itself already induced B cell influx. Nevertheless, this effect is negligible when compared to the infiltration in response to the bacterium. Inflammatory cells recruited into the gastric mucosa of *H. pylori*-infected ACH6-treated mice were found to form foci within the tissue with a highly structured organization that

resembled that of tertiary lymphoid organs (TLO). TLO formation was described to be a hallmark of *H. pylori* infection (Genta et al., 1993; Kobayashi et al., 2004; Shomer et al., 2003)

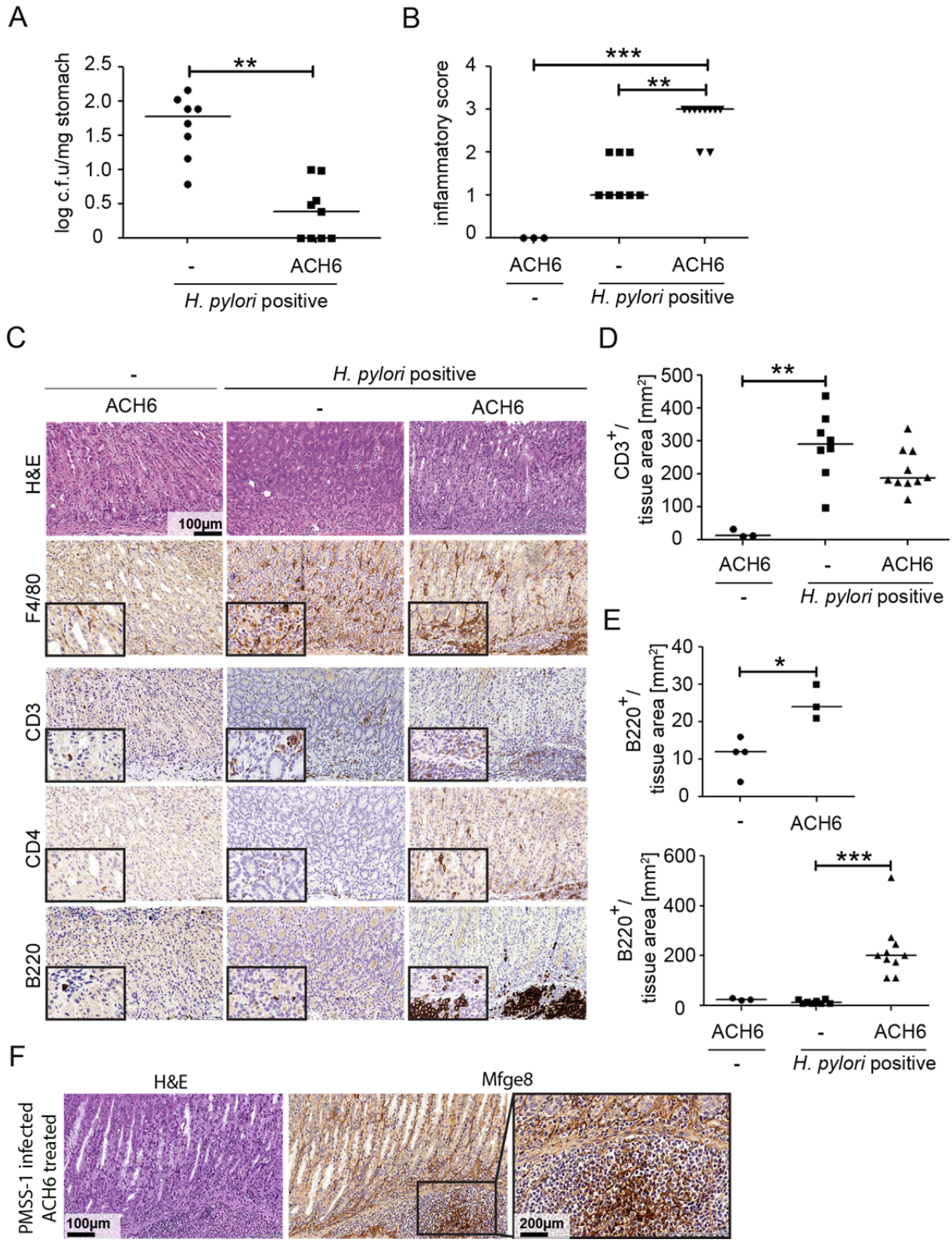

Figure 28: Agonizing of LTβR signaling during *H. pylori* infection enhances gastric inflammation
(A) Colony-forming units (c.f.u) per mg of stomach. (B) Inflammatory score. Activity and chronicity of infection was evaluated in antrum and corpus of murine stomach samples according to the updated Sydney system. (C) Murine stomach samples were stained for $F4/80^+$, $CD3^+$, $CD4^+$ and $B220^+$ cells by IHC. Representative pictures are shown. (scale bar represents 100µm and 200µm for magnifications. (D-F) $CD3^+$ and $B220^+$ cells were calculated per area of tissue (mm^2). *p≤0.05, **p≤0.01, ***p≤0.001 (ANOVA Kruskal-Wallis, Dunn's multiple comparison test). (F) Murine stomach samples were stained for Mfge-8^+ FDCs. Representative pictures are shown. (scale bar represents 100µm and 200µm for magnifications.

As was noted above, mice treated with LTβR-Ig lack active LTβR signaling in response to *H. pylori* infection, as demonstrated by the absence of FDC networks which can be visualized by FDC-M1, PNA and CD21/35 staining of the spleen. FDC-M1 was demonstrated to be identical to the milk fat globule epidermal growth factor 8 (Mfge8) protein (Kranich et al., 2008). To confirm that the inflammatory foci observed in ACH6-treated *H. pylori*-positive mice were similar to TLO structures (defined among others by compartmentalization of B and T cells and FCDs), samples were stained with Mfge8 which displayed Mfge8 positive cells inside of the foci (Figure 28F).

Enhanced activation of LTβR-mediated alternative NF-κB signaling during *H. pylori* infection led to a strong inflammatory immune response. Formerly described results obtained from *in vitro* experimental approaches provided evidence for *H. pylori*-stimulated upregulation of a broader chemokine response in the context of blocked canonical NF-κB signaling. In addition, LIGHT, the alternative ligand for the LTβR was increased in *H. pylori* positive human gastritis tissue when compared to pathogen-negative inflammatory biopsy samples. Therefore, as a final proof of principle that colonization of *H. pylori* can exacerbate gastric pathology, cytokines and chemokines were analyzed in *H. pylori* positive ACH6-injected mice.

Remarkably, increased levels of the chemokine CXCL13 could be detected in H. pylori positive ACH6-treated mice when compared to *H. pylori* positive control mice (Figure 29). CXCL13 is a B cell chemoattractant specifically controlled by LTβR signaling. Interestingly, the infection alone did not seem to be sufficient to induce the high influx of B cells, but active alternative NF-κB signaling via the stimulated LTβR must be provided. Remarkably, also LIGHT was observed to

be significantly up-regulated during chronic *H. pylori* infection, when receptor signaling was triggered by ACH6, confirming the important role of this ligand in *H. pylori* pathology.

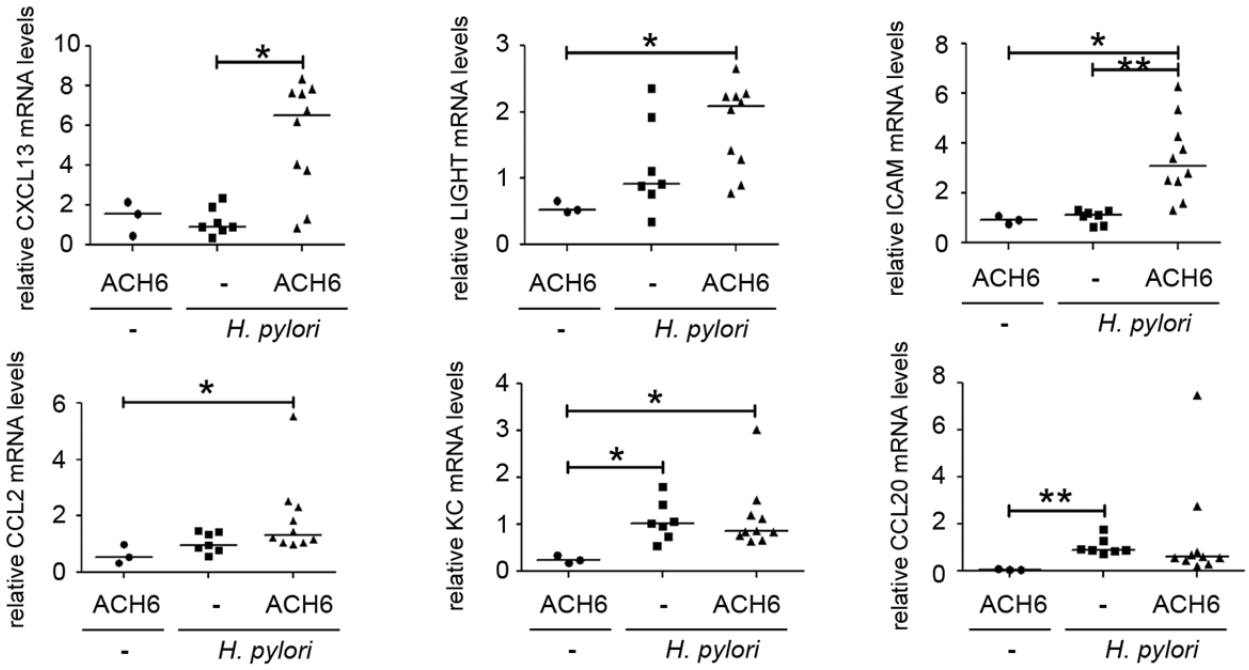

Figure 29: Stimulated LTβR signaling during *H. pylori* infection broadens the cytokine milieu.
Relative mRNA expression levels of CXCL13, LIGHT, ICAM, CCL2, KC and CCL20 in the stomach of control, *H. pylori*-infected mice and *H. pylori*-infected mice and treated with ACH6. Ct values were normalized to GADPH. Statistical significance was calculated by using One-way ANOVA Kruskal-Wallis with Dunn's multiple comparison test. *p≤0.05, **p≤0.01.

In addition other NF-κB target genes involved in leukocyte trafficking (ICAM) and inflammatory cell recruitment (CCL2) were increased. Interestingly, the cytokines CCL20 and KC that were down-regulated when LTβR signaling was blocked showed no further increase during enhanced LTβR signaling in *H. pylori* positive mice. These results suggest that CCL20 and KC are not important for the LTβR-mediated exacerbation of *H. pylori*-induced pathology.

Together, these results indicate that enhanced activation of alternative NF-κB signaling via the LTβR is an important driver in aggravating *H. pylori*-induced gastric pathology and might further be used by the bacterium to keep the inflammation ongoing.

6 Discussion

Chronic *H. pylori* colonization of the gastric mucosa can cause chronic active gastritis, an inflammatory response to the pathogen that – if untreated – results in tissue damage and might progress to gastric precancerous lesions and gastric cancer. One of the major signaling pathways activated during *H. pylori* infection is the canonical NF-κB signaling pathway. *H. pylori*-triggered early activation of this pro-inflammatory signaling pathway and the underlying molecular signaling events leading to gastric carcinogenesis were and still are the focus of intense research (Glocker et al., 1998; Lamb et al., 2009; Li et al., 2014; Seo et al., 2013; Sharma et al., 1998). The alternative NF-κB signaling pathway which gets activated via the ligands $LT\alpha_1\beta_2$, LIGHT, CD40L, TWEAK and BAFF (Dejardin et al., 2002) is less well characterized in the context of *H. pylori* colonization. Both lymphoid neogenesis and formation of tertiary lymphoid organs (TLOs) have been observed during chronic *H. pylori* infection (Genta et al., 1993; Kobayashi et al., 2004; Shomer et al., 2003), and these histological phenotypes have been described to be triggered and maintained by LTβR-mediated alternative NF-κB-signaling in several other tissues (Drayton et al., 2006), including pancreas (Bistrup et al., 2004) and liver (Heikenwalder et al., 2005). However, definitive proof for a linkage between chronic *H. pylori* infection and activation of this signaling pathway is still missing. The alternative NF-κB-specific target gene CXCL13 was shown to be highly upregulated in biopsy samples of *H. pylori*-infected patients when compared to *H. pylori* negative biopsies (Galamb et al., 2008; Nakashima et al., 2011), supporting an involvement of LTβR-stimulated alternative NF-κB signaling in the inflammatory response to chronic *H. pylori* infection. NIK is a key downstream molecule of LTβR signaling which was shown to be involved in *H. pylori*-induced NF-κB activation in gastric epithelial cells (Maeda et al., 2000; Neumann et al., 2006). However, further downstream p100/p52 processing during *H. pylori* infection, an indicator of active/ongoing alternative NF-κB signaling, was so far only reported in lymphocytes (Ohmae et al., 2005). Ohmae and colleagues

could show p100 processing to p52 specifically in B cells whereas testing of the gastric cancer cell line AGS was negative for processing. In addition, *H. pylori*-infected human gastric samples displayed weak cytoplasmic staining which was more pronounced in infiltrating lymphocytes thus leading to the conclusion that alternative NF-κB signaling was provided by lymphocytes instead of gastric epithelial cells (Ohmae et al., 2005). The study here, provides proof for the activation of the alternative NF-κB pathway in immune cells but also gastric epithelial cells in *H. pylori*-induced gastric pathology of patient samples (visualized by RelB translocation into the nucleus, p100/p52 processing and specific target genes e.g. CXCL13 and LIGHT *in vivo* and *in vitro*).

LTβR is expressed on stromal and parenchymal cells and was shown in this study to be expressed on gastric epithelial cells independent of gastric pathology. The LT system (LTβR and ligands LTα1β2 and LIGHT) is essential for maintenance of the lymphoid microenvironment (Gommerman & Browning, 2003; Ware, 2005). To gain a better understanding of the impact of *H. pylori* infection on epithelial cells in the context of alternative NF-κB signaling, mice were infected with a human pathogenic *H. pylori* strain and treated with LTβR-Ig, a murine receptor-human IgG1 fusion protein which scavenges the ligands of the LT system and therefore inhibits LTβR signaling. Notably, whereas LT expression was affected by the bacterial infection blocking of LTβR signaling did not alter expression levels, confirming the obtained *in vitro* data that LT expression is controlled independently of LTβR-mediated alternative NF-κB signaling. On the other hand, *H. pylori* infected mice exhibited a reduced number of lymphocyte infiltrates within gastric tissue when LTβR-mediated NF-κB signaling was inhibited, resulting in increased bacterial burden and reduced gastritis. However, it has to be noted that administration of LTβR-Ig acts systemically and therefore likely influences the secondary lymphoid organs (i.e. spleen and lymph nodes) which are the main sites for the priming of a *Helicobacter*-specific T cell response. Thus, induction of a functional T_H1 polarization of responding T cells in the gut-draining mesenteric lymph nodes (Toller et al., 2010), which is required for pathogen control, might be altered upon systemic LTβR-Ig administration. To confirm that the observations made

in LTβR-treated *H .pylori*-infected mice were specifically related to a disruption in *H. pylori*-induced regulation of the LT system and not due to secondary effects of LTβR-Ig treatment on the secondary lymphoid organs, a second mouse model approach was set up by using the agonistic LTβR antibody ACH6, which specifically triggers downstream LTβR signaling, but does not affect the lymph nodes (Lukashev et al., 2006). Analyses of ACH6-treated *H. pylori*-positive mice showed a completely reversed phenotype, including reduced bacterial burden and increased gastric inflammation, supporting the hypothesis of a direct correlation between the *H. pylori* infection and induction of the LT system. TLO-like structures observed in ACH6-treated pathogen-infected mice further supported a specific effect of alternative NF-κB signaling in response to the microbe, since FDCs were not observed in *H. pylori* infected control mice. TLOs have already been described during *H. pylori* infection (Genta et al., 1993; Kobayashi et al., 2004; Shomer et al., 2003), but final identification in the mouse model at hand needs to be provided since characterization of TLOs include additionally to FDCs, DCs as antigen-presenting cells, compartmentalization of adaptive immune cells, stromal cells (Stranford & Ruddle, 2012) and a highly organized vascular system of HEVs and lymphatic vessels (Link et al., 2011; Penaranda et al., 2010).

Former use of transgenic mouse models already pointed at an involvement of alternative NF-κB signaling in gastric pathology. Ishikawa and colleagues could show that mice which lack the terminal ankyrin domain of NF-κB2 and constitutively express NF-κB2/p52 develop gastric hyperplasia without *Helicobacter* colonization present (Ishikawa, H. et al., 1997). In line with this, analyses on *Nfkb2*$^{-/-}$ mice demonstrated the requirement of NF-κB2-mediated signaling in *H. felis*-induced gastric pathology (Burkitt et al., 2013). In the study presented here, evidence for *H. pylori*-mediated activation of the alternative NF-κB signaling pathway and the effect on gastric pathology could be provided by an *H. pylori* mouse model as well as with *in vitro* analyses confirming the involvement of this signaling pathway in *H. pylori* pathology. In addition to the results obtained from the mouse model, this study provides *ex vivo* proof of alternative NF-κB

signaling in epithelial cells during chronic *H. pylori* infection. Analyses of human gastric patient samples in progressing stages of *H. pylori*-induced pathology corroborated the important role of *H. pylori* infection and following activation of alternative NF-κB signaling in both immune cells and epithelial cells.

The intensive crosstalk between the canonical and alternative NF-κB signaling pathways is not yet fully understood. However, crosstalk at key points of both signaling pathways were already described, e.g. p100 or LTβ expression which are both controlled by canonical NF-κB (de Wit et al., 1998; Kuprash et al., 1996; Subrata et al., 2012; Voon et al., 2004), but are essential for the execution of alternative NF-κB signaling. In line with these observations, *H. pylori*-induced enhanced expression of p100 and ligands LTα and LTβ could be reduced via blocking of canonical NF-κB activation. However, p100 processing could still be detected, which indicates that p100 overexpression alone is insufficient for processing, as was suggested by a previous study in various mammalian cell lines (Xiao et al., 2001). Processing of p100 in a canonical NF-κB-independent manner implies the involvement of another LTβR ligand capable of activating the alternative NF-κB signaling pathway. Besides LTs, the only LTβR ligand identified to be able to activate this signaling pathway so far is LIGHT. Indeed, cell culture experiments revealed increased levels of LIGHT when canonical NF-κB was blocked through administration of the IKKb-inhibitor TPCA-1, whereas treatment with LTβR-Ig and therefore scavenging of all LT system ligands (LIGHT, LTα and LTβ) fully disabled active alternative NF-κB signaling. These results state a necessary interaction of canonical and alternative signaling during *H. pylori* infection and pathology. Furthermore, LT up-regulation and p100 expression provided by canonical NF-κB with subsequent activation of alternative NF-κB signaling indicates a secondary loop of alternative NF-κB activation upon *H. pylori* infection.

When analyzing the impact of *H. pylori* virulence factors in activation of alternative NF-κB signaling, the T4SS was observed to be essential for canonical NF-κB-triggered LT expression

and subsequent LTβR downstream signaling. In contrast, the CagA protein itself, which was described to be an important contributor to the inflammatory response (Sokolova et al., 2014) was found to be dispensable. These results are in agreement with earlier studies demonstrating that CagA-deficient *H. pylori* strains with a functional T4SS stimulate NF-κB activation almost as efficiently as wild type bacteria (Schweitzer et al., 2010; Sokolova et al., 2013). However, the exact mechanism of T4SS-dependent NF-κB activation still needs to be elucidated (Backert & Naumann, 2010). The CagA protein is present in close to 100% of East Asian *H. pylori* stains and around 60-70% in Western stains (Alm et al., 1999; Censini et al., 1996; Tomb et al., 1997). The CagA protein in the individual *H. pylori* strain was demonstrated to correlate with more severe gastric pathology in Western countries (Shiota et al., 2013a; Shiota et al., 2013b). Yet, the data provided in this thesis show that CagA-negative *H. pylori* isolates are still capable of initiating pro-inflammatory signaling pathways like alternative NF-κB which regulate gastric inflammatory cell influx and subsequent pathology. The provided *ex vivo*, *in vivo* and *in vitro* data in the thesis at hand indicate that both NF-κB pathways are activated in the course of *H. pylori* infection. *H. pylori* activated canonical NF-κB signaling precedes alternative NF-κB signaling, p65/p50 translocation and target gene expression, thereby controlling the induction of a feed-forward loop through LT expression, which is primarily provided by gastric epithelial cells (Figure 30, top panel). Upon ligand engagement to LTβR on epithelial cells, p100/p52 processing is induced followed by subsequent activation of chemokine expression important for immune cell recruitment (CXCL13, CXCL10 and CCL20) and further stimulation of LTβR signaling (LIGHT) (Figure 30, middle panel). Gastric influx of inflammatory cells ($CD4^+$ T cells and later B cells), which in turn also express LTs and LIGHT leads to a feed-forward loop thereby maintaining and reinforcing gastric inflammation (Figure 30, lower panel). This is confirmed by the observations made in human gastric tissue, where expression of LTs and target genes is linked to the presence of *H. pylori* and correlates with the inflammatory score of the lesions.

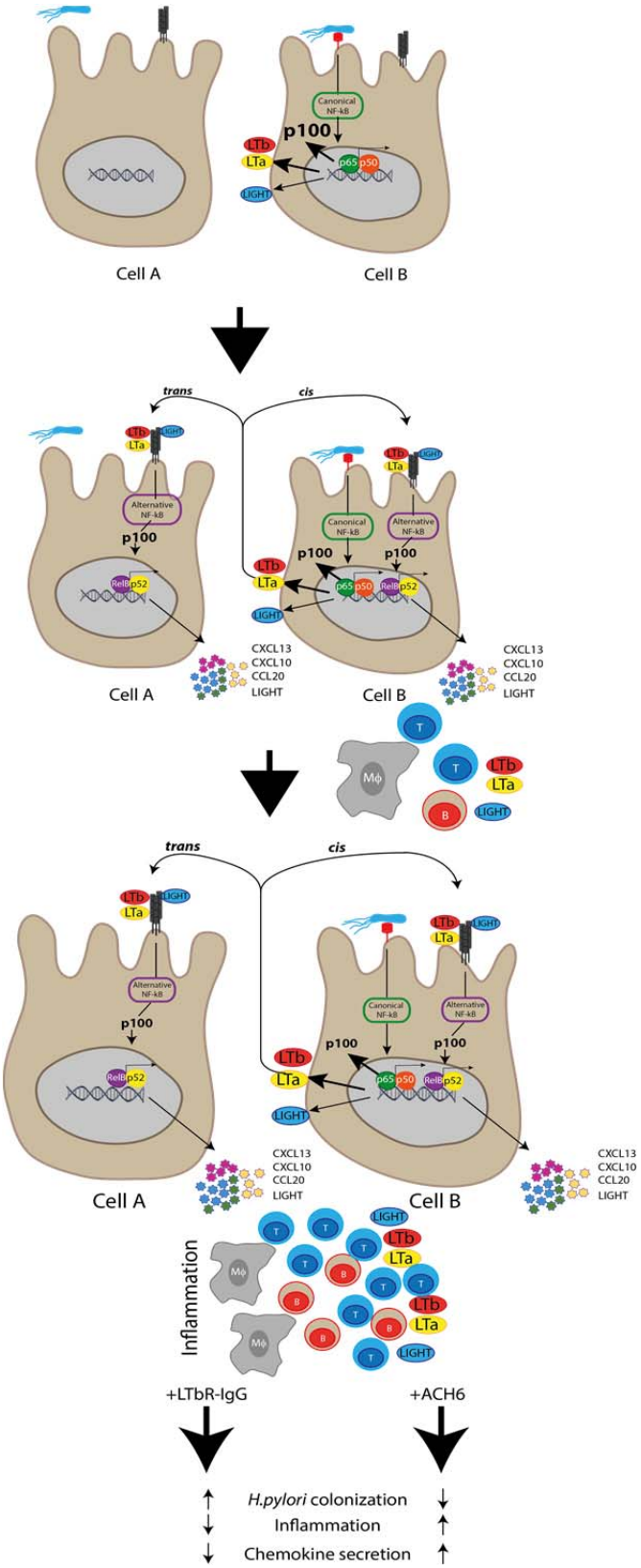

Figure 30: Schematic model of *H. pylori*-induced gastric pathology exacerbated by both early canonical and persistent alternative NF-κB signaling.

Inhibition of canonical NF-κB has been suggested as a therapeutic strategy to reduce *H. pylori*-induced gastric inflammation. *In vitro* studies showed reduction in NF-κB activation and IL-8 secretion in gastric epithelial cells by natural substances as capsaicin, which is the main pungent ingredient of chilli pepper and hot red chillies (Lee et al., 2007), antiulcer drugs as ecabet sodium (Kim et al., 2003) or probiotics (Yang et al., 2012).

Furthermore, when canonical IKKβ was inhibited in an animal model of *Mongolian gerbils* gastric infiltration of neutrophils and mononuclear cells was reduced which otherwise had no effect on bacterial burden (Yanai et al., 2008). This study implies an insufficient control of infection by inhibition of canonical NF-κB signaling alone. Based on the finding that inhibition of the canonical NF-κB signaling pathway may on the one hand reduce expression of LTs yet on the other hand may induce a compensatory up-regulation of LIGHT upon *H. pylori* challenge, it is tempting to speculate that sole blocking of canonical NF-κB signaling might not be a sufficient therapeutic option to counteract gastric inflammation and ameliorate pathology induced by the pathogen. Subsequent effects following *H. pylori*-stimulated blocking of canonical NF-κB signaling, e.g. LIGHT-mediated LTβR signaling activation leading to exacerbation of the inflammatory response through enhanced expression of target genes such as B-cell chemoattractant CXCL13 should be therefore considered in future as therapeutic approaches against the pathogen.

The confirmation that blocking of the canonical NF-κB signaling pathway with resulting loss of LT expression is compensated by the pathogen through upregulation of LIGHT supports the hypothesis that alternative pathway signaling is an important trigger of progressive inflammation which may facilitate bacterial survival.

Since this study provides significant evidence that blocking of LTβR signaling during *H. pylori* infection in mice highly reduced gastric inflammation and infiltration of inflammatory cells to the stomach, additional blocking of alternative NF-κB should be considered in supplement to a preceding antibiotic treatment or in combination with canonical NF-κB inhibitors. This is

supported by observations that - once alternative signaling is turned on, it can potentially be maintained even in the absence of *H. pylori* or further stimulation of canonical NF-κB through persistence of inflammatory cells within self-sustaining TLOs.

In conclusion, the findings provided in this thesis describe a novel role for LTβR signaling as an executor of the immune response to *H. pylori* infection. The data indicate that activated alternative NF-κB signaling contributes to the persistence and exacerbation of gastric inflammation and development of gastric lesions and suggest that it might be considered as a potential therapeutic target - maybe as treatment in combination with additional drugs - for the management of gastric pathology associated with *H. pylori* infection

Further work however will be needed to address how *H. pylori* regulates the crosstalk between canonical and alternative NF-κB signaling, including the question as to how the pathogen switches from LT-driven to LIGHT-driven signaling in later stages of gastric pathology. This switch in the usage of LTβR ligands was evident in human *ex vivo* samples, the mouse model with strongly stimulated LTβR signaling and *in vitro* data with *H. pylori* infected gastric cancer cell lines where specific pathogen-mediated upregulation of LIGHT was observed after canonical NF- κB blocking.

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