TECHNISCHE UNIVERSITÄT MÜNCHEN

Fakultät für Medizin

Institut für Medizinische Mikrobiologie, Immunologie und Hygiene

The diverse roles of NLRP3 and NLRC4 inflammasomes during *Helicobacter pylori* infection

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Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. Radu Roland Rad

Prüfer der Dissertation:

1. Univ.-Prof. Dr. Markus Gerhard

2. apl. Prof. Dr. Thilo M. Fuchs

Die Dissertation wurde am 15.03.2016 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 06.07.2016 angenommen.

Parts of this thesis have already been published or manuscript is in preparation:

***Helicobacter pylori*-Induced IL-1beta Secretion in Innate Immune Cells Is Regulated by the NLRP3 Inflammasome and Requires the Cag Pathogenicity Island**

Semper, R. P., Mejias-Luque, R., Gross, C., Anderl, F., Muller, A., Vieth, M., Busch, D.H., Prazeres da Costa, C., Ruland, J., Gross, O., Gerhard, M.

Journal of Immunology *193*, 3566-3576, 2014.[1]

***Helicobacter pylori* exploits the Nlrc4 inflammasome to dampen host defenses**

Semper, R. P., Mejias-Luque, R., Vieth M., Gerhard, M.

In preparation

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Abstract

Infection with *Helicobacter pylori* is one of the most prevalent bacterial infections worldwide, affecting 50 % of the world's population. Although an immune response against *H. pylori* is triggered, the bacterium is not eliminated. The coevolution of *H. pylori* and humans has driven *H. pylori* to develop evasion mechanisms by escaping and manipulating immune responses, making them ineffective in eliminating the bacterium. However, the long-term presence of this inflammatory milieu in the stomach may predispose gastric cells to become cancerous. Although *H. pylori* infection does not cause disease in most infected people, it is the main risk factor for gastric cancer. The pro-inflammatory cytokine IL-1β plays a crucial role in the development of gastric tumours and polymorphisms in the *IL-1* gene cluster leading to increased IL-1β production have been associated with increased risk for gastric cancer. IL-1β is expressed as inactive pro-form. To be active, pro–IL-1β must be cleaved by the inflammasome, an intracellular multiprotein complex involved in physiological and pathological inflammation. In the present study, it was shown that *H. pylori* activates the inflammasome in different cells, such as dendritic cells, macrophages and neutrophils, as well as epithelial cells. Also, different inflammasome types, bacterial virulence factors, and molecular mechanisms which regulate the *H. pylori*-induced IL-1β secretion could be identified. *In vivo* experiments in mice showed that different inflammasome types are involved in the *H. pylori*-induced gastric IL-1β production. The activation of the inflammasome was important for the induction of an adaptive immune response, which contributed to control the bacterium. On the other hand, other immune responses were suppressed in an inflammasome-dependent manner which prevented the clearing of the bacterium but resulted in inflammation. By activating different inflammasome types in different cell types, *H. pylori* controls IL-1β production and takes advantage of the beneficial properties of IL-1β such as enabling corpus colonisation, while inhibiting negative host responses. Thus, the regulation of the inflammasome possibly represents a step by which *H. pylori* manages to influence immune responses so that it prevents its clearing, and enables its chronic colonisation of the

stomach. However, this chronic inflammation and persistent production of IL-1β can cause stomach pathologies.

Zusammenfassung

Eine Infektion mit *H. pylori* ist eine der häufigsten bakteriellen Infektionen. Etwa 50% der Weltbevölkerung sind mit diesem Magenkeim infiziert. Obwohl eine Immunreaktion gegen *H. pylori* induziert wird, ist diese nicht in der Lage das Bakterium zu entfernen. Die Ko-Evolution von *H. pylori* und dem Menschen führte dazu, dass *H. pylori* Mechanismen entwickelte die Immunreaktion zu manipulieren und dieser zu entgehen. Die Jahrzehnte lange Besiedelung des Magens mit *H. pylori* und die dadurch resultierende chronische Entzündung des Magens, macht die Zellen anfällig zu entarten. Auch wenn die Mehrheit der *H. pylori*-infizierten Personen trotz jahrelanger Besiedelung keine Erkrankungen entwickelt, eine Minderheit (<1%) entwickelt Magenkrebs. Das pro-inflammatorische Zytokine IL-1β spielt eine entscheidende Rolle bei der Magenkrebsenwicklung. So sind beispielsweise Polymorphismen die zu einer erhöhten Menge an IL-1β führen mit einem höheren Risiko Magenkrebs zu entwickeln verbunden. IL-1β wird als eine inaktive Vorform gebildet. Um funktionell zu sein, muss es gespalten werden. Die Spaltung wird meist durch einen Multiproteinkomplex, genannt Inflammasom, durchgeführt. Dieses ist an zahlreichen physiologischen und pathologischen Entzündungen beteiligt. Ob *H. pylori* das Inflammasom aktiviert war bislang unbekannt. In der vorliegenden Studie konnte gezeigt werden, dass *H. pylori* das Inflammasom in unterschiedlichen Zellen, wie Dendritische Zellen, Makrophagen und Neutrophilen, aber auch Epithelzellen aktiviert. Auch konnten unterschiedliche Inflammasomtypen, bakterielle Virulenzfaktoren sowie erstmalig molekulare Mechanismen identifiziert werden, welche die *H. pylori*-induzierte IL-1β Sekretion regulieren. *In vivo* Experimente in Mäusen zeigten, dass bei der *H. pylori*-induzierte IL-1β Produktion im Magen unterschiedliche Inflammasomtypen beteiligt sind. Die Aktivierung des Inflammasoms war einerseits wichtig für die Induktion einer adaptiven Immunreaktion, welche zur Entzündung und Bekämpfung des Bakteriums beitrug,

andererseits wurden Inflammasom-abhängig andere Immunreaktionen unterdrückt. Durch die Aktivierung unterschiedlicher Inflammasomtypen in unterschiedlichen Zelltypen schafft es *H. pylori* die IL-1β Produktion so fein zu steuern, dass es die positiven Eigenschaften von IL-1β für sich nutzen kann die Besiedelung auszuweiten, während es die negativen Folgen begrenzen kann. Somit stellt die Regulation des Inflammasoms möglicherweise einen Schritt dar, durch den *H. pylori* es schafft Immunreaktionen so zu beeinflussen, dass es seine Beseitigung verhindert. Allerdings kann durch diese chronische Entzündung und andauernde Produktion von IL-1β zur Entwicklungen von Pathologien wie Magenkrebs kommen.

Abbreviations

AIM2	Absent in melanoma 2
APC	Antigen presenting cell
APC	Allophycocyanin
APS	Ammonium persulfate
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BabA	blood group antigen binding adhesin
BMDC	bone marrow derived dendritic cell
BMDM	bone marrow derived macrophage
BSA	Bovine serum albumin
C2TA	MHC class 2 transcription activator
Cag	Cytotoxin-associated gene
CagPAI	Cag Pathogenicity Island
CARD	caspase recruitment domain
CD	*cluster of differentiation*
CFU	Colony forming units
CT	Cholera toxin
DAMPs	Damage Associated Molecular Patterns
DC	Dendritic cells
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethylene glycol tetra acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	forkhead box P3
gGT	γ-Glutamyltranspeptidase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HET-E	incompatibility locus protein from Podospora anserine
HRP	Horseradish peroxidase
IFN	Interferons
Ig	Immunoglobulin
IL	Interleukin
kDa	kilo Dalton
Knockout	Knockout
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MALT	Mucosa Associated Lymphoid Tissue
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MOI	Multiplicity of infection
NACHT	acronym for NAIP, C2TA HET-E and TP1 (telomerase-associated protein)

NAIP	NLR family, apoptosis inhibitory protein
NAP	Neutrophil-activating protein
NBD	Nucleotide binding domain
NLR	Nucleotide binding domain (NBD) and leucine rich repeat (LRR) containing NOD-like receptors
NLRC	NLR family, CARD domain containing
NLRP	NLR family, pyrin domain containing
NLRX	NLR family member X
NOD	nucleotide-binding oligomerization domain containing
PAMPs	Pathogen-associated molecular patterns
ON	Over night
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
PMN	polymorphonuclear cell
PMSS1	Pre-mouse Sydney strain 1
PRR	Pattern-recognition receptor
PVDF	Polyvinylidene fluoride
RNA	ribonucleic acid
ROS	Reactive oxygen species
Rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SabA	Sialic acid binding adhesion
SD	Standard deviation
SS1	Sydney strain 1

Syk	Spleen tyrosine kinase
T3/4SS	Type 3/4 secretion system
TGF	Transforming growth factor
T_H cell	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TP1	telomerase-associated protein
T_{reg} cell	Regulatory T cell
Ure	Urease
VacA	Vacuolating cytotoxin A
v/v	Volume per volume
Wt	Wild type
w/v	Weight per volume

1 Introduction

1.1 *Helicobacter pylori*

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic bacterium with curved to spiral shape, which selectively colonises the human stomach.

The presence of spiral-shaped microorganisms in human stomach mucosa was described in men more than 100 years ago by W. Jaworski (1). He already believed that these bacteria might play a pathogenic role in gastric diseases. In the following years, several reports described spirochetes in human stomachs with ulcers or carcinomas (2-4). The major problem to demonstrate the role of these spiral bacteria in human gastric pathology was the persistent failure to culture these organisms *in vitro*. Finally, in 1982, Warren and Marshall were successful. They isolated spirochetes from gastric biopsies from patients with chronic gastritis and peptic ulcers, and could cultivate these spirochetes in pure culture *in vitro* (5, 6). To fulfil the third Koch's postulate, Marshall himself ingested a bacterial culture and developed gastritis. Thereafter, the bacterium was reisolated from his biopsy (5). Thus, all four Koch's postulates were fulfilled in 1984.

Initially, this bacterium was called *Campylobacter pyloridis*, and subsequently re-named *Campylobacter pylori*, because it is mainly found near the pylorus of the stomach. Later, the bacterium was characterised by 16S ribosomal ribonucleic acid (rRNA) analysis and classified as *Helicobacter pylori* (*H. pylori*) (*helico* [gr.]: spiral shaped) (7).

1.1.1 Epidemiology of *H. pylori*

H. pylori infection is one of the most prevalent infections worldwide. More than half of the global population is infected with this bacterium (8). The prevalence of *H. pylori* is highly variable in relation to geography, ethnicity, age and socioeconomic status especially during childhood (9, 10). Thus, over 90 % of the population in

developing countries are infected, whereas in industrialized counties the prevalence is steadily decreasing and ranges from 20 to 50 % (11, 12).

1.1.2 Transmission of *H. pylori*

Infection with *H. pylori* mainly occurs in early childhood. The mode of transmission is not completely clear. Person-to-person transmission of *H. pylori* through oral/oral or faecal/oral exposure seems most likely. Intrafamilial clustering of infection with genetically identical strains (13, 14) and presence of *H. pylori* in drinking water (15) support this theory. On the basis of these likely transmission routes, *H. pylori*-infected family members, low socioeconomic status, density of housing, number of siblings, and lack of running water – often found in developing countries – have been linked with higher infection rates (16). The improvement of hygienic standards has contributed to decrease its prevalence in many countries in the recent decades.

1.1.3 *Helicobacter pylori*-induced pathology

Following infection with *H. pylori,* a transient acute gastritis with infiltration of polymorphonuclear cells occurs in all individuals. Only in a minority of people, particularly in childhood, the bacterium may be spontaneously cleared and the gastric mucosa recovers (17). In the majority of infected persons, the host immune response fails to clear the infection and a chronic active gastritis develops. Most infected individuals remain clinically asymptomatic despite lifelong persistence of *H. pylori* infection. However, after decades of chronic gastric inflammation some infected persons develop disease. In around 10-15 % of them, the infection leads to peptic ulceration, which can affect the stomach or the duodenum depending on the location of the inflammation, while 1-2 % develop gastric adenocarcinomas. The combination of bacterial, host, and environmental factors influences the outcome of infection. Further, the clinical outcome depends on the distribution pattern of *H. pylori*-induced inflammation. An antrum-predominant gastritis results in increased acid production and consequently increased acid load in the proximal part of the duodenum (18). This may lead to gastric metaplasia, the replacement of duodenal cells with gastric epithelial cells. This gastric metaplastic tissue might be colonised by *H. pylori.* The subsequent inflammation of the small intestine can lead

to the development of duodenal ulcer (19). In contrast, a corpus-predominant gastritis is associated with reduced acid secretion (20) due to the loss of acid-secreting cells (atrophy). The acid-secreting parietal cells are replaced with mucus-secreting cells, pyloric-type glands, and fibrous tissue. Proliferation of the mucous glands with an intestinal phenotype leads to intestinal metaplasia, which can progress to dysplasia. This lesion is characterised by cellular atypia like nuclear pleomorphism, abnormal cell development, growth and differentiation, and abnormal distribution of glands and crypts. Intestinal metaplasia and dysplasia are considered as early precursor lesions that can develop to gastric adenocarcinoma (21). This precancerous cascade of histologic changes from atrophy to adenocarcinoma of the intestinal type is known as Correa´s cascade. Since the infection with *H. pylori* increases the risk of adenocarcinomas, the pathogen was classified as a Class-I carcinogen by the World Health organisation in 1994 (22). Furthermore, *H. pylori* is known to be involved in the development of mucosa-associated lymphoid tissue (MALT) lymphoma (23), a malignant transformation of lymphocytes. Although the causal relationship between *H. pylori* infection and gastric MALT lymphoma is well established, the exact mechanisms how *H. pylori* triggers the development of MALT lymphoma is not fully understood. In general, MALT lymphomas are B cell lymphomas that arise from lymphoid aggregates in the lamina propria as part of an adaptive immune response to a chronic inflammatory stimulus. Here, the infectious agent does not directly transform lymphoid cells but is a constant source of antigen stimulation. Sagaert et al. proposed a model, in which *H. pylori* infection leads to the recruitment of T and B cells, and neutrophils into the stomach, where *H. pylori* triggers sustained lymphoid proliferation. This permanent stimulation and proliferation of these cells, as well as neutrophil-mediated release of reactive oxygen species (ROS) may then induce oncogenic events like activation of intracellular survival pathways, and resistance to apoptosis, which leads to the progression of the lymphoma (24).

1.1.4 Pathogenicity and virulence factors of *H. pylori*

In general, human surfaces are protected by epithelia. Epithelial cells constitute barrier surfaces that separate mammalian hosts from the external environment. Infections can only occur when pathogens can cross or colonise this barrier. For protection against colonisation, the gastric mucosa - like other mucosal epithelia - secretes mucus. Microorganisms stuck in the mucus are prevented from adhering to the epithelium, and are expelled by the mucus flow. In the stomach, mucus serves additionally as a shield to the acid, and protects the gastric mucosa from autodigestion. The gastric mucosa is more than just a physical barrier; it also produces antimicrobial substances. In particular, the acid pH of the stomach and the digestive enzymes serve as a chemical barrier against infections.

In order to inhabit the hostile environment of the human stomach and to avoid to be cleared by the immune system, *H. pylori* has developed several pathogenicity and virulence factors by which it breaks through the defence barrier of the stomach and evades the immune system. Its pathogenicity and virulence factors not only help the bacterium to colonise the stomach but they also contribute to gastric inflammation and pathology.

Although infection with *H. pylori* usually results in chronic active gastritis, most infected patients do not develop clinical symptoms. This suggests that some *H. pylori* strains may be more virulent than others. Several epidemiological and animal studies show a strong correlation between the development of ulcers and adenocarcinomas, and the presence of specific virulence factors of *H. pylori* (25-27). The most well characterized virulence factors Cag pathogenicity island (CagPAI), vacuolating cytotoxin A (VacA) and the γ-glutamyltranspeptidase (gGT) will be described in the following sections.

1.1.4.1 Bacterial factors that facilitate gastric colonisation

Upon transmission, *H. pylori* has to reach its unique ecological niche in the human stomach. How *H. pylori* archives and survives in the hostile gastric environment will be discussed in the following sections.

1.1.4.1.1 Urease

The first challenge for *H. pylori* is to cross the acidic gastric lumen to reach the more neutral mucus layer and the underlying gastric epithelial cells. Therefore, one key factor for the survival of *H. pylori* in the acidic gastric lumen is the production of urease. The enzyme urease consists of two subunits, UreA and UreB. Urease is a cytoplasmic enzyme, but due to cell lysis it is also found on the surface of the bacterium (28). Urease catalyses the hydrolysis of urea (CH_4N_2O) to ammonia (NH_3) and carbon dioxide (CO_2). NH_3 and CO_2 diffuse into the periplasm, where NH_3 prevents the accumulation of protons by consuming a proton and generating NH_4^+. CO_2 is hydrated to H_2CO_3 by the periplasmic enzyme alpha-carbonic anhydrase. Then H_2CO_3 dissociates to H^+ and HCO_3^- (29). This H^+ is again buffered by the urease activity. The external urease generates a cloud of ammonia around the bacterium. This neutral microenvironment protects the bacterium against the gastric acid.

The urease activity is regulated by the external pH. At acid pH UreI, a proton-gated urea channel in the cytoplasmic membrane, opens and urea diffuses into the cytoplasm (30). To avoid alkalisation, UreI is inactive at neutral pH. The urease activity is crucial for colonisation, since *H. pylori* mutants lacking urease failed to colonise the mouse stomach (31).

In addition to urease, *H. pylori* possesses other ammonium-producing enzymes, such as the γ-glutamyltranspeptidase (gGTs), two aminases AmiE and AmiF, which hydrolyse short-chain amides to ammonia and the corresponding organic acids (32, 33) and the arginase RocF, which hydrolyses L-arginine to L-ornithine and urea (34), which is then converted by urease to NH_3.

1.1.4.1.2 Flagella

The low gastric pH and the constant shedding of the mucus layer represent a continuous challenge for the bacterium to avoid being washed out by the mucus flow or being killed by the gastric acid. Therefore, as a next step, *Helicobacter pylori* has to move through the viscous mucus to get to the epithelium. With the help of its polar flagella and its spiral-shaped form, *H. pylori* can penetrate the viscous mucus to reach the epithelial layer. Thus, non-motile *H. pylori* strains are not able to colonise the stomach (35). For precise spatial orientation *H. pylori* uses the pH gradient in the mucus (36), but also chemotactic compounds such as amino acids, urea, sodium bicarbonate, and sodium and potassium chloride serve for orientation (37). The chemotactic motility of *H. pylori* has been shown to depend on proton motive force. It is proposed that chemotactic movement toward urea may supply the substrate and then the urease-driven hydrolysis of intracellular urea may provide the proton motive force (38, 39).

Moreover, the motility of the bacteria also influences the inflammatory response, since non-motile, coccoid bacteria cause lower IL-8 secretion by gastric epithelial cells compared to motile, bacillary *H. pylori* (40). In general, flagellin is a pathogen-associated molecular pattern (PAMP) and is recognised by receptors of the innate immune system (see section 1.1.6). Further, it serves as a trigger for NLRC4 inflammasome activation (see section 1.2.2).

1.1.4.1.3 Adhesion molecules

Another essential factor enabling colonisation of the stomach is the adhesion to the epithelial surface. Although most *H. pylori* appears to be free-living within the mucus, approximately 20 % of *H. pylori* in the stomach are found adhered to the surfaces of mucus epithelial cells (41). The adherence to the gastric mucosa is assumed to play a substantial role in initial colonisation and long-term persistence in the human stomach. Upon infection, the bacterial binding occurs first via the neutrophil-activating protein (Nap) to mucins, which cover the epithelium and generally prevents microbial colonisation (42, 43). This binding causes degradation of mucins and leads to decreased mucosal viscosity. After penetrating the mucin layer *H. pylori* can adhere to gastric epithelial cells by specific adhesins: such as

sialic acid binding adhesin (SabA) (44), *Helicobacter pylori* adhesin A (HpaA) and Nap, which bind to sialic acids like sialyl-Lewis x or a (45). In contrast, blood group antigen binding adhesin (BabA) binds specifically to Lewis b blood group antigen (46). *Helicobacter* outer membrane porin Q (HopQ) is the specific interaction partner of CEACAMs (47). In addition, other surface-exposed components of *H. pylori* such as adherence associated lipoproteins A and B (AlpA and B) (48) and *Helicobacter* outer membrane porin Z (HopZ) (49) have been shown to be involved in adherence. The exact host receptors of these adhesins are not identified yet.

At the molecular level, adhesins mediate adherence to gastric epithelial cells, but they can further influence the course of disease through aggravation of inflammatory responses in the stomach. Thus, BabA-positive strains induce stronger inflammation in the mucosa (50, 51), and they are associated with increased risk for gastritis, ulcer disease, gastric cancer, and MALT lymphoma (52). Besides its binding to epithelial cells, SabA also binds the sialylated carbohydrates on granulocytes and induces an oxidative burst in these cells, which contributes to inflammation (53).

1.1.4.2 Cytotoxin-associated gene A (CagA) und Cytotoxin-associated pathogenicity island (CagPAI)

The cytotoxin-associated pathogenicity island (CagPAI) is a 40 kb gene locus, which contains approximately 30 genes. Most likely, it was acquired by horizontal gene transfer (27). The CagPAI codes for the cytotoxin-associated gene A (CagA) and a type-4-secetion system (T4SS), which translocates bacterial products directly into the host cytoplasm. The only known factors, which are delivered by the *H. pylori* T4SS, are CagA (54) and peptidoglycan (55).

After translocation of CagA into the host cell, CagA becomes phosphorylated by the host cell Src-kinase (56), which triggers different cell signalling pathways. This results in cytoskeletal changes (hummingbird phenotype) (57) and cell spreading (58), dysregulation of cell growth (59), induction of cell proliferation by triggering transcription factors (60), reduced cell viability, and attenuated apoptosis (58). Further, CagA triggers loss of cell-cell contact (tight junctions) resulting in loss of barrier function (61). Furthermore, CagA-positive strains are linked with higher

levels of pro-inflammatory cytokines and IL-8 than CagA-negative strains (62). Therefore, it is not surprising that patients with severe gastric diseases including chronic active gastritis, peptic ulcer disease, MALT lymphoma and gastric cancer, are almost always infected with CagA-positive *H. pylori* strains (58, 63).

1.1.4.3 Vacuolating cytotoxin A (VacA)

VacA is a pore-forming cytotoxin produced as a large 140 kDa pre-polypeptide. Upon sequential proteolytic processing 88 kDa mature monomers are secreted. These monomers form hexamers. Although VacA is expressed by all *H. pylori* strains, there are different variances of the *vacA* gene, which differ in two regions of the gene: in the so called s allele in the N-terminal signal region, of which two allelic forms *s1* and *s2* occur; and in the allele m in the middle region of the *vacA* gene, which is present as *m1* and *m2* (26).

VacA is a multifunctional enzyme, which acts on different cell types and in different manners. VacA inhibits Ii-dependent antigen processing (64), phagosome maturation (65), and dendritic cell maturation (66). In T cells, VacA affects proliferation and activation by blocking IL-2 secretion and IL-2 receptor expression (67, 68). VacA induces cytochrome c release from mitochondria (69, 70) and, by the induction of caspase-3, apoptosis in epithelial cells (71). Moreover, VacA forms hexameric pores, which are selective to anions and small neutral molecules including urea. This urea permeability may be important to acid survival by providing a substrate for urease (72). Further, VacA leads to cell vacuolation and increase of trans-epithelial conductivity (73).

Through the short extension *vacA s2*-strains show slower and less effective pore formation (74), abolished vacuolating activity (75) and induction of apoptosis (71). Although *vacA m2* strains have a change in the binding region of the *vacA* gene, they can still bind to and vacuolate some cells (76).

H. pylori genotypes with strong vacuolating activity (*s1m1 vacA*) are usually associated with the presence of the *cagA* gene. Infection with these *cagA/s1m1vacA*-positive strains, also called type I strains, is associated with the

occurrence of ulcers or gastric cancer (77). In contrast, type II strains have only a weak vacuolating activity and do not possess a *cagA* gene (78, 79).

1.1.4.4 γ-Glutamyltranspeptidase (gGT)

γ-glutamyltranspeptidase (gGTs) is an enzyme which catalyses the transpeptidation and hydrolysis of γ-glutamyl moieties to peptides or water. gGT is synthesized as a proenzyme that is autocatalytically cleaved. Its subunits build heterodimers that form the active enzyme. Until now, the localization of this enzyme is not clear. Busiello et al. described the gGT as a secreted enzyme (80) whereas Shibayama et al. defined it as a periplasmic protein (81).

H. pylori gGT (HPgGT) has a strong hydrolysis activity with strong affinity to glutamine and glutathione. Thus, it converts glutamine to glutamate and ammonia, and glutathione to glutamate and cysteinylglycine (82). Since *H. pylori* cannot take up extracellular glutamine and glutathione directly, the main physiological role of gGT is to enable the bacterium to use extracellular glutamine and glutathione as sources of glutamate to incorporate it into the tricarboxylic acid cycle and to use it for glutamine biosynthesis (82). The ammonia produced is further used as nitrogen source for the bacterium and to buffer the gastric acid.

In addition, HPgGT induces the production of ROS, since the extracellular ammonia and the consumption of extracellular glutathione may alter the redox balance of the host's gastric mucosa. This can result in host DNA damage, increased cytokine production (83), cell cycle alterations, and cell death (81, 84).

HPgGT not only affects gastric epithelial cells but also immune cells. HPgGT inhibits dendritic cell (DC) maturation and contributes to DC tolerization and induction of regulatory T cells (66). In addition to indirect effects on T cells by reprogramming dendritic cells, HPgGT has also direct effects on T cells. Therefore, gGT works as an inhibitor of T cell proliferation by inducing a cell cycle arrest (85). Wüstner at al. could show that the inhibition of T cell proliferation is caused by glutamine deprivation due to the enzymatic activity of HPgGT (86). There are high glutamine levels in the plasma, however under conditions of inflammatory responses it

becomes limited. While resting T cells derive most of their energy from oxidative phosphorylation, upon stimulation T cells change their metabolic pathways to aerobic glycolysis and glutaminolysis to meet the growing demand of energy during proliferation (87, 88). In addition, glutamine is an important precursor of nucleotides, proteins, and for glutathione biosynthesis during this process (89, 90). Thus, it is not surprising that in the presence of HPgGT, T cells stop to proliferate due to the missing energy source and the metabolic precursor glutamine.

The effects of HPgGT – tolerization of DCs and inhibition of T cell mediated immunity – might represent the molecular basis of the decreased bacterial load in mice infected with HPgGT knockout strains (66, 91, 92). Although virtually all clinical isolates produce gGT, its activity differs. Isolates from patients with ulcer disease and gastric cancer display increased gGT activity (83, 93). Therefore, HPgGT might also be an important factor in the induction of gastric pathology.

1.1.5 Immune response against *H. pylori*

In general, the immune system protects against a wide range of agents using a variety of cellular mechanisms to recognise and kill pathogens or tumour cells. The two arms of the mammalian immune system consist of the innate and the adaptive immune response: The former response is a universal non-specific defence, which is fundamental for the immediate recruitment of immune cells to the site of infection but it does not confer protective and long-lasting immunity. The innate immune system recognises common structural features of pathogens. These so-called damage- or pathogen-associated molecular patterns (DAMPs or PAMPs) are recognised by pattern recognition receptors (PRR) which are expressed on innate immune cells like neutrophils, macrophages and dendritic cells but also on epithelial cells. In contrast, the cells of the adaptive immune system – B and T cells – generate a long-lasting specific immunity including immunological memory. Lymphocytes recognise, by their specialised B or T cell receptor, respectively, specific antigens (94).

1.1.5.1 Innate immune response

Multiple studies provided evidence that the gastric epithelium plays a key role in initiating the *H. pylori*-induced inflammation and immune response. Upon contact of *H. pylori* with the gastric epithelium NFκB expression is upregulated, which results in the production of pro-inflammatory cytokines, and chemokines such as IL-8. This leads to the infiltration of other immune cells (95). During the acute phase of inflammation neutrophils are one of the first cell types that migrate towards the site of infection. The main function of neutrophils is to engulf microorganisms by phagocytosis and to destroy them in intracellular vesicles using antimicrobial peptides and reactive oxygen species (ROS). During phagocytosis, the enzyme NADPH oxidase becomes activated. However, *H. pylori* disrupts NADPH oxidase targeting, which results in release of superoxide anions in the extracellular space instead of accumulation in phagosomes. Thereby, *H. pylori* evades phagocytic killing and the accumulation of superoxide anions contributes to gastric mucosa damage. Upon progressive damage of epithelial integrity, macrophages, which cannot traverse epithelial barrier, engulf invading *H. pylori*. Macrophages are actively phagocytic cells, that are recruited by *H. pylori*-derived products and signals from epithelial cells to the side of infection. However, *H. pylori* has developed mechanisms to avoid phagocytic killing by macrophages. Thus, *H. pylori* can inhibit or delay its uptake (96, 97) by arresting phagosome maturation (65). Instead, *H. pylori* induces the formation of megasomes, which enable bacterial survival (97). Further, *H. pylori* impairs antimicrobial activity and production of nitric oxide by its catalase and arginase activity (98, 99). However, macrophages have another crucial function in host defence, they orchestrate the adaptive immune response by secreting signalling proteins that activate and recruit other immune cells. Further, in particular situations they can act as antigen-presenting cells (APCs). However, the specialized and most effective APCs are dendritic cells (DCs). By antigen presentation and secretion of cytokines that direct lymphocyte differentiation, DCs represent the critical bridge between innate and adaptive immune response. During *H. pylori* infection, DCs are recruited into the gastric mucosa (100, 101), where they are found near the surface epithelium. Furthermore, DCs can send their dendrites

transepithelially to the lumen (100). Therefore, DCs can have direct contact with *H. pylori* and together with epithelial cells, they initiate the immune response to *H. pylori* infection. Stimulation of macrophages or DCs with *H. pylori* or bacterial products *in vitro* results in the secretion of pro-inflammatory cytokines like IL-1, TNFα, IL-6, IL-10, IL-12, IL-23 and TGFβ (100, 102-107) and also upregulation of maturation marker (105). However, in contrast to stimulation with dead *H. pylori* or LPS, which led to full DC maturation, infection with live *H. pylori* resulted in a semi-mature state, characterised by only a slight upregulation of maturation marker CD83, and costimulatory molecules CD80 and CD86. *H. pylori* can even dampen DC maturation, since cells which were infected with live *H. pylori* prior to stimulation with LPS or dead bacterium showed this semi-mature phenotype, too (106, 107). These semi-mature DCs secreted high levels of anti-inflammatory cytokines such as IL-10 and low levels of pro-inflammatory cytokines such as IL-12 (100, 107).

1.1.5.2 Adaptive immune response

H. pylori-infected persons develop humoral as well as cellular immune responses towards the bacterium. Thus, *H. pylori*-specific antibodies and T cells can be found in the gastric mucosa of infected individuals. Further, infected patients show increased gastric expression of IFNγ, IL-10, IL-12, IL-17, and TNF compared to uninfected controls (62, 108-110). How these cells and cytokines contribute to immunity and pathology is hard to dissect in humans. Therefore, mouse studies are used to elucidate the role of the different components.

Mice, such as $Rag^{-/-}$ (lacking B and T cells), SCID (severely deficient in functional B and T lymphocytes) or nu/nu mice (lacking thymus, therefore unable to produce T cells) that are not capable to elicit an adaptive immune response are higher colonised than immunocompetent animals (111-114). In contrast to wild type mice, these B- and T cell-deficient mice display no sign of gastritis. Adoptive transfer of lymphocytes in these mice resulted in gastritis and reduced colonisation (113-115). These results demonstrate that adaptive immune responses are crucial for bacterial clearing but also inducing immunopathology during *H. pylori* infection.

1.1.5.2.1 T cell responses

Upon antigen presentation through APCs, T cells become activated and differentiate to diverse T cell populations. While $CD8^+$ T cells all become $CD8^+$ cytotoxic T cells (CTLs), which destroy infected cells directly, $CD4^+$ T cells differentiate into several effector T cells with various functions, namely T_H1, T_H2 and T_H17 T cells.

Upon *H. pylori* infection $CD4^+$ and $CD8^+$ T cells infiltrate the human gastric lamina propria (116, 117). These infiltrating T cells secrete, when stimulated with *H. pylori* antigens, IFNγ (118). In experimental *H. pylori*-infected mice both T cell subtypes contribute to control colonisation, since absence of either MHC-I or MHC-II led to similar increase of bacterial burden (119).

In many studies, the accumulation of gastric *H. pylori*-specific $CD4^+$ cells have been proposed to contribute to pathogenesis in mice and humans (113, 120, 121). Further characterisation showed that these $CD4^+$ cells are IFNγ-producing T_H1 and IL-17-secreting T_H17 cells, whereas only few IL-4-secreting T_H2 cells were found (116, 122-124).

T_H2 cells mainly produce IL-4, IL-5 and IL-13, and mediate humoral responses against extracellular pathogens. Since *H. pylori* is a non-invasive bacterium, a T_H2 response would be expectable, but this is not the case. In *H. pylori*-infected persons hardly any expression of IL-4 can be detected (125, 126). That T_H2 response plays a minor role during *H. pylori* infection is substantiated as studies with IL-4-deficient mice showed that the lack of IL-4 did not affect bacterial burden or inflammation (112, 127).

In vaccination settings, T_H2 response may not be crucial for protection, since immunisation of IL-4- and IL-5 knockout mice conferred the same protection as in wild type mice (127, 128). Thus, T_H2 response does not play a major role in the immune response against *H. pylori*.

Instead of the Th2 response, T_H1 response is found towards *H. pylori* in infected humans (126). **T_H1** cells elicit cellular immunity, generally directed against intracellular pathogens. T_H1 type is characterised by secretion of TNFα, IL-2 and IFNγ, and is induced by IL-12 and IL-18 released by DCs or macrophages. Multiple

mouse studies analysing T_H1 response focused on IFNγ, as surrogate marker of T_H1 cells. However, one has to keep in mind that also other cell types such as $CD8^+$ T cells, macrophages and natural killer cells (NK) might produce IFNγ. Indeed, IFNγ expression is increased in stomach of *H. pylori*-infected humans and mice (112, 126). Since IFNγ is considered to mediate T_H1 function, IFNγ-deficient mice have often been used to elucidate the role of T_H1 cells. IFNγ expression was positively correlating with inflammation, and inversely with bacterial load in mice (129), suggesting a protective role for IFNγ. Thus, IFNγ knockout mice or mice treated with an IFNγ-neutralising antibody showed similar or higher bacterial burden and less histopathology compared to wild type mice (112, 113, 127, 129, 130), highlighting that IFNγ is required for induction of histopathology and to control bacterial load, at least to some extent. Adoptive transfer of IFNγ-deficient $CD4^+$ cells or spleenocytes into immunodeficient mice resulted in milder gastritis and higher bacterial loads compared to wild type cells (113, 120). Transfer of wild type $CD4^+$ cells into RAG/γ-chain-deficient mice (NK, T and B cell deficient, innate immune and cytokine defects, e.g. innate immune cells do not produce IFNγ) resulted in lower inflammation compared to RAG knockout mice (120). Further, transfer of T-bet-deficient $CD4^+$ cells still induced gastritis (131). Consequently, IFNγ from $CD4^+$ cells contribute to immunopathology but also in its absence some gastritis occurs, since firstly other cytokines from $CD4^+$ cells contribute to inflammation, and secondly IFNγ from other cells induces inflammation.

Immunisation studies in IFNγ-deficient mice are contradictory: While Sawai et al. and Garhart et al. reported that IFNγ knockout mice are equally protected as wild type mice, Akhiani et al. and Sayi et al. observed less or no protection (127, 129, 130, 132). Regarding inflammation, these studies also described different results: In the work of Garhart immunized and challenged IFNγ knockout mice had similar inflammation as wild type mice. In contrast, Akhiani reported less severe gastritis in the knockout mice (127, 132). Nevertheless, the level of IFNγ-induced gastritis correlated with bacterial burden. Thus, IFNγ seems to be an important factor to control infection by inducing inflammation also upon immunisation.

Another T_H cell subset which is characterised by production of IL-17 are **T_H17** cells. T_H17 cells respond to extracellular bacteria and fungi by recruiting immune cells such as neutrophils. T_H17 differentiation is induced by IL-6, IL-1β and TGFβ produced by DCs and macrophages. IL-23 is crucial for expansion and maintenance of T_H17 cells. IL-17 is highly upregulated upon *H. pylori* infection in human and mice (110, 133, 134). Surprisingly, inflammation and bacterial load did not correlate inversely but directly when IL-17 was absent during *Helicobacter* infection. IL-17-deficient mice showed lower colonisation accompanied by less inflammation, which was due to less infiltration of neutrophils (134, 135). Compared to adults, *H. pylori*-infected children displayed lower T_H17-associated cytokine expression in the stomach followed by less neutrophil infiltration and inflammation (136), supporting that T_H17 cells induce gastritis. In accordance, neutralisation of IL-17 by antibody treatment caused a reduction in bacterial load and inflammation, whereas adenoviral overexpression of IL-17 resulted in increased gastritis and *H. pylori* burden (134). In addition, transfer of IL-17-deficient $CD4^+$ cells into immunodeficient mice showed a similar picture (120), encouraging that IL-17 contributes to gastritis but may support *H. pylori* colonisation.

During immunisation, IL-17 is induced and correlates inversely with bacterial load (137). $CD4^+$ cells from immunised mice secrete much higher amounts of IL-17 than cells of only infected mice (138). In addition, IL-17 was strongly associated with neutrophil infiltration. When neutrophils were depleted by an antibody, protective immunity was lost, indicating that IL-17 contributes to neutrophil infiltration that confer protection (138). The same group later reported, that IL-17-deficient mice showed same protective immunity as wild type mice, which questions the role of IL-17 in protective immunity (139). However, when IL-17 was neutralised during challenge phase, protection against infection was lost (137). Thus, IL-17-producing T cells are induced upon immunisation and confer protection, but if IL-17 is not present other immune cells seem to compensate the absence of IL-17.

Several studies analysed interaction or compensatory mechanisms between T_H1 and T_H17 cells. In IL-17/IFNγ-double knockout mice, the lack of IL-17 and IFNγ resulted in mild gastritis upon *H. pylori* infection, less than in the single-deficient mice.

However, inflammation in IL-17-single knockout mice was greater as in IFNγ-single knockout mice. Likewise, the transfer of IL-17/IFNγ-double deficient $CD4^+$ cells resulted in less inflammation than transfer of single-deficient cells, and transfer of IL-17 knockout cell resulted in greater gastritis than transfer of IFNγ knockout cells (120). Collectively, these data emphasise that both cytokines contribute to gastritis, but IFNγ does so to a higher extent. IL-12 and IL-23 share their p40 subunit, mice lacking p40 are considered to be defective in T_H1 and T_H17 responses. However, when IL12/IL-23p40 knockout mice were infected with *H. pylori*, surprisingly they were equally colonised than wild type mice and showed similar or not significantly reduced levels of inflammation (127, 132). IL12/IL-23p40 knockout mice were not only similar colonised as wild type mice, but also IL-12p35 knockout and IL-23p19 knockout mice were equally colonised as wild type animals (140, 141), which argues against compensatory mechanisms of T_H17 and T_H1 in clearing the infection.

Vaccination of IL-12/IL-23p40-deficient mice failed to confer protection, although either IL-12p35 or IL-23p19 were protected as wild type mice (127, 132, 141). This suggest, that either IL-12 or IL-23 pathway during immunisation is sufficient for protective immunity. This is supported, since mice which were treated with a neutralizing antibody against IL-12/IL-23p40 during immunisation phase were not protected toward challenge with *H. pylori* (141). Further studies to dissect the role of these T cell subsets during immunisation were performed by depleting mice from the main effector cytokines IL-17 and IFNγ. Neutralisation of IL-17 and IFNγ resulted in higher colonisation similar to the one seen after neutralisation of IL-17 alone (137). This suggest that vaccine-induced IL-17 might be more important than IFNγ for inducing protective immunity after vaccination. This is in contrast to the more important role of IFNγ in the infection only scenario.

In summary, these studies indicate that IFNγ in general and in particular the one produced by T_H1 cells strongly contributes to inflammation and infection control, whereas after prophylactic immunisation T_H17 cells seem to be crucial for protection.

In contrast to effector T cells, T_{reg} cells suppress T cell response and limit immune reactions, and prevent autoimmune responses (94). Apart from T_H1 and T_H17 T cells, *H. pylori*-infected individuals present increased levels of $CD4^+CD25^{high}FoxP3^+$ regulatory T cells. The frequencies of $CD4^+CD25^{high}$ cells are particularly increased in the stomachs of *H. pylori*-infected patients with gastric adenocarcinoma, predominantly in cancer-affected tissues, compared to individuals with asymptomatic *H. pylori* infections or *H. pylori*-positive patients with duodenal ulcers (142). Mouse studies support a role for T_{reg}-mediated immunosuppression towards *H. pylori*. Thus, the elimination of $CD4^+CD25^+$ T_{reg} cells led to enhanced cytokine expression and development of gastritis. Further, the increased inflammatory response was accompanied by reduced bacterial load (143). Consequently, it is accepted that regulatory T cells may suppress immune response leading to persistence of *H. pylori*, but the permanently induced inflammation by other immune cells leads to damage of epithelial cells and pathology.

Studies analysing the T cell response mainly focused on the induction of $CD4^+$ T cells, although within the epithelium of *H. pylori*-infected individuals $CD8^+$ T lymphocytes predominate over $CD4^+$ lymphocytes. Further, human studies emphasize a potential clinical relevance of $CD8^+$ cytotoxic T lymphocytes in the context of *H. pylori* infection (144-146). In mouse studies, the influence of $CD8^+$ lymphocyte was only analysed in immunodeficient mice lacking $CD4^+$ T cells (147, 148). Results from these studies indicate that $CD8^+$ T cells also contribute to the development of gastric lesions. The authors suggested that the regulatory role of $CD4^+$ T cells may be important for suppressing tissue-damaging $CD8^+$ T cell responses (148). More recently, it was shown in pigs that $CD8^+$ T cells were infiltrating upon infection. However, these $CD8^+$ cells might be ineffective in clearing *H. pylori* but contribute to tissue damage (149).

1.1.5.2.2 Humoral responses

H. pylori-infected subjects show a strong humoral response with the formation of antibodies against *H. pylori*, especially against adhesion proteins, flagellin, urease, and CagA (150). However, antibodies alone appear ineffective at limiting

colonisation, they can even inhibit clearing, and do not provide protection against reinfection. Thus, B-cell-deficient mice display similar (151) or even lower bacterial burdens than wild type mice (152). *H. pylori*-infected patients with gastric cancer showed less IgA and IgG in the gastric mucosa than patients with chronic gastritis (153, 154).

Nevertheless, there was correlation between antibody levels and protection upon immunisation. Thus, protected mice showed higher antibody levels (155, 156), but antibodies seemed not to be essential for protection upon immunisation, since there was a similar decrease of bacterial burden in vaccinated B cell-deficient mice as in wild type mice (151, 152, 157).

Collectively, the humoral response toward *H. pylori* may only play a minor role, during infection and vaccine-induced immunity. Instead, cellular immune responses might be more important for pathogenesis and protection during *Helicobacter* infection or upon vaccination.

1.1.6 PRR-mediated *H. pylori* recognition

Cells of the innate immune system recognize pathogens by PAMPs via PRRs, such as cell surface or endosomal membrane bound toll-like receptors (TLRs), membrane bound C-type lectin receptors (CLRs), cytosolic nucleotide binding domain (NOD) and leucine rich repeat containing proteins (NLRs), and intracellular RIG-like receptors (RLRs) or AIM2-like receptors (ALRs).

The recognition of *H. pylori* by TLRs on epithelial and immune cells was addressed in several studies. These studies focused largely on TLR2, TLR4 and TLR5, which recognise bacterial lipoproteins, lipopolysaccharide and flagellin, respectively. Several studies showed that TLR2 seems to be the important TLR in *H. pylori* recognition. The requirement of TLR4 (158-161) and TLR5 (160, 162) is controversial.

The production of different chemokines, particularly GROα or IL-8, and cytokines such as IL-1, IL-6 and TNFα, by gastric epithelial cells upon *H. pylori* infection depends mainly on TLR2 (159, 160). Remarkably, *H. pylori* LPS activates not only the

normal LPS-sensor TLR4 (158) but also TLR2 (160, 163-165). This might be because the O-antigen, the outer part of LPS, is structurally related to lewis-antigen, and lipid A chain of *H. pylori* has different acetylation patterns than the LPS from other gram-negative bacteria (166-168). Due to a modification at the N-terminus of its flagellin, *H. pylori* also escapes recognition by TLR5 (169).

In macrophages, the secretion of IL-1β, IL-6, IL-10 and IL-12 depends on TLR2 (170). DCs maturation and production of cytokines such as TNFα, IL-1β, IL-6, IL-10, IL-12, and IL-23 are TLR2 dependent, too (170-175). In DC-T-cell coculture experiments, *H. pylori*-stimulated BMDCs from TLR2 knockout mice secreted less cytokines, which resulted in lower T_{reg} and T_H17 responses, but a higher IFNγ response compared to *H. pylori*-stimulated BMDCs from wild-type mice (173). In human DCs blockage of TLR2 led to lower cytokines secretion, especially of IL-6. When these cells were co-incubated with naive T cells, blocking of TLR2 on DCs led to an attenuated T cell response, with much lower secretion of IL-17, the release of IFNγ and the expression of Foxp3 were not significantly decreased. *In vivo,* deletion of TLR2 led to reduced bacterial density (173, 175, 176) and more severe gastric immunopathology. This was due to the increased *H. pylori*–specific T_H1 response and less T_{reg} and T_H17 responses (173, 175).

In macrophages and dendritic cells, secretion of IL-1β, IL-6, IL-10 and IL-12 secretion depends further on TLR4 (170, 171, 174). Blockage of TLR4 signalling in DCs led to significantly decreased amounts of IL-6, IL-12p70 and IL-10, which resulted in lower secretion of IL-17 and IFNγ, and reduced levels of Foxp3-expressing and IL-10-secreting T cells in co-culture experiments (174).

Neutralization of TLR5 in DCs did not alter cytokine secretion from DCs or co-incubated T cells (174). Also IL-1β expression and secretion was not altered in TLR5-deficient DCs (175).

DNA from phagocytosed *H. pylori* stimulates TLR9-dependent cytokine production in DCs (171) but not in macrophages (170). Thus, IL-6 and IL-12 levels were lower in TLR9-deficient BMDCs (171) but not IL-1β (175) upon infection with *H. pylori*. *In vivo*,

TLR9 knockout mice showed no difference in colonisation but stronger cytokine response. The authors proposed that TLR9 signalling suppresses T_H1 differentiation via induction of IFNα (177).

RIG-I is one other type of PRRs, belonging to the RLR-family. This receptor recognizes RNA of *H. pylori* and induces expression of type I interferon and interferon-stimulated genes (ISGs) (171).

The role of NLRs in the context of *H. pylori* was addressed only in the recent years. In epithelial cells *H. pylori* peptidoglycan injected via T4SS (55) or peptidoglycan in outer membrane vesicles shed from bacterium (178) were sensed by NOD1, which led to expression of type I interferon, ISGF3 activation (179), and induction of antimicrobial peptides, such as β-defensins (180). *In vivo*, NOD1 knockout mice showed higher bacterial burden (55, 179). The authors proposed that this was caused by less IFNγ, less type I IFN and blocked ISGF3 signalling (179). In murine DCs *H. pylori* induced, via NOD2, the expression of Nlrp3 (172, 175), also in macrophages Nlrp3 was upregulated upon *H. pylori* infection (181). Further, expression of Nlrp3 was increased in the stomach of *H. pylori*-infected mice (182). In human THP-1 cells, a human monocytic cell line, Li et al. saw induction of Nlrp3 expression (183), in contrast Castano-Rodriguez et al. did not observe any upregulation in these cells (184). Still, they saw downregulation of Nod2, Nlrc4, Nlrc5, Nlrc9, Nlrp12, and Nlrx1 upon *H. pylori* infection (184). In contrast, in murine BMDM Nod2 and Nlrc5 were upregulated as Nod1. In line with the results in human THP-1 cells, also in murine macrophages Nlrx1 was downregulated upon infection (181). Nlrp1, Nlrp4, Nlrp5 and Nlrp6 were not regulated by *H. pylori* in THP-1 cells (184) but NLRP1b and Nlrc3 were downregulated upon *H. pylori* infection in murine macrophages. NLRP12 and Nlrx1 are negative regulators of canonical and non-canonical NFκB signalling (185-188). Castano-Rodriguez et al. postulate that Nlrx1 may dampen *H. pylori*-induced immunopathology by downregulating *NFKB1* and NFκB target genes such as pro-inflammatory cytokines, chemokines and cancer-associated genes (184). Beside its effect on NFκB, Nlrx1 might also influence IFNγ and ROS production from macrophages, since Nlrx1-deficient cells infected with *H. pylori* showed higher IFNγ

and ROS levels. These higher levels were accompanied with a faster clearing of the bacterium from BMDMs. *In vivo*, Nlrx1-deficient mice showed much lower gastric bacterial load than wild type mice (181). That NODs and NLRs might play a role in *H. pylori* infection and *H. pylori*-induced pathology is further substantiated since polymorphism in NOD/NLRs (e. g. *NOD1*, *NLRP12*, *NLRP3*, *NLRX1*) or NLR-signalling (e. g. *CARD8*, *CASP1*) are associated with the development of gastric pathologies (184, 189, 190).

1.2 The inflammasome

Tschopp and colleagues first used the term inflammasome in 2002 to describe a multiprotein complex formed in the cytosol, which serves as a platform to recruit and activate inflammatory caspases as caspase-1 (191). Caspase-1 is necessary to cleave pro-IL-1β and pro-IL-18 in their functional forms.

1.2.1 Structure of inflammasomes

Several inflammasome complexes - NLRP1 (NALP1), NLRP3 (NALP3), NLRP6, NLRP7, NLRP10, NLRP12, NLRC4 (IPAF) and AIM2 - have been described to date.

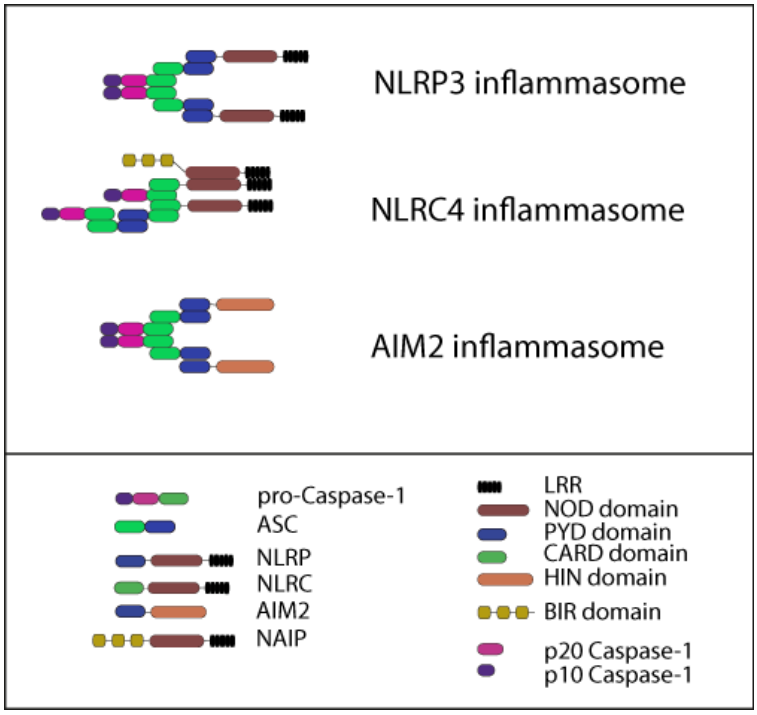

Figure 1: Composition of the three inflammasome types NALP3, NLRC4 and AIM2:

ASC (apoptosis-associated speck-like protein containing a CARD), NLRP (NLR family, pyrin domain containing), NLRC (NLR family, CARD domain containing), AIM2 (Absent in Melanoma 2), LRR (leucine-rich repeats), NOD (nucleotide-binding oligomerization domain), PYD (pyrin domain), CARD (caspase activation and recruitment domain), HIN (hematopoietic interferon-inducible nuclear antigens).

The inflammasome consists of different proteins (Figure 1). The respective inflammasome is named after its scaffolding protein. Most of these scaffolding proteins are members of the NLR (nucleotide-binding domain and leucine-rich repeat containing) superfamily. NLRs are defined by their tripartite structure:

(1) The C-terminal leucine-rich repeats (LRR) of NLRs are believed to have two functions: first, LRRs may be required to maintain NLRs in an inactive state. Second, analogue to their function in TLRs, they are supposed to mediate recognition of PAMPs or DAMPs.

(2) The central domain called nucleotide-binding oligomerization domain (NOD) or NACHT domain (NACHT stands for domain present in NAIP (neuronal apoptosis inhibitor protein), C2TA (MHC class 2 transcription activator), HET-E (incompatibility locus protein from *Podospora anserina*) and TP1 (telomerase-associated protein)), which mediates self-oligomerisation occurring during activation.

(3) The N-terminal part of NLRs consists of a protein-protein interaction domain, either a caspase activation and recruitment domain (CARD), a pyrin domain (PYD), or a baculoviral apoptosis inhibitory protein repeat containing domain (BIR). By this N-terminal domain NLRs mediate downstream protein-protein interactions.

The NLR protein signals by its CARD with caspase-1 directly or by PYRIN via the PYRIN-CARD-containing adaptor protein ASC indirectly. In contrast to the NLRP and NLRC inflammasomes, the AIM2 inflammasome does not consist of an NLR protein, but has a PYRIN-HIN 200 protein (192-194).

In addition to NLRs, ASC and Caspase-1, murine caspase-11 (195-197), the murine orthologue of the human caspase-4 (198) and caspase-5 (191), as well as NAIPs can be part of the inflammasome complex (199, 200).

An overview of the best-analysed inflammasome types and their way of activation are depicted in the following section.

1.2.2 Activation of the inflammasome

In general, two signals are needed for inflammasome activation and secretion of mature IL-1β and IL-18. As a first signal, NFκB activation by TLRs, NOD1/2 or cytokine receptor stimulation leads to transcription and accumulation of precursor cytokines IL-1β and IL-18 but also of NLRP3. Interestingly, pro-IL-1β can also be induced by IL-1β itself, by triggering IL-1R-signaling. The second signal leads to the assembly of the inflammasome complex and is mediated by DAMPs and PAMPs.

Because of inflammasome assembly pro-caspase-1 (~45 kDa) is autocatalytic cleaved in its 10 and 20 kDa subunits. These subunits form the active caspase-1 (201). The active caspase-1 can cleave pro-IL-1β (~31 kDa) and pro-IL-18 (~24 kDa) into bioactive IL-1β (~17 kDa) and IL-18 (~18 kDa) (202). The active forms are secreted by a mechanism not yet fully understood, which may involve exocytosis of endocytic vesicles (203), shedding of plasma membrane microvesicles (204), and release via transporters (205) or multivascular bodies containing exosomes (206). Apart from cleavage of pro-IL-1β and pro-IL-18, caspase-1 activates a type of cell death called pyroptosis, which is characterized by an increase in cell size due to osmotic swelling

and subsequent rupture of cytoplasmic membrane followed by a release of cellular contents into the extracellular environment. Pyroptosis is an efficient mechanism to clear intracellular bacteria (207-209).

1.2.2.1 The AIM2 inflammasome

The AIM2 inflammasome is the only inflammasome that does not belong to the NLR superfamily. For activation of AIM2 inflammasome two signals are required: Transcriptional priming of *Aim2* downstream of type I IFN signalling (first signal) and cytosolic double stranded DNA (second signal). AIM2 is activated by different viruses as murine cytomegalo virus (CMV) or Vaccinia (210, 211), and intracellular replicating bacteria such as *Listeria monocytogenes* (209, 210), *Legionella pneumophila* (212) or *Francisella tularensis* (210, 213). In the case of bacterial infections, DNA is released upon lysis into the cytosol. AIM2 works as direct receptor for double-stranded DNA in the cytosol. Binding of the DNA leads to conformational changes and oligomerization of AIM2 around the DNA molecule. Upon recruitment of ASC and Caspase-1 the inflammasome is assembled (214, 215) and the pro-cytokines are cleaved.

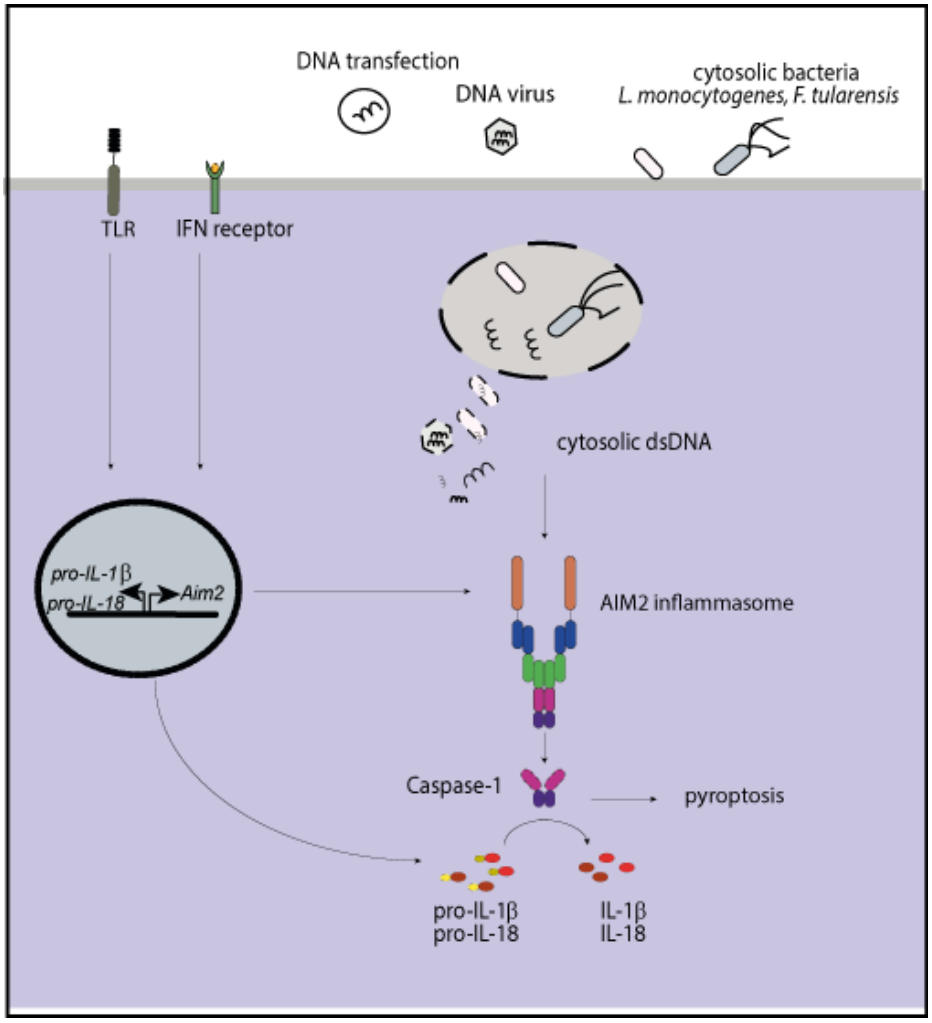

Figure 2: Activation of AIM2 inflammasome:

Activation of AIM2 inflammasome requires two signals: Transcriptional induction of *Aim2* downstream of type I IFN signalling (1st signal) and cytosolic double stranded DNA (2nd signal). The expression of the pro-IL-1β and pro-IL-18 is induced via NFκB upstream of TLRs. Adapted from (192, 216).

1.2.2.2 The NLRP1 inflammasome

Humans as well as mice can form an NLRP1 inflammasome, but its composition and activators differ. The human NLRP1 is triggered by the cell wall component muramyl dipeptide (MDP) (217) but not the murine one, which is activated by the lethal toxin of *Bacillus anthracis* (218, 219) and by *Toxoplasma gondii* (220). In contrast to the murine one, the human NLRP1 inflammasome has a C-terminal extension containing a CARD domain. Thus, the human NLRP1 has both a PYRIN and a CARD domain, whereas the mouse NLRP1 lacks the PYRIN domain. Due to its CARD and PYRIN domains the human NLRP1 can interact directly with caspase-1 or indirectly via ASC. In addition of caspase-1, caspase-5 can bind the human NLRP1 complex (191).

Furthermore, NOD2 association is required for MDP-induced inflammasome activation (221). For NLRP1 inflammasome activation ASC is not strictly required, but the caspase-1 activation is enhanced in the presents of ASC (222).

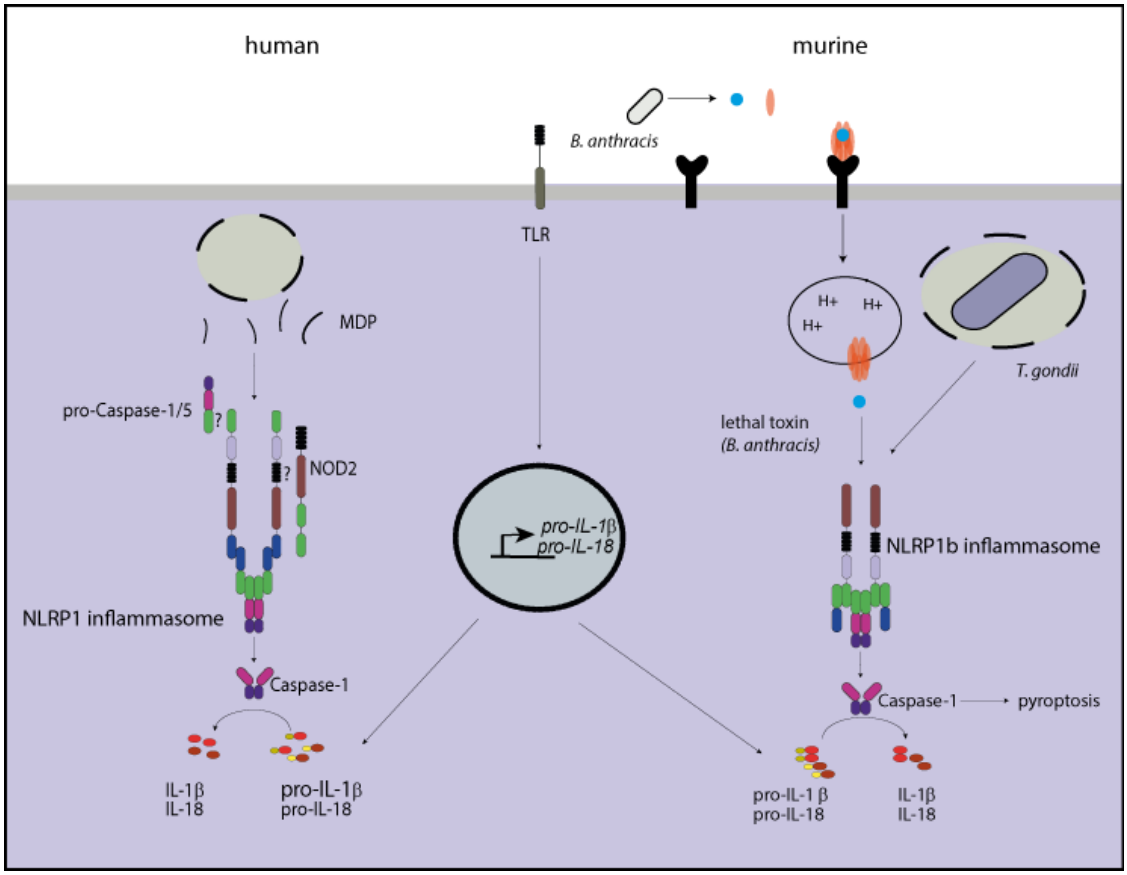

Figure 3: Activation of NLRP1 inflammasome:

The human NLRP1 inflammasome is activated by MDP. In contrast, the murine NLRP1b inflammasome is activated by the lethal toxin of *B. anthracis* and by *Toxoplasma gondii*. The human NLRP1 has both a CARD and a PYRIN domain, whereas the murine one lacks the PYRIN domain. Adapted from (192, 216, 223, 224).

1.2.2.3 The NLRC4-inflammasome

NLRC4-dependent IL-1β secretion is triggered by several gram-negative bacteria including *L. pneumophila* (200, 208, 225), *Pseudomonas aeroginosa* (226-228), *Salmonella typhimurium* (199, 229, 230), *Shigella flexneri* (231, 232), *Aeromonas veronii* (233), *Chromobacterium violaceum* (200), *Escherichia coli* (232), *Burcholderia thailandensis* (200, 232) and *Yersinia pestis* (234) but also by the gram-positive bacterium *L. monocytogenes* (235, 236). The NLRC4 inflammasome specifically responds to functional type III or IV secretion systems (T3SS/T4SS) and bacterial

flagellin (200, 232). In murine immune cells, cytosolic delivery or expression of flagellin are sufficient to trigger NLRC4 inflammasome activation (237). Upon bacterial infection, flagellin can enter the host cell by translocation-associated secretion systems (238). Therefore, flagellin-mediated stimulation of the inflammasome by bacterial strains harbouring genetic mutations that disrupt T3SS is usually reduced or absent (239). Despite not expressing a secretion system, the flagellated bacterium *L. monocytogenes* can also trigger NLRC4 inflammasome activation. In this case, flagellin gains access to the cytosol when *Listeria* escapes the phagosome to replicate in the cytosol (235). In murine cells secretion-system-dependent NLRC4 inflammasome activation is triggered by basal body rod components of T3SSs such as PrgJ (*S. typhimurium*), BsaK (*Burkholderia pseudomallei*), EprJ and EscI (*E. coli*), MxiI (*S. flexneri*), and PscI (*P. aeruginosa*) (232). These rod proteins share a sequence motif that is essential for detection by NLRC4; a similar motif is also found in flagellin (232). During NLRC4-dependent inflammasome activation NAIP (NLR family, apoptosis inhibitory protein), proteins serve as sensors. In murine cells, flagellin triggered inflammasome activation depends on NAIP5 and NAIP6 (199, 200, 240). In contrast, secretion system rod proteins are bound by NAIP2 (199, 200, 232), while the needle proteins interact with NAIP1 (241). Humans possess only one NAIP protein, which detects flagellin and TSS needle proteins, such as CprI of *Chromobacterium violaceum* or the respective homologues of EHEC, *P. aeruginosa*, *S. flexneri* and *S. typhimurium* (200, 242). NAIPs serve as sensors and interact with NLRC4 for inflammasome activation (see Figure 4).

The activation of the NLRC4 inflammasome seems to be a biphasic mechanism: First, cytoplasmic flagellin or secretion system components trigger phosphorylation of Ser533 of NLRC4 by PKCδ (protein kinase C delta). Second, NAIPs sense flagellin or components of bacterial secretion systems. Upon sensing, the ligand-bound NAIP interacts with $NLRC4^{S533P}$ and induces a conformational change of NLRC4. This activated NLRC4 recruits and activates an additional NLRC4 molecule. In this domino-like reaction around ten NLRC4 proteins together with one NAIP molecule form a disc or wheel-like structure. Upon this clustering the oligomerisation of the NLRC4-CARD enables the recruitment of ASC and caspase-1 (243, 244). The

phosphorylation of NLRC4 may facilitate these conformational changes and promote NLRC4 inflammasome assembly (245, 246). NLRC4 phosphorylation and inflammasome activation are independent, since stimulation of Naip5-deficient BMDMs with flagellin resulted in normal NLRC4 phosphorylation but abolished caspase-1 activation, cytokine secretion and pyroptosis (246); and lack of caspase-1 or inhibition of caspase-1 activity did not abolish NLRC4 phosphorylation (245, 246). Further, flagellin of *H. pylori* triggered NLRC4-phosphorylation but did neither activate caspase-1, nor IL-1β secretion or pyroptosis (246).

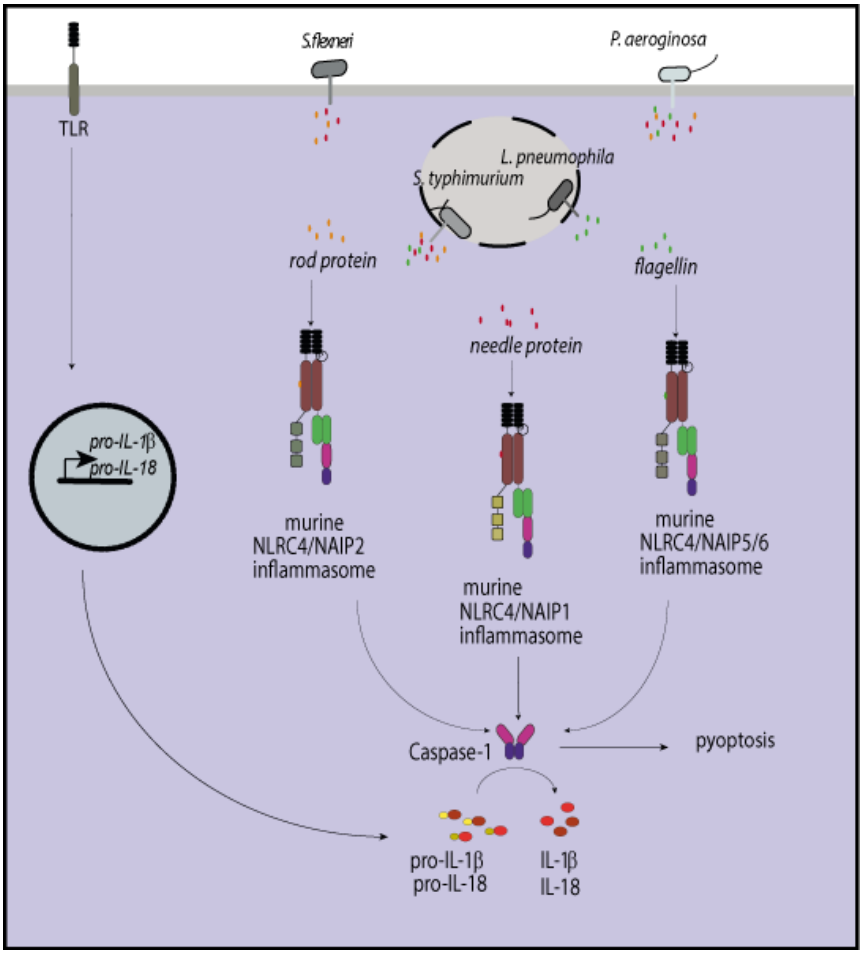

Figure 4: Activation of NLRC4 inflammasome:

NLRC4 is activated by a biphasic mechanism: NLRC4 is phosphorylated (1st step) upon ligand stimulation. NAIP molecules binds its specific ligand and the ligand-bound NAIP interacts with NLRC4, which leads to clustering of ~10 Nlrc4 molecules and one NAIP (2nd step). NAIP/NLRC4 inflammasome are activated by flagellin or proteins of bacterial secretion system. NAIP proteins determine ligand specificity: murine NAIP5 and NAIP6 detect flagellin, NAIP2 recognises rod proteins, and needle proteins interact with NAIP1. Human NLRC4/NAIP inflammasome is activated by needle proteins and flagellin. Adapted from (192, 193, 200, 223, 243-246).

1.2.2.4 The NLRP3 inflammasome

The NLRP3 inflammasome is activated by a broad range of stimuli, such as viruses (*encephalomyocarditis virus*, *vesicular stomatitis virus* (247), *human respiratory syncytial virus* (248), *varicella zoster* (249), *Sendai virus* (250), *influenza virus* (251, 252) and *adenovirus* (253)), bacteria (*Clamydia pneumonia* (254, 255) *Mycobacterium tuberculosis* (256) *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *L. monocytogenes*), fungi (*Candida albicans* (257), *Aspergillus fumigatus* (255), *Paracoccidiodes brasiliensis* (258)), and parasites (*Schistosoma mansoni* (259) and

Dermtophagoides pteronyssinus (260)), but also by danger signals and environmental irritants (ATP (261), UV (262), monosodium urate crystals (263), amyloid-β aggregates (264), asbestos (265), silica and alum (266)).

Multiple studies have been performed in order to clarify the mechanism of NLRP3 inflammasome activation, but until now a unifying mechanism of NLRP3 activation remains elusive. NLRP3 inflammasome is activated by a wide array of stimuli and conditions. Due to the structural diversity of NLRP3 inflammasome stimuli, it is believed that this inflammasome monitors host-derived DAMPs that are produced or released upon disruption of host physiology. Different models of NLRP3 inflammasome activation have been proposed (see Figure 5):

One model postulates a role for reactive oxygen species (ROS) generated by ATP signalling through P2X7 receptor (267), by NADPH-oxidase during phagocytosis (265), or by mitochondria (268, 269). The ROS-model remains controversial since ROS inhibitors may only block priming (270) and cells deficient in NADPH-oxidase subunits (271, 272) or pannexin channel (273) showed normal inflammasome activation. Therefore, mitochondrial-derived ROS is now considered the responsible ROS for NLRP3 inflammasome activation. Mitochondrial ROS can induce oxidation of thioredoxin (TRX) and causes its dissociation from thioredoxin-interacting protein (TXNIP). The released TXNIP binds directly to and activates NLRP3 (274). Further, altered mitochondrial transmembrane potential and mitochondrial dysfunctions trigger NLRP3 inflammasome activation (268).

The second model – the lysosomal rupture model - suggests that during lysosomal destabilisation lysosomal enzymes (e.g. cathepsin B) (266) or bacterial mRNA released from lysosomes during degradation of phagocytised bacteria (275) trigger NLRP3 inflammasome activation.

The third model, the ion flux model proposes that NLRP3 inflammasome activation is triggered by ion fluxes through ATP/P2X7 receptor-induced channel formation (276) or pores formed by bacterial toxins (233, 261, 277, 278). ATP- or toxin-induced membrane permeabilization leads to a decrease in cytoplasmic potassium, which promotes NLRP3 inflammasome activation (261, 279). Recently, also calcium

signalling was found to be critical in NLRP3-dependent inflammasome activation (280), since high extracellular calcium triggers via plasma membrane coupled receptors (e. g. GPCR6A) or calcium sensors (e. g. CASR) the release of calcium from endoplasmic reticulum calcium stores (281, 282), or via cation channels (e.g. TRPM2/7) the influx of calcium (283). The resulting increased intracellular calcium levels cause mitochondrial damage and mtROS production, which trigger NALP3 activation in part through suppression of NALP3 inhibitor cAMP (281).

The different proposed models for NLRP3 inflammasome activation are also linked, since ROS production is associated with ER stress and calcium signalling. Calcium mobilization from ER results in mitochondrial damage and subsequent loss of membrane potential, increased mitochondrial ROS (mROS) production and release of mitochondrial DNA (mtDNA) into the cytosol. All these factors were implicated in NLRP3 inflammasome activation (254, 268, 283). Thus, activation of the NLRP3 inflammasome by several ER stress inducers is dependent on potassium efflux and ROS production (284). Further, phagocytosis and extracellular calcium can trigger potassium efflux (285). Recent studies identified an upstream kinase, spleen tyrosine kinase (Syk) as a critical mediator of NLRP3 inflammasome activation (255, 257, 286). Syk regulates ROS production (287, 288) and lysosomal activity (289, 290), two major signals for NLRP3 inflammasome activation. Furthermore, Syk phosphorylates ASC promoting inflammasome assembly (291).

NLRP3-induced IL-1β secretion is occasionally also dependent on bacterial secretion systems (233, 292). It is suggested, that effector proteins are injected into the cytosol via the secretion system where they activate the inflammasome (292).

In the context of NLRP3-dependent inflammasome activation and cytokine secretion not only caspase-1 may be involved but also other inflammatory caspases, such as caspase-8, which cleaves IL-1β upon fungi and mycobacteria infections (293). Recently, it was further shown that Caspase-11 (human orthologous caspase-4 and 5) works upstream of caspase-1 to potentiate NLRP3 inflammasome activation. Here, TRIF signalling and certain lipid A moiety of LPS may be involved (294). Caspase-11 activation is important for IL-1β secretion upon infection of many gram-

negative bacteria such as *Salmonella* (295) *Vibrio cholera* (296), *Legionella* (195), *E. coli*, *Citrobacter rodentium* and *S. flexneri* (197, 294). Independent of assisting to caspase-1-dependent IL-1β secretion, caspase-11 can induce pyroptosis (197, 295). Extracellular LPS cannot activate caspase-11 (296), rather LPS has to be in the cytosol (294). Thus, gram-negative bacteria, which escape vacuoles or are lysed, are sensed by caspase-11 (297). Caspase-11 (and its human homologues caspase-4/5) binds the lipid A of LPS directly via its CARD (298). LPS from bacteria with a conserved lipid A (penta-acylated or hexa-acylated lipid A) activates caspase-11, in contrast, similar to TLR4 activation, LPS with an attenuated lipid A evades caspase-11 sensing. LPS from *H. pylori*, which has a tetra-acylated lipid IV, does not trigger caspase-11-dependent caspase-1 activation, it even has an antagonistic effect and inhibits this non-canonical inflammasome activation (294).

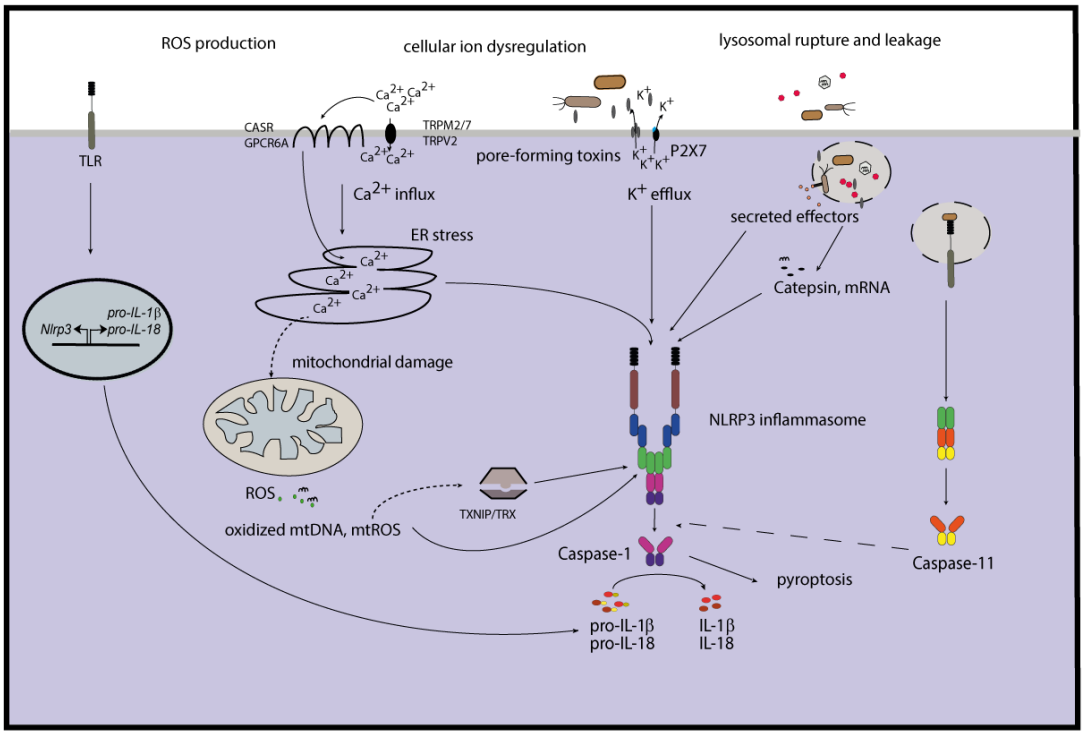

Figure 5: Activation of NLRP3 inflammasome:

Activation of NLRP3 inflammasome requires two steps: Transcriptional induction of *Nlrp3* and oligomerisation of NLRP3. To explain the mechanism underlying the second step, different three models are discussed: The first model propose that production of ROS from different sources lead to NLRP3 inflammasome activation. The second model suggest, that efflux or influx of ions starts NLRP3 inflammasome assembly. The third model proposed that lysosomal disintegration leading to leakage of lysosomal content is sensed and triggers NLRP3 assembly. Adapted from (192, 193, 224).

1.2.2.5 NLRP6 and NLRP12 inflamamsomes

In addition, NLRP6 and NLRP12 can form inflammasomes. Both inflammasomes seem to be important for maintenance of intestinal homeostasis by regulating commensal gut microbiota (299), intestinal inflammation and tumorigenesis (187, 300, 301). Further, they serve as negative regulators of innate immunity during certain bacterial infections by down regulating canonical and non-canonical NFκB, ERK and MAPK signalling (186, 187, 302). In addition, NLRP12 is important for controlling *Y. pestis* infection (303).

1.2.3 Biological effects of IL-1β and IL-18

In unstimulated human or murine hematopoietic cells, expression of IL-1β is absent (202, 304-306). IL-1β is mainly secreted by DCs, monocytes, macrophages and

neutrophils upon stimulation. IL-1β had been described to affect the immune response directly and indirectly. IL-1β induces gene expression and synthesis of enzymes involved in metabolic pathways. These include cyclooxygenase type 2 (COX-2), type 2 phospholipase A and inducible nitric oxide synthase (iNOS). Activation of these enzymes results in fever, lowered pain threshold, vasodilatation and hypertension (306). Additionally, IL-1β increases expression of adhesion molecules on endothelium and induces chemokines promoting infiltration of immune cells (94). Further, IL-1β is an important angiogenesis factor and therefore affects tumour metastasis and blood vessel formation (307). IL-1β is also important for initiating adaptive immune responses. Together with IL-6, IL-23 and TGFβ it induces and sustains T_H17 cells (308). However, during asthma development IL-1β contributes to T_H2 polarized immune response (309). Finally, IL-1β is crucial for B cell activation and antibody production (310).

Another member of the IL-1 family is IL-18. In contrast to pro-IL-1β, pro-IL-18 is constitutively expressed in immune cells (such as macrophages and DCs), endothelial cells, keratinocytes, and epithelial cells throughout the gastrointestinal tract (202, 304-306, 311, 312). Macrophages and DCs are the primary producers of the active IL-18. IL-18 together with IL-12 contributes to T_H1 cell differentiation, NK cell activation and IFNγ secretion (308). In the absence of IL-12 it can also induce T_H2 cells (313). Further, IL-18 can induce T_{reg} cells (106) and can counteract IL-1β responses (314, 315). Other than immune cells, IL-18 is also produced by epithelial cells – especially in the stomach and intestine –, and may be important for epithelial integrity.

1.2.3.1 IL-1β, IL-18 and inflammasome in *H. pylori* infection

IL-1β and IL-18 are cytokines that are induced in response to gastric *H. pylori* infection (316-319) and have been linked to gastric cancer development. Thus, polymorphisms in *IL-1B* and *IL-18* genes that result in higher cytokine production increase the predisposition to gastric cancer in *H. pylori* infected individuals (320-325). Transgenic mice that overexpress IL-1β in the stomach developed spontaneous inflammation, metaplasia, dysplasia and carcinoma of the stomach, demonstrating

that increased levels of IL-1β can be sufficient to induce neoplasia (326). Further, IL-1β may also play an important role in metastasis by activation of NFκB-dependent genes involved in proliferation, apoptosis and angiogenesis (327). Further, IL-1β is a strong inhibitor of acid secretion and enables *H. pylori* to colonise the gastric corpus. The corpus-predominant gastritis is linked to the development of gastric tumours (328). Therefore, it is not surprisingly, that IL-1 receptor knockout (IL-1R$^{-/-}$) mice were protected from *H. felis*-induced gastritis (315). In contrast, IL-18 knockout mice showed enhanced immunopathology upon infection with *H. felis* (315) or *H. pylori* (106). In contrast, in human biopsies, high IL-18 levels correlate with increased immune cell infiltration (319).

Studies analysing the impact of IL-1β, IL-18, and caspase-1 on *H. pylori* colonisation and *H. pylori*-induced inflammation showed conflicting results: Kim et al. saw higher *H. pylori* colonisation in the stomach of IL-1β and IL-1R knockout mice (172). Deficient IL-1R signalling was accompanied by less metaplastic changes (329). In contrast, in a study from Hitzler et al. IL-1R knockout mice showed a similar immune response accompanied with the same bacterial burden as wild type mice (315). Studies analysing the contribution of IL-18 to *H. pylori*-induced inflammation and clearing of the bacterium displayed an inconsistent picture: Two studies reported that there are no differences in colonisation between wild type and IL-18 knockout mice (127, 176). In contrast, two other studies, which were performed from the same group, reported lower colonisation in IL-18 knockout mice. This was due to a stronger T_H17 response (106, 315). The same group reported that also IL-18R knockout mice were colonised lower with *H. pylori*, which was accompanied with stronger inflammation (106). Studies from the same group described that caspase-1 knockout mice showed lower colonisation due to their higher gastric IL-17 expression (315). However, in the study from Kim et al. Caspase-1 deficiency resulted in a higher colonisation compared to wild type mice (172).

H. pylori could activate caspase-1 and induced IL-1β and IL-18 secretion in BMDCs (172, 315) and in some gastric epithelial cell lines (318, 330, 331). Also in human THP-1 macrophages caspase-1 activation accompanied by IL-1β and IL-18 secretion was triggered by *H. pylori* (183) or its LPS (183, 332). However, in murine BMDCs

H. pylori-LPS failed to induce caspase-1 activation and secretion of mature IL-1β. Pre-treatment with *H. pylori*-LPS was even inhibiting secretion of mature IL-1β induced by other bacteria (294).

1.3 Objectives of the study

H. pylori is the main cause for gastric cancer development. The production of IL-1β and IL-18 is highly induced in *H. pylori*-infected individuals. Both cytokines have been extensively linked to gastric carcinogenesis. To be active, these cytokines have to be cleaved by caspase-1, the effector protein of the inflammasome.

The aim of this project was to analyse how *H. pylori* activates the inflammasome to induce secretion of active Il-1β and IL-18. In particular, the study aimed to elucidate the inflammasome type, and cellular mechanisms involved in the *H. pylori*-induced inflammasome activation, as well as the *H. pylori* factor acting as activating signal of the inflammasome assembly. Further, to analyse the *in vivo* role of NLRP3 and NLRC4 inflammasomes on bacterial clearing and *H. pylori*-induced gastritis.

2 Material and Methods

2.1 Material

2.1.1 Bacterial strains

Helicobacter pylori G27 (333)

Helicobacter pylori G27 ΔBabA provided by T. Boren, Umeå University, Sweden

Helicobacter pylori G27 ΔBabASabA provided by T. Boren, Umeå University, Sweden

Helicobacter pylori G27 ΔCagA (107)

Helicobacter pylori G27 ΔCagE (334)

Helicobacter pylori G27 ΔgGT (85)

Helicobacter pylori G27 ΔSabA provided by T. Boren, Umeå University, Sweden

Helicobacter pylori G27 ΔUreA/B (107)

Helicobacter pylori G27 ΔVacA (66)

Helicobacter pylori PMSS1 (335)

Helicobacter pylori PMSS1 ΔCagA provided by R. Haas; Pettenkoferinstitut, Munich, Germany

Helicobacter pylori PMSS1 ΔCagE (335)

Helicobacter pylori PMSS1 ΔgGT, generated in this study (66)

Helicobacter pylori PMSS1 ΔVacA (66)

Helicobacter pylori SS1 (336)

Salmonella typhimurium provided by MIH, Munich, Germany

2.1.2 Cells

Murine bone marrow derived dendritic cells (BMDCs)

Murine bone marrow derived macrophages (BMDMs)

Murine neutrophils isolated from bone marrow

Human peripheral blood mononuclear cells (PBMCs)

Human monocytes-derived DCs (MoDCs)

Human B cells

Human natural killer (NK) cells

Human T cells

2.1.3 Mice

C57Bl6 Harlan Laboratories (Germany) or bred in-house (MIH).

ASC knockout mice (B6.129-*Pycard*^{tm1Vmd}) provided by V. Dixit (Genentech, South San Francisco, USA) (229).

Caspase-1 (and Caspase-11) knockout mice (B6.129S2-*Casp1*^{tm1Flv}/J) provided by O. Gross (Klinikum rechts der Isar, Munich, Germany; originally provided by R. Flavell through The Jackson Laboratories) (337).

NLRP3 knockout mice (B6-*Nlrp3*^{tm1Tsc}/Siec) provided by J. Tschopp (University of Lausanne, Switzerland) (263).

NLRC4 knockout mice (*Nlrc4*^{tm1Vmd}, unknown mixed background), provided by F. Greten (Klinikum rechts der Isar, Munich, Germany; originally provided by V. Dixit (Genentech, South San Francisco, USA)) (229).

2.1.4 Chemical inhibitors

APDC ((2*R*,4*R*)-4-Aminopyrrolidine-2,4-dicarboxylate)	(Sigma-Aldrich)
Bafilomycin A	(LC Laboratories)
Cytochalasin D	(Sigma-Aldrich)
Ebselen	(Enzo)
KCl	(Roth)
NAC (*N*-Acetylcysteine)	(Sigma-Aldrich)
R406	(Salleck)
Z-YVAD-FMK	(Calbiochem)

2.1.5 Media, Buffers and solutions

2.1.5.1 Reagents for bacterial culture

Brucella broth (BB) Medium	14.5 g (Oxoid), 500 ml aqua
Brain heart infusion (BHI)-Medium	36 g BHI (MP Biomedicals), 0.25 % (w/v) yeast extract, 5 % (v/v) fetal calf serum, 1 l aqua
Wilkins Chalgren (WC)-dent	21.5 g WC-Agar, 500 ml aqua, 10 % (v/v) inactivated horse serum, 0.2 g potassium nitrate, Dent-Supplement
Horse serum	(Invitrogen)
Dent-Supplement	10 mg/l Vancomycin, 5 mg/l Trimethoprim, 5 mg/l Amphotericin B, 5 mg/l Cefsulodin (Oxoid)
Special-Supplement	200 mg/l Bacitracin, 10 mg/l Nalidixic acid, 3 mg/l polymyxin B
Freezing medium for *H. pylori*	BHI-medium, 20 % (v/v) Glycerol, 20 % (v/v) FCS
Freezing medium for *S. typhimurium*	BB-medium, 20 % (v/v) Glycerol
Kanamycin	50 µg/ml (Sigma-Aldrich)
Chloramphenicol	50 µg/ml (Sigma-Aldrich)

2.1.5.2 Reagents for cell culture and stimulation

RPMI 1640 + L-Glutamine (Gln)	(Invitrogen GIBCO)
OPTIMEM	(Invitrogen GIBCO)
DMEM	(Invitrogen GIBCO)
DMEM/Ham's F12 1:1	(Invitrogen GIBCO)
Fetal Bovine Calf Serum (FCS)	(Sigma-Aldrich or PAA)
Penicillin (Pen)	(Invitrogen GIBCO)
Streptomycin (Strep)	(Invitrogen GIBCO)
Ciprofloxacin	(Fresenius Kabi)
Primocin	(InvivoGen)
Gentamycin	(Sigma-Aldrich)
Recombinant human GM-CSF	(Miltenyi Biotec)
Recombinant mouse GM-CSF	(Miltenyi Biotec)
Recombinant human IL-4	(Miltenyi Biotec)
Recombinant mouse IL-18	(MBL)
Medium for generation of MoDCs	RPMI, 1 % (v/v) Pen/Strep, 20 ng/ml recombinant human GM-CSF, 20 ng/ml recombinant human IL-4
Medium for generation of BMDCs	RPMI, 1 % (v/v) Pen/Strep, 50 µM β-Mercaptoethanol, 20 ng/ml recombinant mouse GM-CSF
Medium for generation of BMDMs	DMEM, 1 % (v/v) Pen/Strep, 50 µM β-Mercaptoethanol, 20 % M-CSF (v/v) containing LCCM supernatant (L929 fibroblasts)
Medium for primary epithelial cells	DMEM/Ham's F12 1:1, 1 % (v/v) Pen/Strep, 100 µl/ml Primocin, 10 µl/ml Ciprofloxacin, 10 µg/ml Gentamycin
PBS	(Invitrogen GIBCO)
Accutase	(Sigma-Aldrich)
β-Mercaptoethanol	(Sigma-Aldrich)

Petri dish	(Greiner)
Cell culture plates	(BD or Falcon)
Ultra-pure LPS	(Sigma-Aldrich)
ATP	(Roth)

2.1.5.3 Reagents for cell isolation

Biocoll	(Biochrom)
Human B Cell Isolation Kit II	(Miltenyi Biotech)
Human Monocyte Isolation Kit II	(Miltenyi Biotech)
Human NK Cell Isolation Kit	(Miltenyi Biotech)
Human Pan T Cell Isolation Kit II	(Miltenyi Biotech)

2.1.5.4 Reagents for SDS-Page and Western blot

Acrylamide solution 40 % (w/v) acrylamide / Bis 19:1	(Ambion)
Nitrocellulose membrane	(GE Healthcare)
PVDF membrane	(Millipore)
Molecular Weight Marker	(PeqLab or Bio-Rad)
Anti-mouse/human IL-1β (H-153)	(Santa Cruz)
Anti-mouse IL-1β (AF-401-NA)	(R&D)
Anti-human IL-1βp17 (Asp116)	(Cell Signalling)
Anti-human IL-1β (3A6)	(Cell Signalling)
Anti-mouse IL-18	(Biovision)
Anti-mouse Caspase-1 (Casper-2)	(Adipogen)
Anti-mouse Caspase-1 (M-20)	(Santa Cruz)
Anti-human Caspase-1 (C-20)	(Santa Cruz)
Anti-phospho-Syk (Tyr525/526) (C87C1)	(Cell Signaling)
Anti-Syk (D3Z1E)	(Cell Signaling)
β-actin	(Sigma)
GAPDH	(Cell Signaling)
Anti-mouse Horseradish peroxidase	(Promega)

(HRP)	
Anti-rabbit HRP	(Promega)
Anti-goat HRP	(Promega)
Pierce ® ECL Western Blotting Substrate	(Thermo Scientific)
SuperSignal® West Pico	(Thermo Scientific)
Clarity™ Western ECL Substrate	(Bio-Rad).
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
Stacking gel	500 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 0.1 % (v/v) TEMED, 0.05 % (w/v) APS
Separating gel	375 mM Tris-HCl pH 8.8, 0.1 % (w/v) SDS, 0.1 % (v/v) TEMED, 0.05 % (w/v) APS
Electrophoresis buffer	25 mM Tris-HCl, 200 mM Glycine, 0.1 % (w/v) SDS, pH 8.3
Transfer buffer (wet)	25 mM Tris, 190 mM Glycine, 20 % Methanol or Ethanol
Transfer buffer (semi-dry)	48 mM Tris, 39 mM Glycine, 0.037 % SDS, 20 % Methanol
Blocking buffer	TBST, 5 % milk powder
TBS	15 mM NaCl, 5 mM Tris-HCl, pH 7.5
TBST (Washing buffer)	TBS, 0.1 % (v/v) Tween 20 (AppliChem)
Milk powder	(Roth)
Ponceau solution	0.5 % (w/v) Ponceau (Sigma-Aldrich), 1 % (v/v) acetic acid
SDS buffer	25 mM Tris-HCl pH 8.3, 192 mM Glycine, 0.1 % (w/v) SDS

2.1.5.5 Reagents for ELISA

ELISA Lysis buffer	50 mM HEPES, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 % (v/v) IGEPAL, 10 % (v/v) Glycerol, 20 mM β-Glycerophosphat, 1 mM $NaVO_4$, 0.4 mM PMSF, 1 mM EGTA, Protease inhibitor (Roche)
Anti-human IL-1β Ready-Set Go Kit	(eBioscience)
Anti-human IL-6 Ready-Set Go Kit	(eBioscience)
Anti-human TNFα Ready-Set Go Kit	(eBioscience)
Anti-mouse IL-1β Ready-Set Go Kit	(eBioscience)
Anti-mouse IL-6 Ready-Set Go Kit	(eBioscience)
Anti-mouse TNFα Ready-Set Go Kit	(eBioscience)
Anti-mouse IL-18 (74)	(MBL)
Biotin labelled anti-mouse IL-18 (93-10C)	(MBL)
Recombinant mouse IL-18	(MBL)
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.4)
Washing buffer	PBS; 0.05 % (v/v) Tween 20
Dilution buffer	PBS; 1 % (w/v) BSA
Stop solution	1 M H_3PO_4

2.1.5.6 Reagents for LDH assay

CytoTox 96 ® Non-Radioactive Cytotoxicity Assay	(Promega)

2.1.5.7 Reagents for flow cytometry

FACS buffer	PBS, 1 % BSA
BSA	(AppliChem)
Ethidium monoazide bromide (EMA)	(MoBiTec)

Foxp3 staining buffer set	(eBioscience)
Anti-mouse CD3 (17A2)	(eBioscience)
Anti-mouse CD3ε (1145-2C11)	(eBioscience)
Anti-mouse CD4 (GK1.5)	(eBioscience)
Anti-mouse CD4 (RM4-5)	(eBioscience)
Anti-mouse CD45 (30-F11)	(eBioscience)
Anti-mouse CD8α (53-6.7)	(eBioscience)
Anti-mouse CD8β (eBioH35-17.2)	(eBioscience)
Anti-mouse CD11b (M1/70)	(eBioscience)
Anti-mouse Ly6G/Gr-1 (RB6-8C5)	(eBioscience)
Anti-mouse γδ-TCR (eBioGL3)	(eBioscience)
Anti-mouse CD19 (eBio1D3)	(eBioscience)
Anti-mouse FoxP3(FJK-16s)	(eBioscience)

2.1.5.8 Reagents for PCR and RT-PCR

Hard-Shell®PCR plates 384	(Bio-Rad)
Fram Star 384 well PCR plate	(4titude)
DNeasy® Blood and Tissue Kit	(Qiagen)
RNAlater	(Qiagen)
GenElute™ Mammalian Total RNA Miniprep Kit	(Sigma-Aldrich)
RNeasy® Mini Kit	(Qiagen)
DNA free	(Ambion)
RNase-Free DNase Set	(Qiagen)
Green Taq	(Promega)
SYBR® FAST qPCR Universal 2x Master Mix	(Kapa Biosystems)
SsoAdvanced Universal SYBR Green Supermix	(Biorad)
M-MLV RT, RNase H Minus	(Promega)
Random Primer	(Promega)
dNTPs	(Promega)

Primer	(MWG)
DNA Ladder	(Promega)
TAE buffer	40 mM Tris, 20 mM acetic acid, 1 mM EDTA

Primer to test PMSS1ΔgGT mutant

HPgGT_fw GCGAGTTACCCCCCCATTA

HPgGT_rv AACCTTTTGCCCGATCTCACTAG

Primer for mouse genotyping

Asc JT 4295: CTA GTT TGC TGG GGA AAG AAC
JT 4296: CTA AGC ACA GTC ATT GTG AGC TCC
JT 4297: AAG ACA ATA GCA GGC ATG CTG G

Nlrp3 JT 6030 Ay: AAG TCG TGC TTC ATG T
JT 6031 Ay: TCA AGC TAA GAG AAC TTT CTC
JT 6032 Ay: ACA CTC GTC ATC TTC A

Nlrc4 JT 5215: ATC GTC ATC ACC GTG TGG AGC
JT 5216: TGT CAC CAG GAC GTC CTC GTT C
JT 5217: CTG GAA CTC ACG TTG TAT AGC

Primer for semi-quantitative PCR

mDef1	TGAGCATAAAGGACGAGCGA
	CTCAGGACCAGGCAGATGTT
mDef3	GTCTCCACCTGCTGCTTTTAG
	AGGAAAGGAACTCCACAACTGC
mFoxp3	AGGAGCCGCAAGCTAAAAGC
	TGCCTTCGTGCCCACTGT
mGAPDH	GCACAGTCAAGGCCGAGAAT
	GCCTTCTCCATGGTGGTGAA
mIFNγ	TCAAGTGGCATAGATGTGGAAGAA
	TGGCTCTGCAGGATTTTCATG

mIL-10	CTAGAGCTGCGGACTGCCTTC
	CCTGCTCCACTGCCTTGCTCTTAT
mIL-17	GCTCCAGAAGGCCCTCAGA
	AGCTTTCCCTCCGCATTGA
mIL-18	ACTGTACAACCGCAGTAATAC
	AGTGAACATTACAGATTTATCCC
mIL-1β	CAACCAACAAGTGATATTCTCCATG
	GATCCACACTCTCCAGCTGCA
mIL-23p19	ATGCTGGATTGCAGAGCAGTA
	ACGGGGCACATTATTTTTAGTCT
mKC	TGCACCCAAACCGAAGTCAT
	TTGTCAGAAGCCAGCGTTCAC
mMIP-2	AGTGAACTGCGCTGTCAATGC
	AGGCAAACTTTTTGACCGCC
mTGFβ	ATCCTGTCCAAACTAAGGCTCG
	ACCTCTTTAGCATAGTAGTCCGC
mTNFα	CGATGGGTTGTACCTTGTC
	CGGACTCCGCAAAGTCTAAG

2.1.5.9 Reagents for H&E Staining

Ethanol	(MRI)
Haematoxylin	(Morphisto)
Eosin	(Morphisto)
Roticlear	(Roth)
DPX	(VWR)

2.2 Methods

2.2.1 Cultivation of bacteria

2.2.1.1 *Helicobacter pylori*

H. pylori glycerol stock was thawed, plated on a WC-dent agar plate and cultivated for 2-3 days at 37 °C under microaerophilic conditions (10 % CO_2, 5 % O_2, 85 % N_2). For experiments *H. pylori* was splitted at least once on a new plate. Isogenic mutant strains were grown on WC-dent plates containing 50 µg/ml of kanamycin or chloramphenicol for selection under the same conditions. Bacteria were harvested, resuspended in cell culture medium and directly used for experiments. The bacterial number was determined by measurement of the optical density (OD_{600}). OD_{600}=1 corresponds to ~2x 10^8 *H. pylori*/ml.

For preparation of aliquots *H. pylori* was harvested from WC-dent agar plates and resuspended in BHI-dent, 20 % FCS, 20 % glycerol. Aliquots were immediately frozen in liquid nitrogen and stored at -80 °C.

2.2.1.2 *Salmonella typhimurium*

Frozen glycerol stocks were thawed, plated on a Columbia plate and cultivated for 1 day at 37 °C under microaerophilic conditions (10 % CO_2, 5 % O_2, 85 % N_2). Bacteria were harvested, resuspended in medium and directly used for experiments. The bacterial number was determined by measurement of the optical density (OD_{600}). OD_{600}=1 corresponds to ~2x 10^9 *S. typhimurium*/ml.

For preparation of glycerol stocks bacteria were harvested from a Columbia plate and resuspended in Brucella Broth medium, 20 % glycerol. Aliquots were immediately frozen in liquid nitrogen and stored at -80 °C.

2.2.2 Treatment of *H. pylori*

For PFA-fixation, *H. pylori* was harvested and resuspended in 4 % paraformaldehyde. After at least 4 h incubation at 4 °C, fixed bacteria were washed with PBS. After final resuspension in medium, bacterial suspension was stored at 4 °C until use.

For heat-inactivation *H. pylori* was harvested and resuspended in medium. Afterwards, *H. pylori* was killed by heating at 90 °C for 5 min.

2.2.3 Isolation and generation of cells

2.2.3.1 Bone marrow-drived dendritic cells (BMDCs)

For generation of murine BMDCs, bone marrow was flushed out of femur and tibia, and filtered. ~8×10^6 cells were plated in 10 ml medium per petri dish. BMDCs were cultivated in RPMI medium, 10 % (v/v) FCS, 1 % (v/v) Pen/Strep, 50 µM β-Mercaptoethanol and 20 ng/ml recombinant GM-CSF. On day 2 5 ml fresh medium supplemented with 20 ng/ml GM-CSF was added. After further 3 days, 5 ml fresh medium including GM-CSF was added. At day 6, cells were harvested, seeded in OPTIMEM medium on cell culture plates (1×10^6 cells per ml), rested for at least one hour and used for experiments.

2.2.3.2 Bone marrow-drived macrophages (BMDMs)

For generation of murine BMDMs, bone marrow was flushed out of femur and tibia and filtered. ~5×10^6 cells were plated in 10 ml medium per petri dish. BMDMs were generated in DMEM medium, 10 % (v/v) FCS, 1 % (v/v) Pen/Strep, 50 µM β-Mercaptoethanol and 20 % LCCM supernatant. On day 3, cells were supplemented with 4 ml medium and 1 ml M-CSF containing supernatant. After 3 days, again fresh medium and 1 ml LCCM supernatant were added. At day 6, cells were harvested by detaching the cells with accutase. Cells were seeded in OPTIMEM medium on cell culture plates (1×10^6 cells per ml), rested for at least one hour and used for experiments.

2.2.3.3 Peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMC) were isolated from *H. pylori* negative donors by density gradient centrifugation with Biocoll (Biochrom, Germany). Therefore, blood was diluted 1:1 with PBS (w/o Ca^{2+} and Mg^{2+}), and Biocoll solution was overlaid with the same volume of PBS-blood and centrifuged for 25 min at room temperature (450 g, without break). Cells from the interphase, containing the mononuclear cells were harvested and washed at least 3 times with PBS (7 min, 300 g).

Cells were resuspended in medium (1×10^6 cells/ml), plated on cell culture plates, and rested overnight before they were used for experiments.

2.2.3.4 Monocytes, B-cells, NK-cells and T-cells

Human monocytes, B cells, NK cells or T cells were isolated from PBMCs by negative selection with the Monocyte Isolation Kit II, B Cell Isolation Kit II, NK Cell Isolation Kit or Pan T Cell Isolation Kit II, respectively (all Miltenyi Biotech). Cell number was determined and cells were washed with cold MACS buffer (PBS (pH 7.2), 0.5 % (w/v) BSA, 2 mM EDTA). After centrifugation (7 min, 300 g), cells were isolated according manufacturer's instructions. Cells were resuspended in medium (1×10^6 cells/ml), plated on cell culture plates, and rested overnight before they were used for experiments.

2.2.3.5 Monocyte-drived DCs (MoDCs)

For generation of MoDCs, ~8×10^6 monocytes were cultivated in 10 ml RPMI medium, 10 % (v/v) FCS, 1 % (v/v) Pen/Strep, 20 ng/ml recombinant GM-CSF and 20 ng/ml recombinant IL-4. The next day cells were supplemented with further 20 ng/ml GM-CSF and IL-4. After 3 days, 10 ml fresh medium was added. At day 6, cells were harvested, plated on cell culture plates, and used for experiments.

2.2.3.6 Primary gastric epithlial cells

Primary gastric epithelial cells were isolated from glandular stomach of 7 to 21 days old mice. After rinsing with PBS, stomach was digested in Hank's medium with 20 mM EDTA (7 min, 37 °C, 100 rpm). Afterwards, tissue was transferred to a petri dish and pressed between two glass slides to release cells. After rinsing the glass slides and petri dish with DMEM/ Ham's F12 1:1 medium (Gibco) to collect the cells, cells were transferred to a Falcon tube. After centrifugation (10 min, 300 g), cells were extensively resuspended in DMEM/ Ham's F12 1:1 medium with 10 % (v/v) FCS, 1 % (v/v) Pen/Strep, 50 µg/ml Gentamycin, 10 µg/ml Ciprofloxacin and 100 µg/ml Primocin. Cells were seeded on rat-tail collagen I (10 µg/cm^2) coated cell culture plates, and incubated overnight at 37 °C.

For coating, wells were incubated with 100 µg/ml rat-tail collagen in 0.02 % acetic acid solution for 1 hour. After removal of the coating solution, plates dried overnight.

Before infection with *H. pylori*, cells were washed with PBS to remove antibiotics. For infection, antibiotic free DMEM/ Ham's F12 1:1 medium was added.

2.2.4 Stimulation and infection of cells

For inflammasome induction studies cells were prestimulated with LPS for 3 hours where indicated. For murine BMDCs and BMDMs 10 ng/ml LPS were used. Murine neutrophils were incubated with 50 ng/ml of LPS. For all human cells (B cells, NK cells, T cells, PBMCs and MoDCs) 5 ng/ml were used. After TLR-priming cells were infected with *H. pylori.* For this, *H. pylori* was harvested and resuspended in cell culture medium. For *H. pylori* infection a multiplicity of infection (MOI) between 1 and 10 was used for human cells and a MOI between 1 and 50 for murine cells. Cells were incubated with *H. pylori* between 0.5 hours and overnight. Control stimulations were performed with 5 mM ATP for 1 h and *S. typhimurium* (MOI 10) for 6 h. In some experiments, BMDCs were stimulated with heat-inactivated (90 °C, 5 min) or PFA-fixed (4 h at 4 °C) *H. pylori*. Chemical inhibitors were added 30 min before stimulation with *H. pylori*, *S. typhimurium*, ATP or Alum. For infection of primary epithelial cells MOI of 10 was used.

For harvesting, plates were centrifuged (5 min, 300 g) and supernatants were transferred to a 96-well plate or to 1.5 ml tubes. Cell free supernatant was stored at -20 °C until use. Cells were lysed in SDS buffer and stored at -20 °C until use. For RNA isolation cells were lysed directly in the lysis buffer of the RNeasy® Mini Kit (Qiagen).

2.2.5 Protein precipitation and cell lysis

2.2.5.1 Trichloroacetic acid (TCA) precipitation

For precipitation and concentration of proteins from cell culture supernatants TCA precipitation was used. One volume of cell free supernatant of the stimulated immune cells was transferred to a 1.5 ml tube and mixed with one volume of 20 % TCA. After at least 1 h incubation on ice, suspension was centrifuged (15.000 g, 20 min, 4 °C), the

supernatant was removed and the protein pellet was washed with 500 µl acetone. After centrifugation and a second wash with acetone, acetone was removed, the pellet was dried at room temperature, and resuspended in 20 µl SDS buffer and 5 µl 5x SDS sample buffer. After 10 min denaturation at 95 °C the protein suspension was frozen at -20 °C or used directly for SDS-PAGE.

2.2.5.2 Cell lysis

For determining pro-IL-β in cell lysates by ELISA, cells were lysed in ELISA lysis buffer for 20 min on ice. After centrifugation (15.000 g, 20 min, 4 °C), supernatant was transferred to a new tube and used immediately.

For Western Blot analysis, the cells were lysed in 1xSDS sample buffer. After 5 min sonication, protein suspension was denaturated for 10 min at 95 °C. The protein suspension was frozen at -20 °C or used directly for SDS-PAGE.

2.2.6 ELISA

For determination of cytokine response upon *H. pylori* infection, cytokine concentrations of culture supernatants and stomach homogenates (preparation see 2.2.14.2.5) were measured by sandwich ELISA. In brief, ELISA plates were coated overnight at 4 °C with the respective capture antibody. After washing with washing buffer, free binding sites were blocked with assay solution for 1 h at room temperature. After washing, standard and diluted samples were added and incubated overnight at 4 °C. Following washing, the biotin-conjugated detection antibody was added for 1 h at room temperature. After removal of unbound antibodies by washing, avidin-HRP was added for 30 min. After washing, substrate TMB was added and the reaction was stopped after 15 min adding 1 M H_3PO_4. The optical density (OD) was measured in the Sunrise ELISA microplate reader at 450 nm with 570 nm correction. After preparing a standard curve by plotting the OD of each standard versus its concentration, the protein concentration of the samples was calculated by comparing the optical density of the unknown samples to the standard curve.

2.2.7 Cytotox assay

For determination of cell death upon *H. pylori* infection, culture supernatants were harvested and the amounts of lactate dehydrogenase (LDH) were measured by CytoTox® 96 Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer's instructions. Therefore, supernatants were put on a 96 well assay plate. Reconstituted substrate mix was added 1:1 to the supernatants. After 30 min incubation in the dark, stop solution was added and absorbance was measured by 490 nm using the Sunrise™ ELISA microplate reader (Tecan).

2.2.8 Bacterial uptake assay

Bacterial uptake of *H. pylori* was evaluated using a gentamicin protection assay. Briefly, BMDCs were infected for 60 min and then incubated for 45 min at 37 °C in medium containing gentamicin (100 µg/ml) to kill extracellular bacteria. Cells were then washed in PBS, lysed in 0.5 % Saponin/PBS, and the number of intracellular bacteria was determined by plating on WC-dent blood agar plates.

2.2.9 BSA assay

To calculate protein contents of gastric homogenates, Pierce™ BCA Protein Assay (Thermo Scientific) was performed according manufacturer's instructions. In short, working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. In addition, a specific BSA standard was prepared in serial dilutions in PBS. 25 µl of each standard or probe was transferred onto a microplate well and 200 µl working reagent was added. After mixing on a plate shaker for 30 s, the plate was incubated at 37 °C for 30 min. After cooling the plate to room temperature, the absorbance was measured at 562 nm using the Sunrise™ ELISA microplate reader. The protein concentration of probes was determined by comparing the absorption of the unknown samples to the plotted absorption of each BSA standard versus its concentration.

2.2.10 Flow Cytometry

2.2.10.1 Surface staining

To analyse the immune response in the stomach, fluorescence-activated cell sorting (FACS) with different fluorophore-conjugated antibodies was performed. To reduce non-specific binding of fluorescence-labelled antibodies and to discriminate live and dead cells, cells were incubated with Fc-block™ (α-CD16/32; 1:500 in FACS buffer) and EMA (1:1000 in FACS buffer) for 20 min on ice under light. After washing (FACS buffer, 5 min, 300 g), staining of surface molecules was performed. For this, cells were incubated with the respective fluorescence-labelled antibodies for 30 min at 4°C in the dark. After washing, cells were either resuspended in FACS buffer/1 % PFA for analysis or prepared for staining of intracellular antigens.

2.2.10.2 Staining of transcription factors

Following the surface staining, cells were washed and resuspended in Foxp3 Fixation/ Permeabilization solution (eBioscience) for 30 min at 4 °C in the dark. After washing with permeabilization buffer, cells were incubated in this buffer with Foxp3 antibody for 30 min at 4 °C in the dark. After washing with permeabilization buffer, cells were resuspended in FACS buffer/1 % PFA and analysed by flow cytometry.

2.2.11 SDS-Polyacrylamide gel electrophoresis and Western Blot

For SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) equal amounts of cell lysate or precipitated supernatant were loaded on a SDS gel and the proteins were separated according to their size. Gels were run at 150 V for 1-1.5 h. Afterwards proteins from cell lysates were transferred to a nitrocellulose membrane at 230 mA (wet blot) or 90 mM/membrane (semi-dry blot) for 2 h. For blotting precipitated proteins from supernatants, PVDF membrane was used (100 mM for 1 h, wet blot). After blocking (5 % milk/TBST, 1 h), membranes were incubated with the appropriate amount of primary antibody in TBST/5 % BSA overnight at 4 °C. After 3-times washing with TBST for 5 min, the HRP-labelled secondary antibody was incubated in TBST/5% milk powder

for 2 h at room temperature. After washing (5-times, 10 min) membranes were developed with Pierce® ECL Western Blotting Substrate, SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) or Clarity™ Western ECL Substrate (Bio-Rad). After incubation, the membrane was exposed to medical x-ray films. X-ray films were developed in the Curix 60 developer or membranes were detected with a digital ECL imager (INTAS science imaging).

To detect another protein on the same membrane, the membrane was washed 3-times with TBST and incubated with Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific) for 15 min. After washing and blocking, the membrane was incubated again with a primary antibody overnight, and detected as described before.

2.2.12 *H. pylori* transformation

For natural transformation of *H. pylori*, genomic DNA of *H. pylori* was isolated with the DNeasy® Blood & Tissue Kit (Qiagen) according manufacturer's instructions.

The acceptor *H. pylori* strain (e.g. wild type strain) was resuspended in BHI/20 % FCS and mixed with 2 µg of donor DNA (e.g. from mutant strain). Following incubation for 60 min at 37 °C, cells were plated onto WC-dent agar medium that contains selective antibiotic. After 2-5 days, single colonies were expended on antibiotic-containing WC-dent plates. After DNA isolation, colonies were tested by PCR, or bacteria were lysed to perform Western Blot.

This technique was used to generate a gGT-mutant in PMSS1 strain. DNA from SS1ΔgGT served as donor DNA.

2.2.13 Polymerase chain reaction (PCR)

2.2.13.1 *H. pylori* genotyping PCR

In order to proof *H. pylori* PMSS1ΔgGT clones, genomic DNA was isolated by DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's instructions and PCR was performed using primers described in section 2.1.5.8. To determine the specific length of the DNA fragment, the PCR product was separated by agarose-gel electrophoresis. Agarose was boiled up in TAE buffer. After cooling down, the fluorescence staining agent Roti-Safe was added to the solution (1:20), and gels were cast in horizontal

chambers. After polymerization of the gel, PCR products were loaded in the gel pockets and DNA was separated by applying 90 V for 60 min. Since Roti-Safe intercalate with the DNA the PCR fragments were detected using UV light.

PCR mix:

Forward Primer [10 µM]	1µl
Reverse Primer [10 µM]	1 µl
Green Taq	7.5 µl
H_2O	4.5 µl
DNA [50 ng]	1 µl

PCR program

Initial denaturation	94 °C	5 min	
Denaturation	94 °C	45 s	35 x
Annealing	55 °C	30 s	
Elongation	72 °C	45 s	
Final elongation	72 °C	5 min	
Storage	4 °C	∞	

Amplification products: 2500 bp mutant allele, 1500 bp wild type allele

2.2.13.2 PCR for mouse genotyping

To genotype the mouse knockout strains PCR reactions were performed. Mouse tails (0.5 cm) were digested in 500 µl tailing buffer (100 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl, 100µg/ml proteinase K) overnight using a thermomixer (56 °C, 650 rpm). The lysate was then centrifuged (13 000 g, 10 min). The supernatant was added 1:1 to isopropanol, which led to the precipitation of the DNA. After centrifugation and removal of isopropanol, the DNA pellet was dried at room temperature. DNA was suspended in water and stored at -20 °C or was directly used

for PCR. After PCR reaction agarose-gel electrophoresis was performed to determine the length of the amplified PCR product.

The following PCR mixture and PCR-programs were used for genotyping the different knockout mice (primers see section 2.1.5.8):

Nlrp3- deficient mice

PCR mix:

Primer JT 6030 [10 µM]	0.5 µl
Primer JT 6031 [10 µM]	0.5 µl
Primer JT 6032 [10 µM]	0.5 µl
Green Taq	7.5 µl
H_2O	5 µl
DNA	1 µl

PCR program

Initial denaturation	95 °C	5 min	
Denaturation	95 °C	45 s	39 x
Annealing	55 °C	30 s	
Elongation	72 °C	40 s	
Final elongation	72 °C	5 min	
Storage	4 °C	∞	

Amplification products: 500 bp mutant allele, 250 bp wild type allele.

Asc-deficient mice

PCR mix:

Primer JT 4295 [10 µM]	1 µl
Primer JT 4296 [10 µM]	1 µl
Primer JT 4297 [10 µM]	1 µl
Green Taq	7.5 µl
H_2O	3.5 µl
DNA	1 µl

PCR program

Initial denaturation	94 °C	5 min	
Denaturation	94 °C	15 s	3 x
Annealing	60 °C	40 s	
Elongation	72 °C	30 s	
Denaturation	94 °C	15 s	36 x
Annealing	57 °C	25 s	
Elongation	72 °C	30 s	
Final elongation	72 °C	5 min	
Storage	4 °C	∞	

Amplification products: 260 bp mutant allele, 450 bp wild type allele.

Nrc4-defiecent mice

PCR mix:

Primer JT 5215 [10 µM]	1 µl
Primer JT 5216 [10 µM]	1 µl
Primer JT 5217 [10 µM]	1 µl
Green Taq	7.5 µl
H_2O	3.5 µl
DNA	1 µl

PCR program

Initial denaturation	94 °C	5 min	
Denaturation	94 °C	45 s	35 x
Annealing	55 °C	30 s	
Elongation	72 °C	45 s	
Final elongation	72 °C	5 min	
Storage	4 °C	∞	

Amplification products: 657 bp mutant allele, 839 bp wild type allele.

2.2.14 *In vivo* experiments

For *in vivo* experiments, 6-10 week old mice were used (description of mouse strains 2.1.3). Mice were bred and housed in the animal facility of the Institut für medizinische Mikrobiologie, Immunologie und Hygiene (MIH). Different knockout and wild type mice were infected with *H. pylori* strains PMSS1 or SS1. After one month of infection, the mice were sacrificed and histological (2.2.14.2.6), immunological (2.2.14.2.2 - 2.2.14.2.5) and bacterial (2.2.14.2.1) parameters were analysed.

2.2.14.1 Infection

Before inoculation with the bacterium, mice were starved for 5 hours. Mice were infected orally using a feeding needle. The infection was repeated on day 3 and 5. *H. pylori*, cultured in microaerobic conditions as described under 2.2.1.1, was harvested immediately before inoculations with a sterile inoculation loop and resuspended in Brucella broth. After determination of the optical density at 600 nm, bacterial cell suspensions were diluted to allow intragastric inoculation of ~10^8 cfu/0.2 ml to each mouse.

2.2.14.2 Analysis

For analysis mice were sacrificed with cervical dissociation or CO_2. After removal of the stomach, stomach was opened along the lesser curvature, washed with PBS and dissected longitudinally into equal strips. Of every stomach, the same section was assigned to the same downstream processing to reduce sampling error.

2.2.14.2.1 Quantification of bacteria in the mouse stomach

For quantitative assessment of *H. pylori* colonisation, one stomach section was homogenized in Brucella broth and serial dilutions were plated on WC-dent agar plates, supplemented with bacitracin (200 µg/ml), nalidixic acid (10 µg/ml) and polymyxin B (3 µg/ml).

The plates were incubated under microaerophilic conditions for 5 days before colonies were counted.

2.2.14.2.2 Isolation of stomach cells for FACS staining

One stomach piece was put in RPMI and placed on ice. After adding collagenase (Sigma-Aldrich) and Dnase I (Roche) in a final concentration of 1 mg/ml and 200 µg/ml, respectively, stomach sections were digested for 30 min (37 °C, shaking at a speed of 200 rpm). After mechanical disruption of the digested tissue between glass slides and filtering through a 70 µm cell strainer, cells were washed at least two times with RPMI medium, before they were resuspended in FACS buffer for staining of surface markers (2.2.10.1) and transcription factors (2.2.10.2).

2.2.14.2.3 mRNA extraction, DNase treatment and cDNA preparation

One stomach piece was put in RNA*later*™ RNA Stabilization Reagent (Qiagen) and stored overnight in the fridge. After transferring the stomach piece to a new tube, sections were stored at -80 °C until RNA isolation. Total gastric RNA was isolated using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) or RNeasy® Mini Kit (Qiagen) according to manufacturer's instructions. For homogenisation, stomach tissues were put into lysis buffer followed by homogenization using a T10 basix ULTRA-TURRAX Disperser. In order to remove genomic DNA, the total RNA extracts were subjected to on column DNase treatment using the RNase free DNase kit (Qiagen) according to the manufacturer's instructions. After elution, the concentration of the RNA was measured using a NanoDrop 1000 spectrophotometer. All samples prepared for one PCR run were adjusted to the same mRNA concentration (between 100 and 250 ng/µl) in a total volume of 35 µl nuclease-free water. RNA was transcribed into complementary DNA using the M-MLV reverse transcriptase RNase H (-) point mutant kit (Promega) following the manufacturer's protocol. For each sample 30 µl total RNA, 9 µl ddH_2O and 3 µl random primers (150 ng/µl) were mixed. The reaction mixtures were incubated at 70 °C for 5 min to melt secondary structures, and subsequently rapidly chilled on ice for 5 min to prevent re-forming of secondary structures. After brief centrifugation, 14 µl were removed and put into a new tube. To this tube 5 µl 5x M-MLV buffer, 1.25 µl dNTPs and 4.75 µl nuclease-free water were added. This mixture serves as a negative control for reverse transcription. To the remaining 28 µl of the first tube 10 µl 5x M-MLV buffer, 2.5 µl dNTPs, 2 µl M-MLV reverse transcriptase RNase H (-) point mutant and 7.5 µl nuclease-free water were added. The samples were then incubated at RT for 10 min, at 50 °C for 50 min and at 70 °C for 15 min. Afterwards, cDNAs were diluted 1:5 in nuclease-free water and either directly used as templates for RT-PCR analysis or stored at -20 °C.

2.2.14.2.4 Semi-quantitative PCR

For semi-quantitative PCR analysis, cDNAs were generated as described above (2.2.14.2.3). For analysis of gene expression, 4 µl cDNA, 0.5 µl forward primer [10 µM], 0.5 µl reverse primer [10 µM] and 5 µl 2x Master Mix (Kapa Biosystems or Biorad) were

mixed. The PCR was performed with Hard-Shell®PCR plates 384 (Bio-Rad) or 4titude Fram Star 384 (4titude) on the CFX384™ Real-Time PCR cycler (Bio-Rad).

For determining gene expression following two-step PCR program was used.

Initial denaturation	95 °C	3 min	
Denaturation	95 °C	10 s	39 x
Annealing	60 °C	30 s	
Melting curve	65-95 °C	0.5 /s	
Storage	12 °C	∞	

Primers used for PCR analysis are listed in 2.1.5.8. Results were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as an endogenous control. For analysis of the resulting data, the $2^{-\Delta\Delta Ct}$ method was used in order to calculate relative changes in gene expression.

2.2.14.2.5 Sample preparation for ELISA

For detection of cytokines from gastric mucosal extracts, stomach pieces were homogenised in PBS with Roche protease inhibitor. After determining whole protein amount with BSA assay (2.2.9), 5 µg protein per well in PBS were used for ELISA. ELISA was performed according to the manufacturer's instructions (see 2.2.6).

2.2.14.2.6 Histology

For assessment of gastric histopathology, stomach stripes were fixed in 4 % phosphate-buffered formalin for at least 24 h in the fridge. After dehydrating, stomach stripes were paraffin-embedded and 3-4 µm sections were cut with a microtome (Leica). Sections were stained with hematoxylin and eosin to analyse immune cell infiltration. In brief, slides were incubated at 60 °C for 20 min before three changes of xylene (each 10 min) were performed. Afterwards, slides were rehydrated in ethanol by progressively less concentrated ethanol (two changes in absolute ethanol, followed by one change in 90 % ethanol, 70 % ethanol and 50 % ethanol for 5 min each). After washing with water, slides were stained with Mayer's hematoxylin (Morphisto) for 5 min. After washing in running tap water tissue was counterstained in eosin solution (Morphisto) for 5 min. After washing with tap water, slides were dehydrated in 80 %

ethanol for 1 min, followed by two changes of absolute ethanol for 5 min. After three changes of xylene for 5 min, each biopsy was mount with DPX mounting medium.

A pathologist (M. Vieth, Bayreuth) then graded the slides according to the updated Sydney system (336) for gastritis, degree of atrophy, and intestinal metaplasia.

3 Results

Parts of the results presented here, have been published in The Journal of Immunology titled as "*Helicobacter pylori*-induced IL-1β secretion in innate immune cells is regulated by the NLRP3 inflammasome and requires the Cag Pathogenicity Island"(338).

3.1 Analysis of *H. pylori* mediated IL-1β and IL-18 induction *in vitro*

IL-1β and IL-18 are cytokines that are highly upregulated upon *H. pylori* infection (316, 319) and linked to the development of gastric malignancies (320, 321, 325). Both cytokines are expressed as precursors and have to be cleaved by the inflammasome. However, to date, the mechanism and virulence factors involved in the induction of mature IL-1β are still unknown.

3.1.1 *H. pylori* induces expression of pro-IL-1β and secretion of mature IL-1β in murine and human immune cells

To distinguish between priming and inflammasome activation, immune cells were prestimulated with LPS which leads to the accumulation of pro-IL-1β and upregulation of Nlrp3 but not caspase-1 activation (339). As a first step, time course experiments were performed, to elucidate the accumulation of pro-IL-1β upon LPS prestimulation over time.

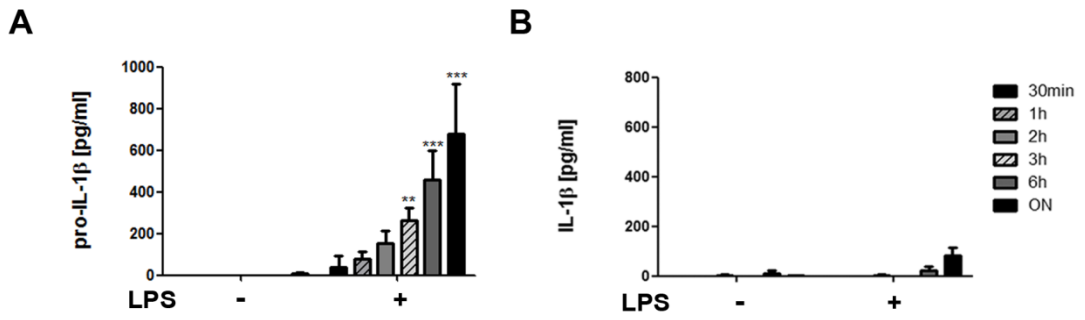

Figure 6: LPS stimulation of BMDCs induces pro-IL-1β but not the secretion of its mature form.

(A) Murine BMDCs were stimulated with LPS (10 ng/ml). Production of pro-IL-1β was measured at the indicated time points in the cell lysate by ELISA. Results are presented as mean±SD of three independent experiments. LPS-stimulated cells were compared to uninfected control cells of the respective time point (asterisks shown on the top of the bars) ($p<0.0001$ ANOVA, **≤0.01, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(B) Murine BMDCs were stimulated with LPS (10 ng/ml). Production of IL-1β was measured at the indicated time points in the supernatant by ELISA. Results are presented as mean±SD of three independent experiments.

Stimulation with LPS led to the induction of pro-IL-1β in a time-dependent manner. After 3 hours of stimulation, significant amounts of pro-IL-1β were produced and increased over time (Figure 6A). In contrast, there was no cleavage to mature IL-1β upon LPS stimulation (Figure 6B). Thus, prestimulation for 3 hours is sufficient to accumulate significant amounts of pro-IL-1β to be cleaved by the inflammasome upon its activation. Therefore, in further experiments cells were prestimulated for at least 3 hours with LPS.

Next, time course experiments with LPS prestimulated cells were performed to analyse *H. pylori*-induced secretion of mature IL-1β.

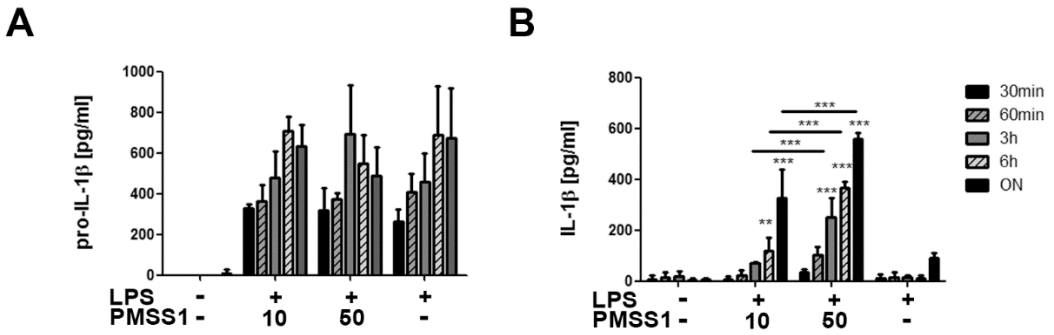

Figure 7: *H. pylori* induces IL-1β secretion in LPS-primed murine BMDCs in a time-dependent manner.

(A) Murine BMDCs were prestimulated with LPS (10 ng/ml for 3 hours) and infected with the *H*. *pylori* strain PMSS1 at MOI 10 and 50. At the indicated time points, amounts of pro-IL-1β were measured in cell lysates by ELISA. Results are presented as mean±SD of three independent experiments.

(B) Murine BMDCs were prestimulated with LPS (10 ng/ml for 3 hours) and infected with the H. *pylori* strain PMSS1 at MOI 10 and 50. At the indicated time points, amounts of mature IL-1β were measured in supernatants by ELISA. Results are presented as mean±SD of three independent experiments. LPS prestimulated and *H. pylori*-infected cells were compared to LPS only-stimulated control cells of the respective time point (asterisks shown on the top of the bars) ($p<0.0001$ ANOVA, *≤0.05, ***≤0.001 Bonferroni-corrected for pairwise comparison).

Upon stimulation, levels of pro-IL-1β were increasing until the cleavage of IL-1β started. After 3 hours (MOI 50) and 6 hours (MOI 10) post *H. pylori* infection, mature IL-1β was secreted, while IL-1β amounts were increasing over time, the levels of the pro-form decreased in parallel (Figure 7A and B). In contrast, in LPS only-stimulated cells, the pro-IL-1β levels did not decline over time as no cleavage occurred (Figure 7A and B).

From 6 hours onwards, mature cytokine was present with both MOIs. In further experiments, IL-1β secretion was analysed after 6 hours or ON stimulation with *H. pylori*.

As a next step, time course experiments were performed, to analyse if *H. pylori* alone can induce IL-1β secretion, meaning that *H. pylori* can provide the first and second signal for inflammasome activation.

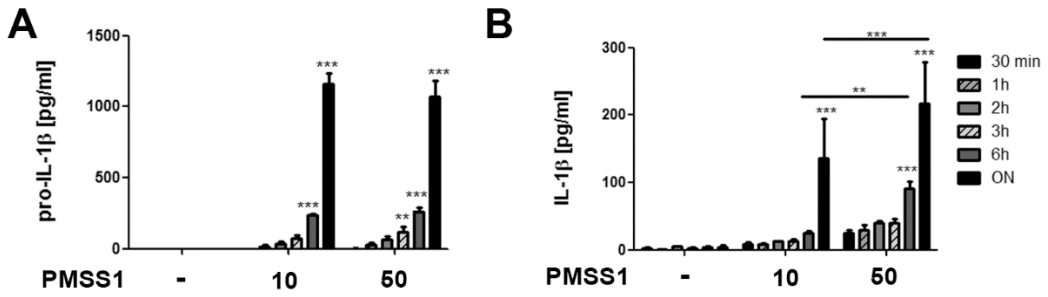

Figure 8: *H. pylori* induces pro-IL-1β and secretion of mature IL-1β in murine BMDCs in a time-dependent manner.

(A) Murine BMDCs were infected with the *H. pylori* strain PMSS1 at MOI 10 and 50. At the indicated time points, amounts of pro-IL-1β were measured in cell lysate by ELISA. Results are presented as mean±SD of three independent experiments. *H. pylori*-infected cells were compared to non-infected control cells of the respective time point (asterisks shown on the top of the bars) ($p<0.0001$ ANOVA, **≤0.01, ***≤0.001 Bonferroni-corrected for pair-wise).

(B) Murine BMDCs were infected with the *H. pylori* strain PMSS1 at MOI 10 and 50. At the indicated time points, amounts of IL-1β were measured in supernatants by ELISA. Results are presented as mean±SD of three independent experiments. *H. pylori*-infected cells were compared to non-infected control cells of the respective time point (asterisks shown on the top of the bars) ($p<0.0001$ ANOVA, **≤0.01, ***≤0.001 Bonferroni-corrected for pair-wise comparison).

Infection of BMDCs with *H. pylori* led to the production of pro-IL-1β in a time-dependent manner (Figure 8A). After 6 hours incubation with *H. pylori* at a MOI50 secretion of mature IL-1β was induced. With a lower MOI, secretion of the mature form started at a later time (Figure 8B).

In LPS-primed cells, secretion of IL-1β was induced earlier and at higher levels compared to none-primed cells (compare Figure 7A and Figure 8A). This means that *H. pylori* induces IL-1β production and can provide the priming and activation signal.

H. pylori could induce IL-1β secretion in BMDCs cells. However, besides BMDCs, *H. pylori* has contact to other immune cells. Therefore, we tested several human and murine immune cells for the secretion of IL-1β upon *H. pylori* stimulation.

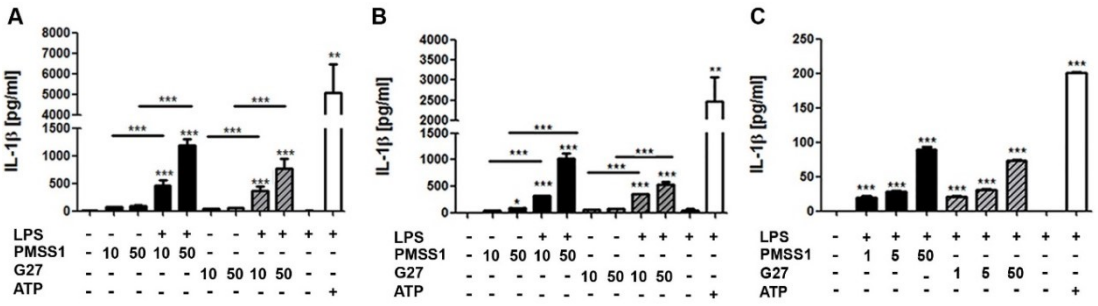

Figure 9: *H. pylori* induces IL-1β secretion in different murine immune cells.

(A) Murine BMDCs were infected with the *H. pylori* strains PMSS1 or G27 overnight at MOI 10 and 50, and the secretion of IL-1β was measured in the supernatants by ELISA. For prestimulation, LPS (10 ng/ml for 3 hours) was used. As a positive control, cells were stimulated with ATP (5 mM) for 1 hour. Results are presented as mean±SD of three independent experiments. *H. pylori*–infected BMDCs were compared with uninfected control cells, whereas infected and LPS-prestimulated cells were compared with LPS only-stimulated control cells (asterisks shown on top of the bars) ($p<0.0001$ ANOVA, **p≤0.01, ***p≤0.001, Bonferroni-corrected for pairwise comparison).

(B) Murine bone marrow derived macrophages (BMDM) were infected with the *H. pylori* strains PMSS1 or G27 o/n at MOI 10 and 50 and the secretion of IL-1β was measured in the supernatants by ELISA. For prestimulation, LPS (10 ng/ml for 3 hours) was used. One-hour stimulation with ATP (5 mM) was used as a positive control. Results are presented as mean±SD, of three independent experiments. *H. pylori*-infected cells were compared to uninfected control cells, while infected and LPS-prestimulated BMDMs were compared to LPS only-stimulated control cells (asterisks shown on top of the bars) ($p< 0.0001$ ANOVA, *p≤0.05, **p≤0.01, ***p≤0.001 Bonferroni-corrected for pairwise comparison).

(C) IL-1β levels detected in the supernatants of neutrophils infected with the *H. pylori* strains PMSS1 or G27 o/n at MOI 1, 5 and 50. LPS (50 ng/ml for 3 hours) was used to prestimulate cells where indicated. As a positive control, ATP (5 mM) was used. Results are presented as mean±SD of one experiment performed in triplicates. *H. pylori*-infected and LPS-prestimulated neutrophils were compared to LPS only-stimulated control cells (asterisks shown on top of the bars) ($p<0.0001$ ANOVA, ***p≤0.001 Bonferroni-corrected for pairwise comparison).

Infection with *H. pylori* induced secretion of mature IL-1β in BMDCs (Figure 9A), BMDMs (Figure 9B) and neutrophils (Figure 9C). The secretion of IL-1β was not restricted to the PMSS1 strain, as infection with the G27 strain triggered IL-1β secretion in an MOI-dependent manner in all three cell types analysed. DCs produced slight higher amounts of IL-1β than macrophages, whereas cytokine production in neutrophils was inferior compared to the other two cell types. These diverse levels of IL-1β secretion were not only seen in *H. pylori*-induced production, but also in ATP-stimulated cells. This is in accordance with other studies (340).

H. pylori induced IL-1β in different murine innate immune cells albeit at different levels. Thus, as a next step, *H. pylori*-induced IL-1β production in human immune cells was analysed.

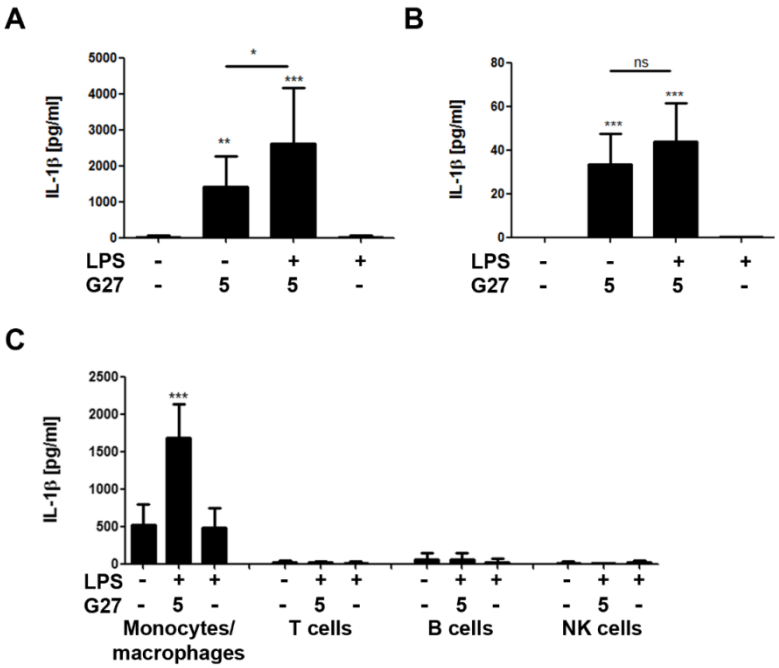

Figure 10: *H. pylori* induces IL-1β secretion in human innate immune cells.

(A) Human PBMCs were infected with the *H. pylori* strain G27 at MOI 5. Cells were prestimulated with 5 ng/ml LPS where indicated. After overnight culture, IL-1β secretion was measured in the supernatants by ELISA. Results are presented as mean±SD from nine different healthy donors. *H. pylori*–infected cells were compared with uninfected control cells, whereas infected and LPS-prestimulated PBMCs were compared with LPS only-stimulated control cells (asterisks shown on top of the bars) ($p < 0.0001$ ANOVA, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, Bonferroni-corrected for pairwise comparison).

(B) Human MoDCs were infected o/n with the *H. pylori* strain G27 at MOI 5. Secretion of IL-1β was measured in the supernatants by ELISA. Cells were prestimulated with 5 ng/ml LPS for 3 hours where indicated. Results are presented as mean±SD of seven different healthy donors. *H. pylori*-infected cell were compared to uninfected control cells, while LPS-prestimulated cells were compared to LPS only-stimulated control cells (asterisks shown on the top of the bars) ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(C) IL-1β release was measured in the supernatants of monocytes/macrophages, T cells, B cells, and NK cells isolated from total PBMCs by MACS. Cells were infected overnight with *H. pylori* G27 at MOI 5. For prestimulation, LPS (5 ng/ml for 3 hours) was used. Results are presented as mean±SD of three different donors. Asterisks on top of the bars indicate significance relative to LPS only-stimulated control cells ($p<0.0001$ ANOVA, ***$p \leq 0.001$ Bonferroni-corrected for pairwise comparison).

H. pylori triggered IL-1β secretion in human PBMCs. As in murine innate immune cells, there were higher amounts when cells were prestimulated with LPS (Figure 10A). Since PBMCs consist of different immune cells, the major source of *H. pylori*-induced IL-1β

secretion should be identified. For answering this, negative selection was performed to isolate monocytes/macrophages, T cells, B cells and NK cells from PBMCs. After prestimulation with LPS and incubation of these cells with *H. pylori*, IL-1β secretion was exclusively observed in monocytes/macrophages (Figure 10C). As there are only quite low amounts of DCs in human blood (1-2 %), DCs generated from monocytes (MoDCs) were used to analyse if human DCs secrete IL-1β in response to *H. pylori*. Upon stimulation of *in vitro* generated MoDCs with *H. pylori*, IL-1β was secreted, albeit in much lower amounts compared to PBMCs and blood monocytes/macrophages (Figure 10B).

These results, indicate that *H. pylori* can provide the first, but more importantly, the second signal for IL-1β secretion in human innate immune cells.

3.1.2 *H. pylori* induces the production of pro- and mature IL-18 in murine BMDCs

In addition to IL-1β, IL-18 is regulated by the inflammasome. Consequently, it was analysed, if also IL-18 was induced in murine BMDCs upon *H. pylori* infection.

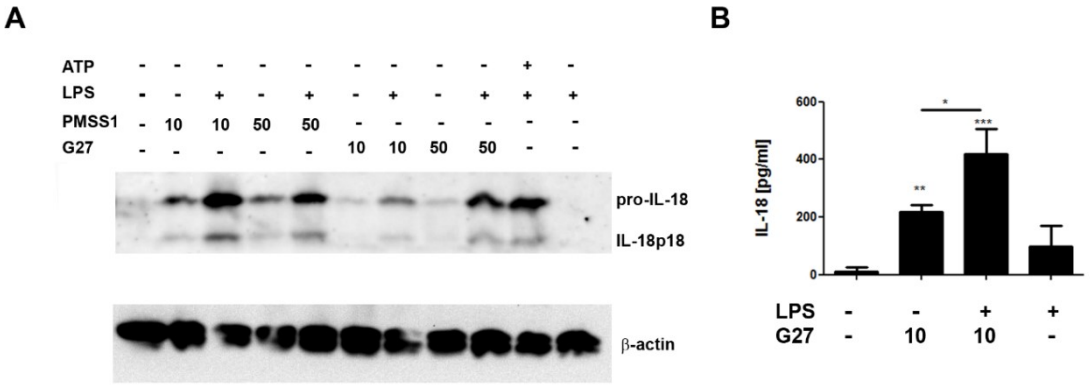

Figure 11: *H. pylori* induces IL-18 in murine BMDCs.

(A) Murine BMDCs were infected the with the *H. pylori* strains PMSS1 or G27 overnight at MOI 10 and 50, and amounts of pro- and mature IL-18 as well as β-actin were detected in cell lysates. For prestimulation, cells were incubated with 10 ng/ml LPS for 3 hours. Stimulation with ATP (5 mM) was used as control. One representative blot of two is shown.

(B) Murine BMDCs were infected with the *H. pylori* strain G27 overnight at MOI 10, and the secretion of IL-18 was measured in the supernatants by ELISA. For prestimulation, cells were incubated with 10 ng/ml LPS. Results are presented as mean±SD of three independent experiments. *H. pylori*–infected BMDCs were compared to uninfected control cells, whereas infected and LPS-prestimulated cells were compared to LPS only-stimulated control cells (asterisks shown on top of the bars) (p=0.0001 ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, Bonferroni-corrected for pairwise comparison).

Infection of BMDCs with *H. pylori* triggered the production of pro-IL-18 and mature IL-18 in murine BMDCs (Figure 11A). Like IL-1β, there were higher amounts of *H. pylori*-induced IL-18 secretion in LPS prestimulated cells (Figure 11B), also the amounts of the pro-form were increased in these cells (Figure 11A). Surprisingly, there was no consecutive expression of pro-IL-18 and also no induction of IL-18 by LPS (Figure 11A).

Thus, *H. pylori* triggers pro- and mature IL-18 production in murine BMDCs, and consequently provides the first and second signal for secretion of mature IL-18.

3.1.3 *H. pylori*-induced IL-1β secretion depends on activation of the inflammasome

To be biologically active, IL-1β has to be cleaved by caspase-1, the effector protease of the inflammasome. To confirm the necessity of caspase-1 in the observed *H. pylori*-

induced IL-1β secretion, presence of active caspase-1 subunit p10 or p20 was detected by Western blot.

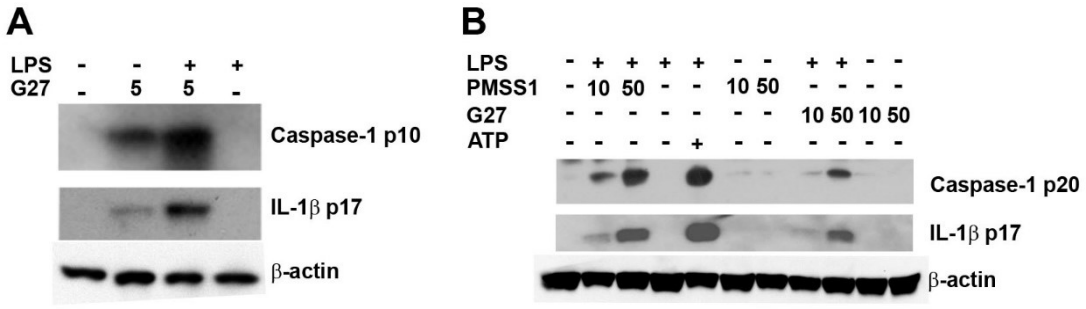

Figure 12: *H. pylori* activates caspase-1.

(A) Human PBMCs were infected o/n with the *H. pylori* strain G27 at MOI 5. Cells were prestimulated for 3 h with 5 ng/ml LPS where indicated. Protein levels of cleaved caspase-1 p10 and mature IL-1β p17 were detected in the supernatant. β-actin levels were measured in the respective cell lysates. One representative blot out of three donors is shown.

(B) Murine BMDCs were infected o/n with *H. pylori* strains PMSS1 or G27 at MOI 10 or 50 or stimulated with ATP (5 mM) for 1 hour. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. Protein levels of cleaved caspase-1 p20 and mature IL-1β p17 were detected in the supernatant. β-actin levels were measured in the respective cell lysates. One representative blot out of three experiments is shown.

Upon infection with *H. pylori*, cleaved caspase-1 was detected in PBMCs (Figure 12A) and BMDCs (Figure 12B). There were higher amounts of the caspase-1 subunit when cells were prestimulated with LPS. Corroborating the ELISA results, mature IL-1β was produced in *H. pylori*-infected cells, but none after stimulation with LPS alone.

These results demonstrate that *H. pylori* activates caspase-1 in human and murine immune cells.

To substantiate that *H. pylori*-induced IL-1β depends on caspase-1 activity, a caspase 1 specific inhibitor was added before cells were infected with *H. pylori*.

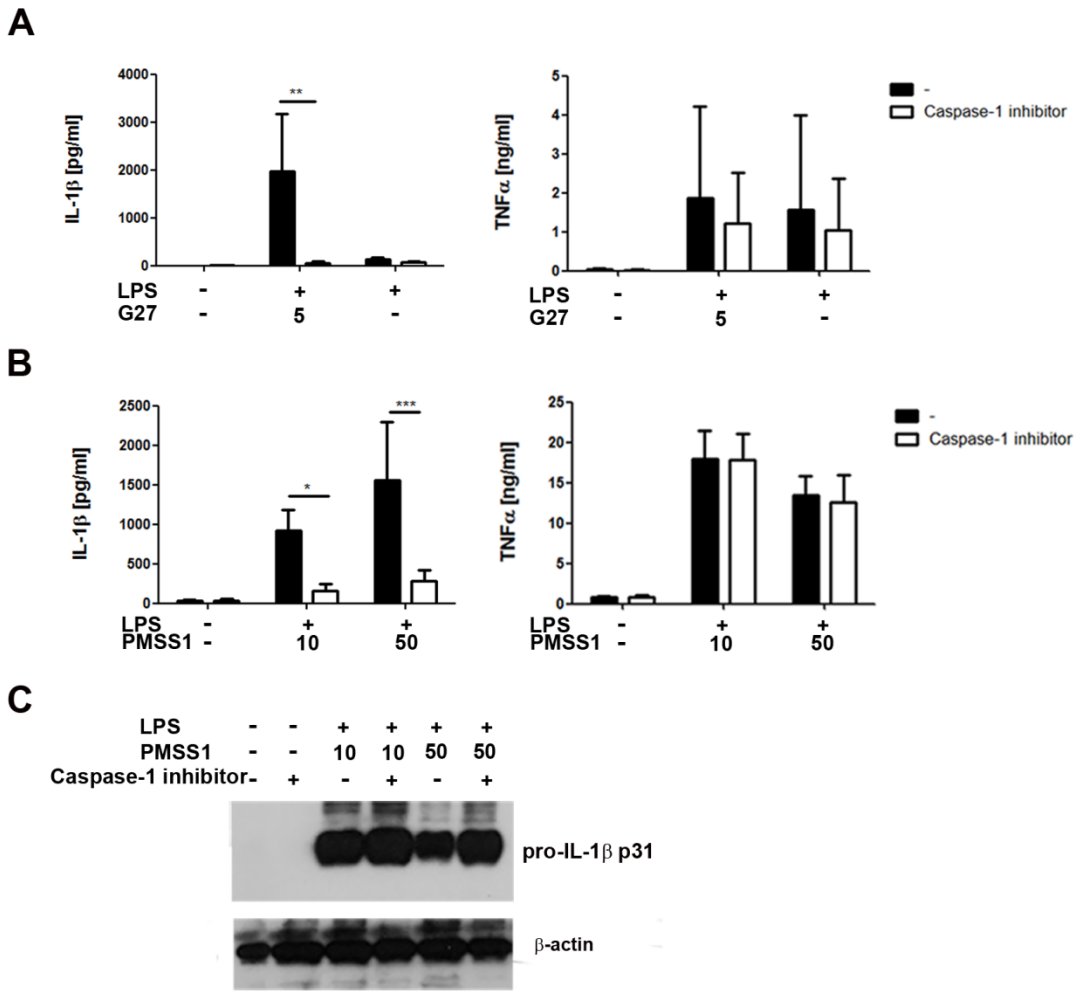

Figure 13: Caspase-1 activity is essential for *H. pylori*-induced IL-1β secretion.

(A) The levels of IL-1β and TNFα were measured in the supernatants of human PBMCs infected o/n with the G27 *H. pylori* strain at MOI 5. Cells were prestimulated with 5 ng/ml of LPS where indicated. The caspase-1 inhibitor (10 µM) was added 30 minutes prior infection. Results are presented as mean±SD from three different donors. Inhibitor-treated cells were compared to non-treated cells (p=0,0032 ANOVA, **p≤0.01 Bonferroni-corrected for pairwise comparison).

(B) Murine BMDCs were infected o/n with the *H. pylori* strain PMSS1 at MOI 10 and 50 and secretion of IL-1β and TNFα was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. Caspase-1 inhibitor (10 µM) was added 30 minutes before infection. Results are presented as mean±SD of three independent experiments. Inhibitor-treated cells were compared to non-treated cells (p=0.0002 ANOVA, *≤0.05, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(C) Murine BMDCs were infected o/n with the *H. pylori* strain PMSS1 at MOI 10 and 50 protein levels of pro-IL-1β and β-actin were measured in cell lysates. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. The caspase-1 inhibitor (10 µM) was added 30 minutes before infection. One representative blot from the experiments shown in (B) is presented.

Incubation of PBMCs (Figure 13A) or BMDCs (Figure 13B) with a caspase-1 inhibitor resulted in reduced secretion of IL-1β, whereas no changes in the level of the inflammasome-independent cytokine TNFα (Figure 13A and B) or accumulation of intracellular pro-IL-1β (Figure 13C) were detected. These results demonstrate that *H. pylori*-induced IL-1β secretion is mediated by the activity of caspase-1.

To corroborate the results obtained using the caspase-1 inhibitor, LPS-primed BMDCs of caspase-1-deficient mice were infected with *H. pylori* and secretion of IL-1β was examined.

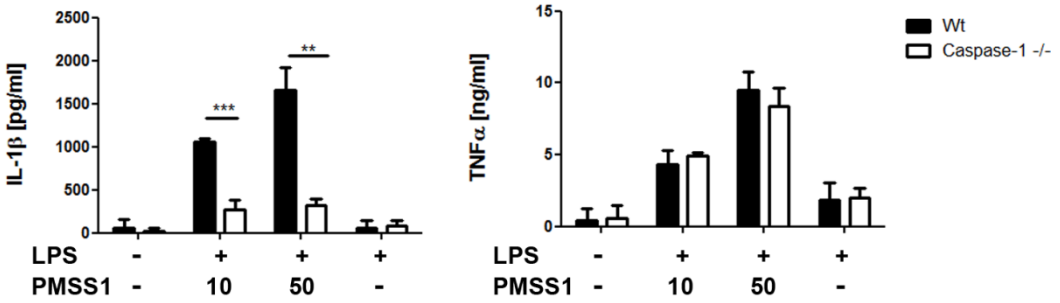

Figure 14: *H. pylori*-mediated IL-1β induction depends on caspase-1.

Wild type and caspase-1$^{-/-}$ BMDCs were primed with 10 ng/ml LPS for 3 hours where indicated and infected o/n with *H. pylori* PMSS1 (MOI 10 or 50). Levels of IL-1β and TNFα were analysed in the supernatant by specific ELISAs. Results are presented as mean±SD of three independent experiments. Cells from Caspase-1$^{-/-}$ mice were compared to wildtype cells ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

Caspase-1-deficient BMDCs showed impaired IL-1β production but normal TNFα secretion upon infection with *H. pylori*.

Together, these results confirmed that IL-1β secretion upon *H. pylori* infection is mediated by caspase-1.

3.1.4 *H. pylori* does not induce pyroptosis

In addition to cleavage of IL-1β and IL-18, inflammasome activation can also lead to cell death called pyroptosis (341). Dying or lysed cells secrete lactate dehydrogenase (LDH), which can be measured in a coupled enzymatic assay. To test whether *H. pylori* can induce pyroptosis, the viability of stimulated BMDCs and PBMCs was analysed by measuring LDH amounts in the cell supernatant.

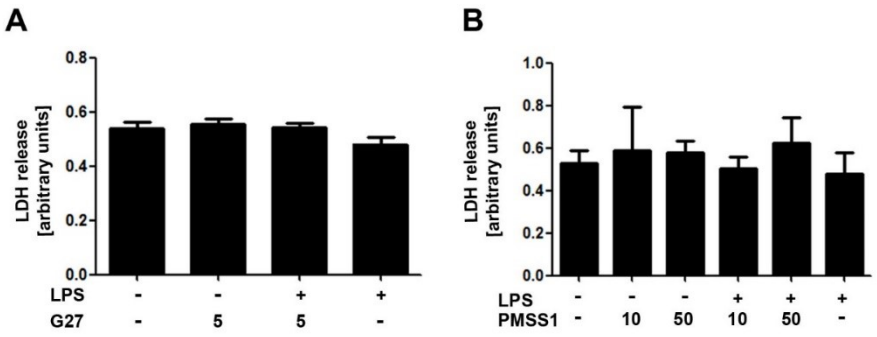

Figure 15: *H. pylori* does not induce pyroptosis.

(A) Human PBMCs were infected o/n with the *H. pylori* strain G27 at MOI 5. Cells were prestimulated for 3 hours with 5 ng/ml LPS where indicated. Cytotoxicity was measured by LDH release within the supernatants. Absorption was measured at 490 nm. Results are represented as mean±SD from four different donors.

(B) Murine BMDCs were infected o/n with the *H. pylori* strains PMSS1 at MOI 10 or 50. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. Cytotoxicity was measured by LDH release in the supernatants. Absorption was measured at 490 nm. Results are represented as mean±SD of three independent experiments.

PBMCs (Figure 15A) and BMDCs (Figure 15B) stimulated with *H. pylori* and/or LPS did not release increased amounts of LDH compared to unstimulated cells, indicating that *H. pylori* did not lead to pyroptosis in innate immune cells at the time points and MOIs investigated. These results indicate that *H. pylori* specifically triggers cytokine secretion upon inflammasome activation.

3.1.5 *H. pylori* activates the NLRP3 inflammasome

The results indicate that IL-1β secretion upon *H. pylori* infection is mediated by inflammasome activation, therefore it was analysed which inflammasome complex is involved. In addition to the effector protease caspase-1, inflammasomes also consist of ASC and a sensor protein such as NLRP3, NLRP1, NLRP6, NLRP12, NLRC4 or AIM2. However, the majority of tested PAMPs and DAMPs, including many bacteria, have been shown to trigger NLRP3 inflammasome. For flagellated bacteria and bacterial secretion systems a stimulation of the NLRC4 inflammasome was shown (see section 1.2.2).

Thus, NLRP3 and NLRC4 inflammasome seemed good for candidates driving *H. pylori*-mediated IL-1β secretion. To confirm this hypothesis, Asc, Nlrp3 and Nlrc4-deficient BMDCs were stimulated with *H. pylori*, and IL-1β secretion was measured.

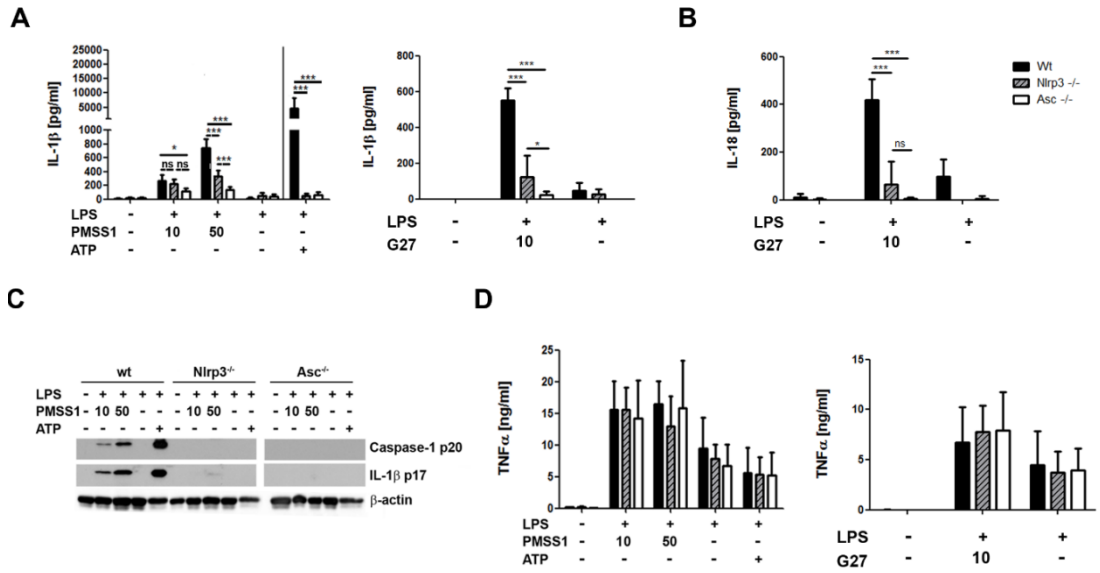

Figure 16: *H. pylori* triggered IL-1β and IL-18 secretion depends on Nlrp3 and Asc.

(A) Wild type, Nlrp3$^{-/-}$, and Asc$^{-/-}$ BMDCs were infected o/n with the *H. pylori* strains PMSS1 or G27 at the indicated MOIs. Cells were primed with 10 ng/ml LPS for 3 hours where indicated. Stimulation with ATP (5 mM) for 1 hour was used as positive control. Levels of IL-1β were analysed in the supernatant by specific ELISA. Results are presented as mean±SD of 4 independent experiments. ($p<0.0001$ ANOVA, *≤0.05, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(B) Wild type, Nlrp3$^{-/-}$, and Asc$^{-/-}$ BMDCs were infected o/n with the *H. pylori* strain G27 (MOI 10). Cells were primed with 10 ng/ml LPS for 3 hours where indicated. Levels of IL-18 were analysed in the supernatant by specific ELISA. Results are presented as mean±SD of 4 independent experiments. ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(C) Wildtype, Asc$^{-/-}$ and Nlrp3$^{-/-}$ BMDCs were infected o/n with the *H. pylori* strain PMSS1 (MOI 10 or 50). Cells were primed with 10 ng/ml LPS for 3 hours where indicated. Stimulation with ATP (5 mM) for 1 hour was used as positive control. Protein levels of mature IL-1β p17 and active caspase-1 p20 were detected in the supernatant. β-actin was measured in the respective cell lysates. One representative blot of the experiment shown in (A).

(D) Wild type, Nlrp3$^{-/-}$, and Asc$^{-/-}$ BMDCs were infected o/n with the *H. pylori* strains PMSS1 or G27 at the indicated MOIs. Cells were primed with 10 ng/ml LPS for 3 hours where indicated. Stimulation with ATP (5 mM) for 1 hour was used as positive control. Levels of TNFα were analysed in the supernatant by specific ELISA. Results are presented as mean±SD of 4 independent experiments.

H. pylori-triggered IL-1β and IL-18 secretion was blocked in BMDCs from Asc$^{-/-}$ animals. Likewise, lack of NLRP3 led to lower IL-1β secretion. Dependency of *H. pylori*-mediated IL-1β secretion on ASC and NLRP3 was not strain specific, since infection with PMSS1

and G27 strain only triggered IL-1β secretion in wild type cells but not in Nlrp3- or Asc-deficient ones (Figure 16A). The secretion of IL-18 was ASC and NLRP3 dependent, too (Figure 16B). Further, Asc- and Nlrp3-deficient BMDCs failed to process the cleaved product of caspase-1 and IL-1β (Figure 16C). In contrast, inflammasome-independent TNFα production was not affected in $Asc^{-/-}$ or $Nlrp3^{-/-}$ cells (Figure 16D).

Since *H. pylori* possesses flagella and a T4SS, both known triggers for NLRC4 inflammasome activation, Nlrc4-deficient BMDCs were infected with *H. pylori* to test if also this inflammasome is involved in *H. pylori*-triggered IL-1β production.

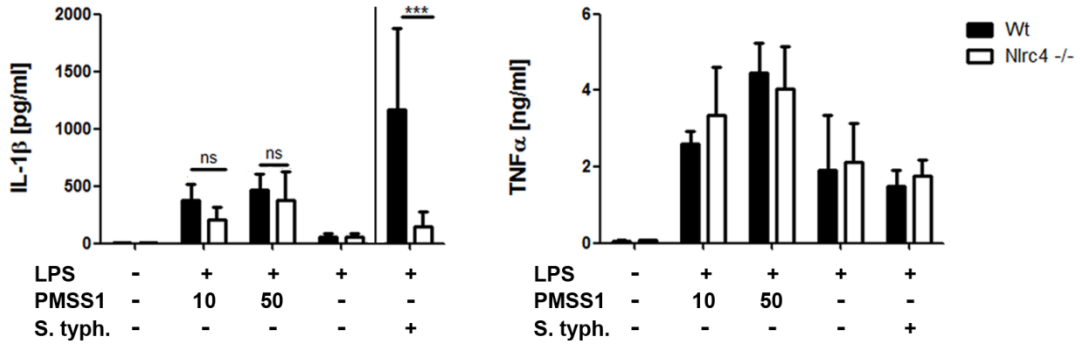

Figure 17: *H. pylori*-triggered IL-1β production by BMDCs is independent of Nlrc4.

Wildtype and $Nlrc4^{-/-}$ BMDCs were infected o/n with the *H. pylori* strain PMSS1 (MOI 10 or 50). Cells were primed with 10 ng/ml LPS for 3 hours where indicated. Stimulation with *S. typhimurium* (MOI 10) for 6 hours was used as positive control. Levels of IL-1β and TNFα were analysed in the supernatant by specific ELISAs. Results are presented as mean±SD of four independent experiments. ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

No significant changes in the *H. pylori*-mediated IL-1β secretion were detected in Nlrc4-deficient BMDCs compared to wildtype cells (Figure 17). As expected, *Salmonella* infection of Nlrc4-deficient cells led to impaired IL-1β secretion, since *S. typhimurium* mainly activates the NLRC4 inflammasome (229). TNFα secretion was not affected by the lack of Nlrc4.

These results indicate that *H. pylori*-induced IL-1β and IL-18 form BMDCs are primarily dependent on activation of the NLRP3 inflammasome.

3.1.5.1 *H. pylori*-mediated NLRP3 inflammasome activation depends on intracellular pottassium efflux

Several upstream events such as potassium efflux, ROS production or lysosomal leakage (e. g. reviewed in (342)) have been linked to NLRP3 inflammasome activation.

First, it was tested if *H. pylori*-induced NLRP3 activation was triggered by K^+ efflux. K^+ efflux was blocked by increased extracellular potassium concentration in the medium.

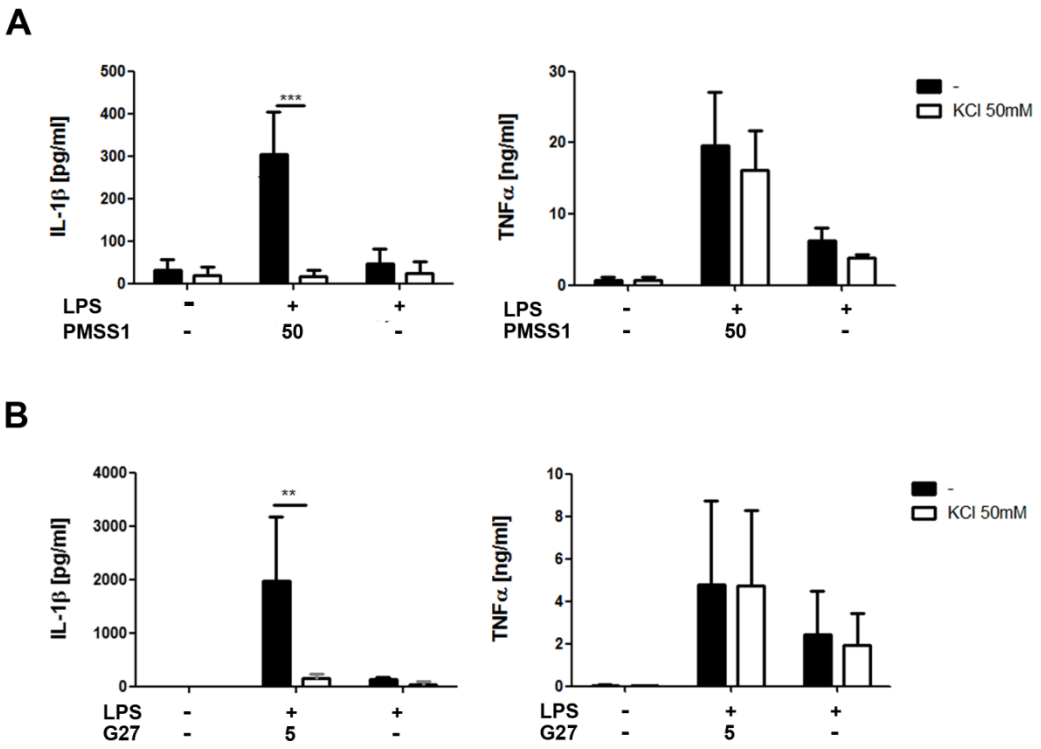

Figure 18: *H. pylori*-mediated NLRP3 inflammasome activation requires potassium efflux.

(A) Murine BMDCs were infected for 6 hours with the *H. pylori* strain PMSS1 at MOI 50 and secretion of IL-1β and TNFα was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. KCl (50 mM) was added 30 minutes before infection. Results are presented as mean±SD of three independent experiments. KCl-treated cells were compared to non-treated cells ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(B) Human PBMCs were infected for 6 hours with the *H. pylori* strains G27 at MOI 5. Secretion of IL-1β and TNFα was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 5 ng/ml LPS where indicated. KCl (50 mM) was added 30 minutes before infection. Results are presented as mean±SD of three different donors. KCl-treated cells were compared to non-treated cells ($p=0.0021$ ANOVA, **≤0.01 Bonferroni-corrected for pairwise comparison).

H. pylori-induced IL-1β secretion in BMDCs was blocked in presence of increased K^+ levels, while the level of TNFα was not significantly altered (Figure 18A). Similar results were obtained in human PBMCs (Figure 18B).

Togerther, these results indicate that potassium efflux is essential for *H. pylori*-induced inflammasome activation.

3.1.5.2 *H. pylori*-mediated NLRP3 inflammasome activation depends on phagocytosis

Phagocytosis as well as subsequent lysosomal damage with release of lysosomal enzymes or nucleic acids are additional mechanisms associated with NLRP3 inflammasome activation. Therefore, next it was analysed whether endocytosis was necessary for *H. pylori*-induced IL-1β production. For this purpose, BMDCs were incubated with bafilomycin A1 or cytochalasin D. Bafilomycin A1 is a potent and selective inhibitor of V-ATPase. It blocks endosomal acidification and thus the fusion between autophagosomes and lysosomes, and lysosomal degradation. Cytochalasin D inhibits actin polymerization and therefore phagocytosis.

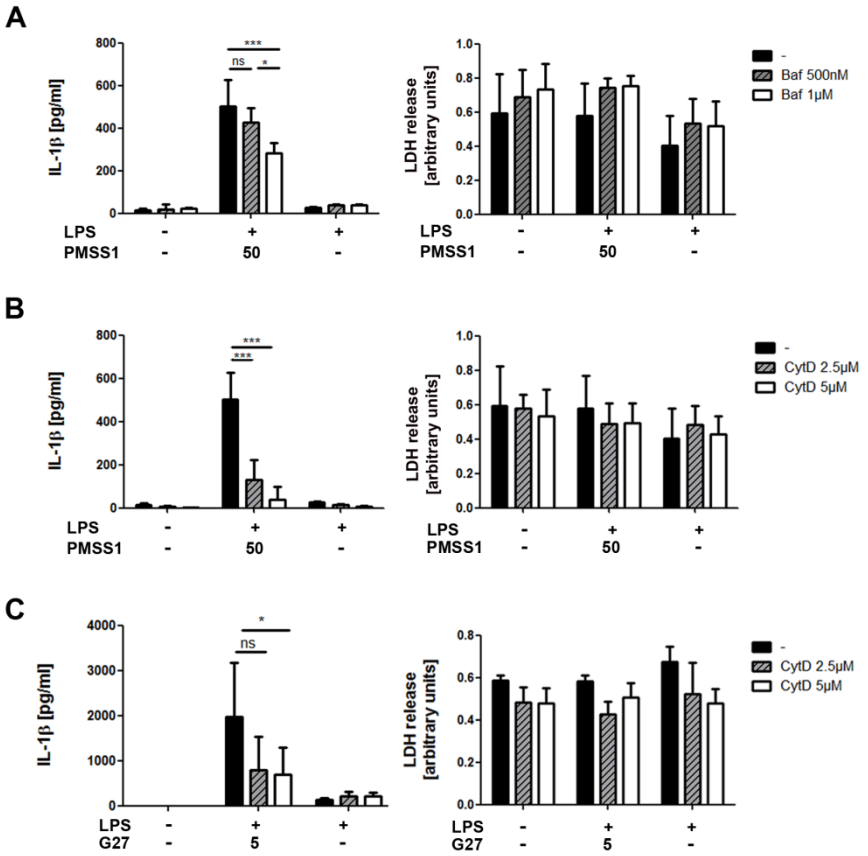

Figure 19: Phagocytosis is required for *H. pylori*-mediated NLRP3 inflammasome activation.

(A) Murine BMDCs were infected for 6 hours with the *H. pylori* strain PMSS1 at MOI 50 and secretion of IL-1β and LDH release were measured in the supernatants by ELISA or LDH assay, respectively. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. Bafilomycin A1 (Baf) was added at the indicated concentrations 30 minutes before infection. Results are presented as mean±SD of three independent experiments. Baf-treated cells were compared to non-treated cells. ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(B) Murine BMDCs were infected for 6 hours with the *H. pylori* strain PMSS1 at MOI 50 and secretion of IL-1β and LDH release were measured in the supernatants by ELISA or LDH assay, respectively. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. Cytochalasin D (CytD) was added at the indicated concentrations 30 minutes before infection. Results are presented as mean±SD of three independent experiments. CytD-treated cells were compared to non-treated cells. ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(C) Human PBMCs were infected for 6 hours with the *H. pylori* strain G27 at MOI 5. Secretion of IL-1β and LDH release were measured in the supernatants by ELISA or LDH assay, respectively. Cells were prestimulated for 3 hours with 5 ng/ml LPS where indicated. Cytochalasin D (CytD) was added at the indicated concentrations 30 minutes before infection. Results are presented as mean±SD of three different donors. CytD-treated cells were compared to non-treated cells. ($p=0.0030$ ANOVA, *≤0.05 Bonferroni-corrected for pairwise comparison).

Following incubation with bafilomycin A1 and cytochalasin D, *H. pylori*-induced IL-1β secretion was decreased with increasing amounts of the inhibitors (Figure 19A and B). To rule out a deleterious effect of the inhibitor used, cellular toxicity was assessed by messurement of LDH. No adverse effects were detected when using bafilomycin or cytochalasin at these concentrations (Figure 19A and B). Similar results were obtained for cytochalasin in human PBMCs (Figure 19C).

Togther, these results indicate that phagocytosis of the bacterium contributes to *H. pylori*- triggered IL-1β secretion.

3.1.5.3 *H. pylori*-mediated NLRP3 inflammasome activation depends on reactive oxygen species (ROS)

It is well known that *H. pylori* induces ROS, however it is yet unclear if ROS activates the inflammasome or rather acts as priming signal leading to increased expression of pro-IL-1β and Nlrp3 protein. Nevertheless, it was analysed if ROS could be involved in *H. pylori*-triggered IL-1β production. To minimize effects due to altered priming, the cells were prestimulated with LPS before addition of the inhibitor. To prevent ROS accumulation, the two antioxidants N-acetyl-L-cysteine (NAC) and Ebselen, and the glutamate receptor agonist and inhibitor of NADPH-oxidase-dependent ROS system (2*R*,4*R*)-4-Aminopyrrolidine-2,4-dicarboxylate (APDC) were used.

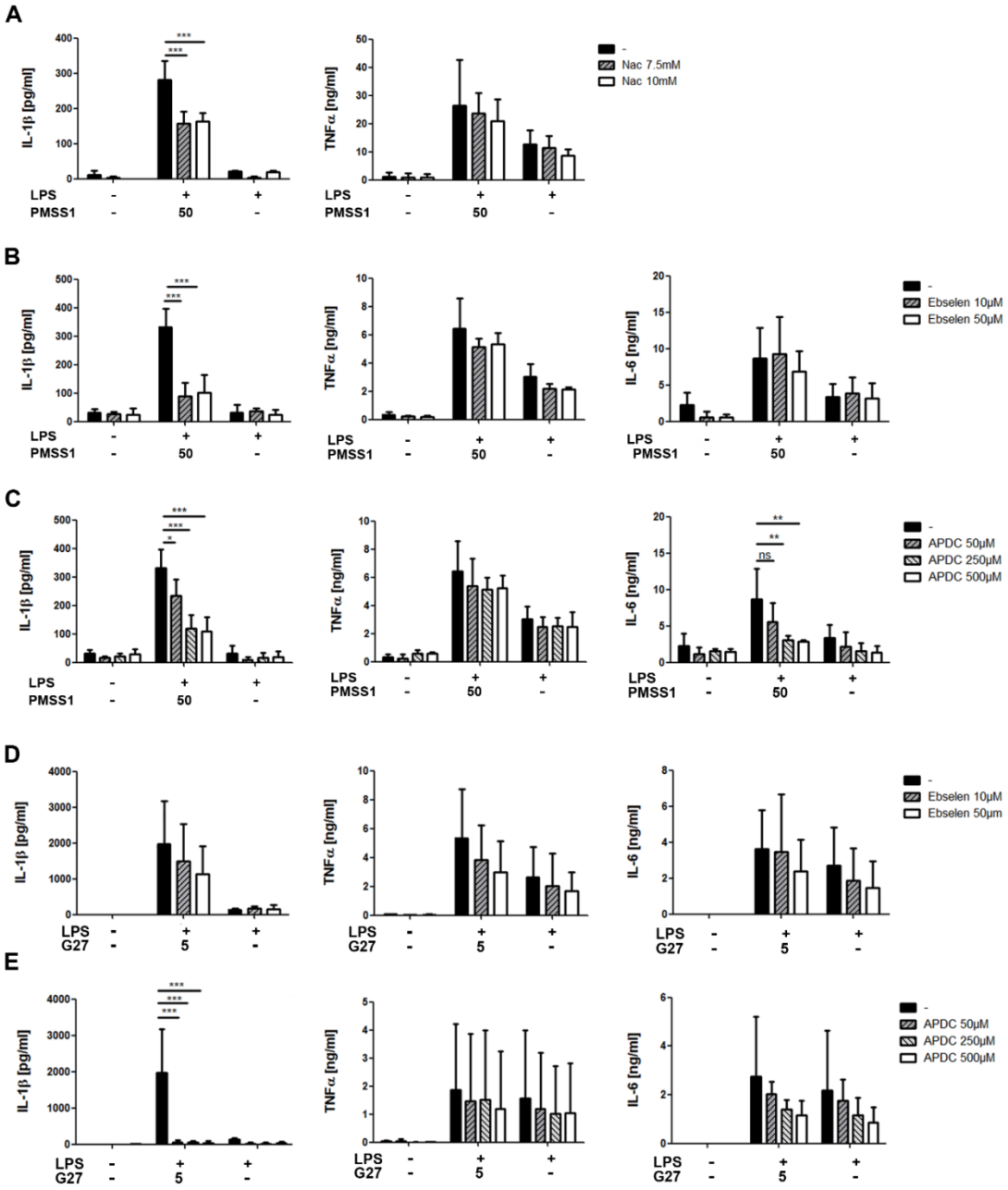

Figure 20: *H. pylori* mediated NLRP3 inflammasome induction involves ROS.

(A) Murine BMDCs were infected for 6 hours with the *H. pylori* strain PMSS1 at MOI 50 and secretion of IL-1β and TNFα was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. N-acetyl-L-cysteine (NAC) was added at the indicated concentrations 30 minutes before infection. Results are presented as mean±SD of three independent experiments. NAC-treated cells were compared to non-treated cells ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(B) Murine BMDCs were infected for 6 hours with the *H. pylori* strain PMSS1 at MOI 50 and secretion of IL-1β, IL-6 and TNFα was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. Ebselen was added at the indicated concentrations 30 minutes before infection. Results are presented as mean±SD of three independent experiments. Ebselen-treated cells were compared to non-treated cells (IL-1β $p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison; IL-6 $p=0,0011$ ANOVA, **≤0.01 Bonferroni-corrected for pairwise comparison).

(C) Murine BMDCs were infected for 6 hours with the *H. pylori* strain PMSS1 at MOI 50 and secretion of IL-1β, IL-6 and TNFα was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. (2*R*,4*R*)-4-Aminopyrrolidine-2,4-dicarboxylate (APDC) was added at the indicated concentrations 30 minutes before infection. Results are presented as mean±SD of three independent experiments. APDC-treated cells were compared to non-treated cells ($p<0.0001$ ANOVA, *≤0.05, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(D) Human PBMCs were infected for 6 hours with the *H. pylori* strain G27 at MOI 5 and secretion of IL-1β, IL-6 and TNFα was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 5 ng/ml LPS where indicated. Ebselen was added at the indicated concentrations 30 minutes before infection. Results are presented as mean±SD of three different donors. Ebselen-treated cells were compared to non-treated cells ($p=0.0026$ ANOVA).

(E) Human PBMCs were infected for 6 hours with the *H. pylori* strain G27 at MOI 5 and secretion of IL-1β, IL-6 and TNFα was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 5 ng/ml LPS where indicated. APDC was added at the indicated concentrations 30 minutes before infection. Results are presented as mean±SD of three different donors. APDC-treated cells were compared to non-treated cells ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

Incubation of BMDCs with any of the three substances that inhibit ROS production resulted in lower levels of *H. pylori*-induced IL-1β secretion, showing that *H. pylori*-mediated ROS production is involved in inflammasome activation (Figure 20A, B and C). The secretion of inflammasome-independent cytokines TNFα and IL-6 was analysed to exclude off-target effects. No changes in TNFα levels were seen after incubation with all three substances (Figure 20A, B and C). IL-6 levels were not affected when using NAC and Ebselen (Figure 20A and B), whereas APDC induced a reduction in IL-6 production in a dose-dependent manner (Figure 20C). This effect could be due to the agonistic activation of glutamate receptors expressed on DCs (343) by APDC, leading to inhibition of cAMP signaling and IL-6 production as previously reported (344).

When analysing the effect of Ebselen and APDC on the *H. pylori*-triggered IL-1β production by human PBMCs, there was a dose-dependent decrease of IL-1β after Ebselen treatment, while APDC completely abolished IL-1β release (Figure 20D and E). TNFα production was not affected by these inhibitors. Like in murine BMDCs, Ebselen did not affect IL-6 secretion (Figure 20D), in contrast to APDC (Figure 20E).

Together, these results indicate that ROS production triggers inflammasome activation by *H. pylori*.

3.1.6 *H. pylori*-mediated inflammasome activation depends on CagPAI

To determine the bacterial factors involved in *H. pylori*-induced inflammasome activation, first it was analysed whether live bacteria are crucial for IL-β secretion. For this, cells were stimulated either with live, or PFA-fixed or heat-inactivated bacteria.

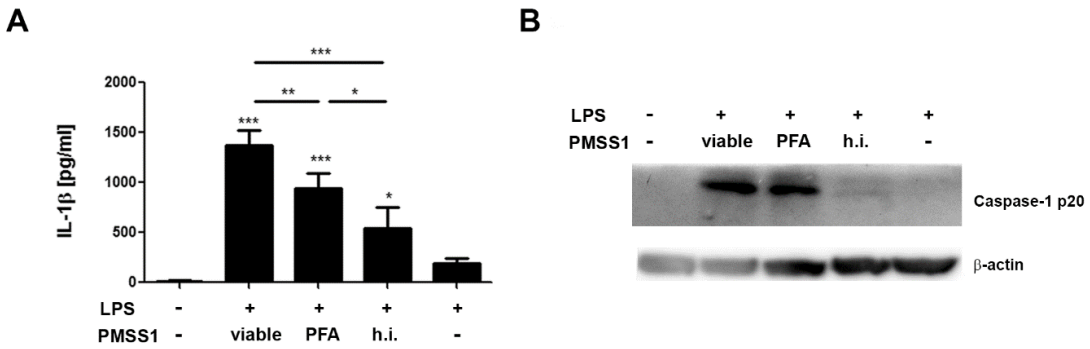

Figure 21: Heat-sensitive factors of *H. pylori* trigger inflammasome activation.

(A) Murine BMDCs were incubated o/n with viable, PFA-fixed or heat-inactivated (h. i.) *H. pylori* strain PMSS1 (MOI 50) and secretion of IL-1β was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. Results are presented as mean±SD of three independent experiments. Cells stimulated with heat-inactivated or PFA-fixed bacterium were compared to cells infected with live bacterium. Asterisks on the top of the bars indicated significance relative to LPS only-stimulated cells ($p<0.0001$ ANOVA, *≤0.05, **≤0.01, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(B) Murine BMDCs were incubated o/n with viable, PFA-fixed or heat-inactivated (h. i.) *H. pylori* strain PMSS1 (MOI 50) and caspase-1 p20 was detected in supernatants. As loading control, β-actin was measured in the respective cell lysates. Cells were prestimulated for 3 h with 10 ng/ml LPS where indicated. One representative blot of the experiments shown in (A).

Reduced IL-1β secretion was detected when cells were incubated with PFA-fixed bacteria (Figure 21A). Heat-inactivation of *H. pylori* led to significantly lower levels of IL-1β, which was accompanied by impaired caspase-1 activation (Figure 21B). This suggests that heat-sensitive bacterial factors such as proteins contribute to *H. pylori*-induced inflammasome activation. *H. pylori* virulence factors might actively regulate inflammasome activation.

To identify virulence determinants involved in *H. pylori*-induced inflammasome activation cells were infected with *H. pylori* strains deficient for several virulence factors. It is well known, that virulence factors of *H. pylori* are important for triggering NFκB. To minimize different priming capacities of the bacterial mutant strains, cells

were prestimulated with LPS to have the same basal priming before infection with *H. pylori*.

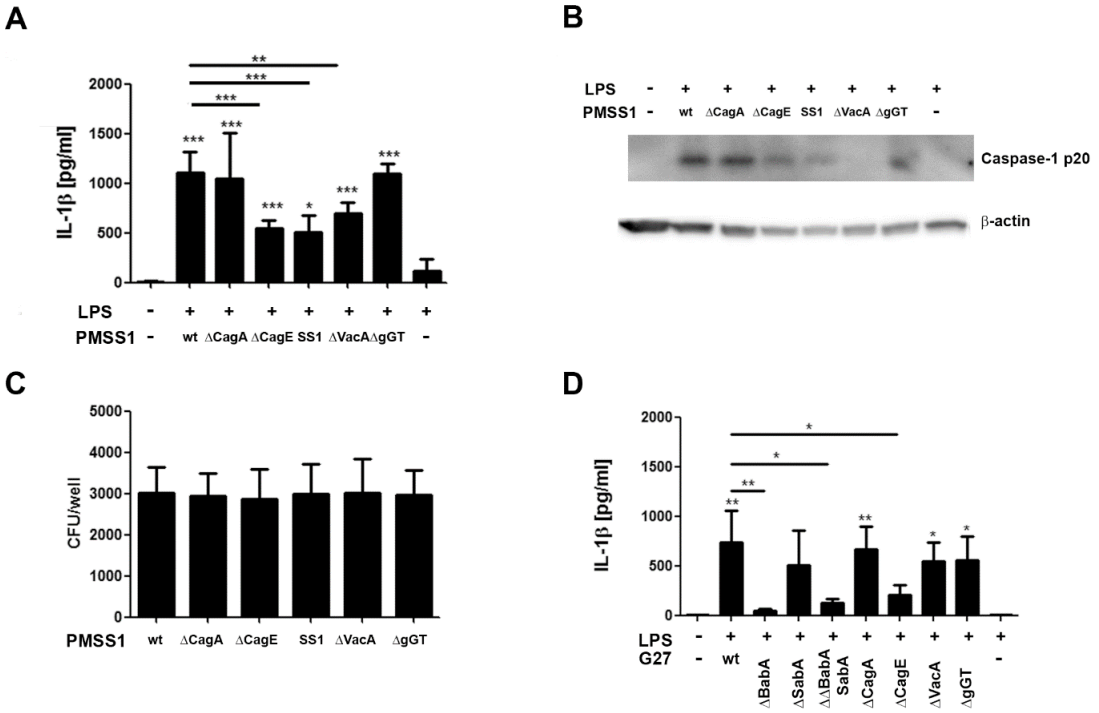

Figure 22: *H. pylori* virulence factors CagPAI and VacA are involved in inflammasome activation.

(A) Murine BMDCs were infected o/n with the wild type strains PMSS1 or SS1, or the PMSS1 isogenic mutant strains deficient for CagA, CagE, VacA or gGT at MOI 50 and secretion of IL-1β was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. Results are presented as mean±SD of three independent experiments. Cells stimulated with knockout strains were compared to cells infected with the wild type strain. Asterisks on the top of the bars indicate significance relative to LPS only-stimulated cells ($p<0.0001$ ANOVA, *≤0.05, **≤0.01, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(B) Murine BMDCs were infected o/n with the wild type strains PMSS1 or SS1, or the PMSS1 isogenic mutant strains deficient for CagA, CagE, VacA or gGT at MOI 50 and caspase-1 p20 was detected in supernatants. As loading control β-actin was measured in the respective cell lysates. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. One representative blot of the experiments shown in (A).

(C) Murine BMDCs were infected with the wild type strains PMSS1 or SS1, or the PMSS1 isogenic mutant strains deficient for CagA, CagE, VacA or gGT at MOI 50 and bacterial uptake was measured in cell lysates after gentamycin treatment. Results (mean±SD) of one representative experiment performed in triplicates is shown (n=3).

(D) Human PBMCs were infected o/n with the wild type strain G27 or the G27 isogenic mutant strains deficient for BabA, SabA, BabASabA, CagA, CagE, VacA, or gGT at MOI 5 and secretion of IL-1β was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 5 ng/ml LPS where indicated. Results are presented as mean±SD of four different donors. Cells stimulated with knockout strains were compared to cells infected with the wild type strain. Asterisks on the top of the bars indicate significance relative to only LPS-stimulated cells ($p<0.0001$ ANOVA, *≤0.05, **≤0.01, ***≤0.001 Bonferroni-corrected for pairwise comparison).

Infection with a CagE-deficient bacterium resulted in lower levels of IL-1β (Figure 22A). CagE codes for the VirB4 ATPase of the T4SS, and is essential for both assembly of the secretion system and transfer of CagA and peptidoglycan. Similar levels of IL-1β were detected when cells were cocultured with SS1 strain, which expresses VacA (345), CagA (346) but has a non-functional CagPAI. Interestingly, the *H. pylori* PMSS1 strain lacking CagA was still capable of inducing IL-1β, indicating that other components of the CagPAI are responsible for inflammasome activation. In addition, an *H. pylori* strain lacking the pore-forming toxin VacA induced lower amounts of IL-1β secretion. Conversely, the absence of gGT did not influence the release of IL-1β. These results correlated with caspase-1 activation. Thus, active caspase-1 p20 was absent or reduced in cells infected with bacterial strains lacking a functional CagPAI or VacA (Figure 22B).

When corroborating these results in human PBMCs, reduced levels of IL-1β upon infection with a CagE-deficient strain were observed, whilst only slight reduced levels were observed when infecting with VacA or gGT mutants (Figure 22D). Importantly, CagA also seemed not be important for IL-1β induction in human PBMCs. Interestingly, adhesion of the bacterium to the cells was important for inflammasome activation because infection with strains deficient in BabA or SabA led to lower levels of *H. pylori*-triggered IL-1β secretion (Figure 22D).

The reduced IL-1β production was not due to impaired uptake of the mutant strains by BMDCs, since comparable amounts of phagocytosed, intracellular bacteria were detected (Figure 22C).

These Results suggest that in murine BMDCs and human PBMCs, the CagPAI, but not CagA, is the main virulence factor involved in *H. pylori*-induced inflammasome activation.

3.1.7 *H. pylori* activates Syk, which mediates IL-1β production

One signalling pathway, which was shown to be involved in NLRP3 inflammasome activation, is the activation of the spleen tyrosine kinase (Syk). Several pathways which are downstream of Syk trigger processes such as ROS production, phagocytosis, and potassium efflux (255, 257, 286). These mechanisms, as shown before, are also crucial for *H. pylori*-triggered IL-1β secretion (section 3.1.5.1 to 3.1.5.3). Furthermore, Syk phosphorylates ASC, which is essential for ASC oligomerization and the recruitment of pro-caspase-1 (347).

To analyse the involvement of Syk in *H. pylori*-mediated activation of the inflammasome, firstly Syk activation was analysed.

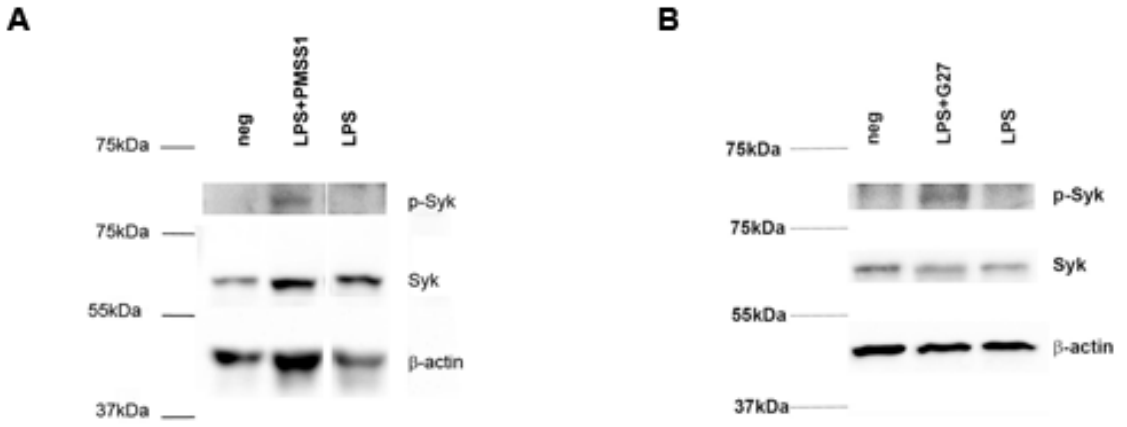

Figure 23: *H. pylori* activates Syk in murine BMDCs and human PBMCs

(A) Murine BMDCs were infected o/n with PMSS1, and phosphorylated and whole Syk was measured in cell lysates by western blot. β-actin was detected as loading control. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. One representative western blot of three independent experiments is shown.

(B) Human PBMCs were infected o/n with G27, and phosphorylated and whole Syk was measured in cell lysates by western blot. β-actin was detected as loading control. Cells were prestimulated for 3 hours with 5 ng/ml LPS where indicated. One representative blot is shown (n=2).

Infection of murine BMDCs and human PBMCs led to the phosphorylation of Syk in its kinase domain (Figure 23A and B). This phosphorylation at Tyr525/526 of human Syk (equivalent to Tyr519/520 of mouse Syk) is essential for Syk function (348, 349).

To analyse if Syk activity is involved in *H. pylori*-induced IL-1β secretion, cells were treated with R406, an inhibitor of Syk kinase activity (350).

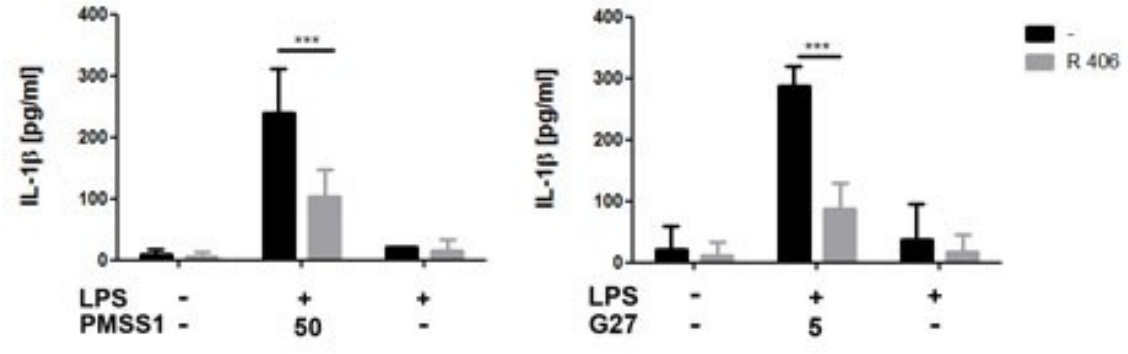

Figure 24: *H. pylori*-induced IL-1β secretion depends on Syk activity.

(A) Murine BMDCs were infected o/n with the *H. pylori* strain PMSS1 at MOI 50 and secretion of IL-1β was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 10 ng/ml LPS where indicated. R406 (1µM) was added 30 minutes before infection. Results are presented as mean±SD of six independent experiments. R406-treated cells were compared to non-treated cells. ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(B) Human PBMCs were infected o/n with the *H. pylori* strain G27 at MOI 5 and secretion of IL-1β was measured in the supernatants by ELISA. Cells were prestimulated for 3 hours with 5 ng/ml LPS where indicated. R406 (1µM) was added 30 minutes before infection. Results are presented as mean±SD of three donors. R406-treated cells were compared to non-treated cells. ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

In R406-treated BMDCs and PBMCs *H. pylori*-induced IL-1β was blocked (Figure 24A and B). This suggests that Syk signalling is involved in *H. pylori*-triggered IL-1β production.

3.2 Role of inflammasomes during *H. pylori* infection *in vivo*

For several pathogens it was shown that inflammasome activation is important for clearing the infection but also for inducing pathology. During *H. pylori* infection the two cytokines IL-1β and IL-18 might have a pivotal role for balancing immune responses. These two cytokines are crucial for induction of immune responses linked to clear *H. pylori* but also to bias inflammation.

In vitro, *H. pylori*-dependent IL-1β and IL-18 production was highly dependent on ASC-NLRP3 inflammasome activation, whereas NLRC4 was not mandatory. By infecting

Asc $^{-/-}$, Nlrp3 $^{-/-}$ and Nlrc4 $^{-/-}$ mice with *H. pylori* the role of NLRP3 and NLRC4 inflammasome activation in the induction of *H. pylori*-induced IL-1β and IL-18 production was analysed *in vivo*.

3.2.1 Lack of ASC does not alter bacterial clearing

The ASC protein is part of most inflammasomes, bridging the scaffolding protein with caspase-1. *In vitro*, *H. pylori*-induced IL-1β secretion and caspase-1 activation was highly dependent on ASC (Figure 16A and C). Thus, it was analysed if the lack of this protein had an impact on the immune response and clearing of *H. pylori in vivo*.

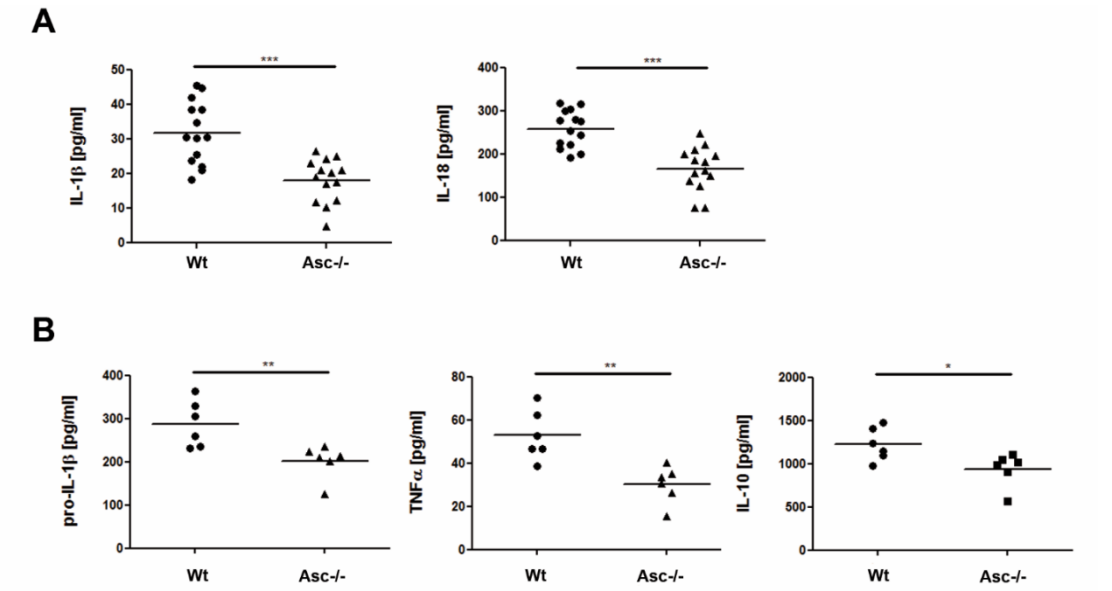

Figure 25: Asc is required for *H. pylori*-triggered mature IL-1β and IL-18 *in vivo*.

(A) IL-1β and IL-18 protein levels detected in the gastric extracts of wild type and Asc$^{-/-}$ mice infected for 1 month with the *H. pylori* strain PMSS1. Results from two pooled experiment are shown. Horizontal lines indicate mean values (***≤0.001, t-test).

(B) Pro-IL-1β, IL-10, and TNFα protein levels detected in the gastric extracts of wild type and Asc$^{-/-}$ mice infected for 1 month with the *H. pylori* strain PMSS1. Results from one experiment are shown. Horizontal lines indicate mean values (*≤0.05, **≤0.01, t-test).

Upon *H. pylori* infection mature IL-1β and IL-18 were induced in gastric lysates of wild type mice (data not shown). *H. pylori*-infected Asc-deficient mice showed significantly lower IL-1β and IL-18 levels in the stomach (Figure 25A), corroborating the *in vitro* findings. Hence, *H. pylori*-induced mature IL-1β and IL-18 production *in vivo* depends on ASC.

When analysing inflammasome independent cytokines, surprisingly, there was also less pro-IL-1β, TNFα and IL-10 in the stomach of *H. pylori*-infected ASC knockout mice (Figure 25B).

Upon infection immune cells are recruited to the side of infection. Since cytokines contribute to recruitment and differentiation of immune cells, amounts of lymphocytes and T cells in the stomach were analysed. These cell types are involved in bacterial clearing and induction of gastritis.

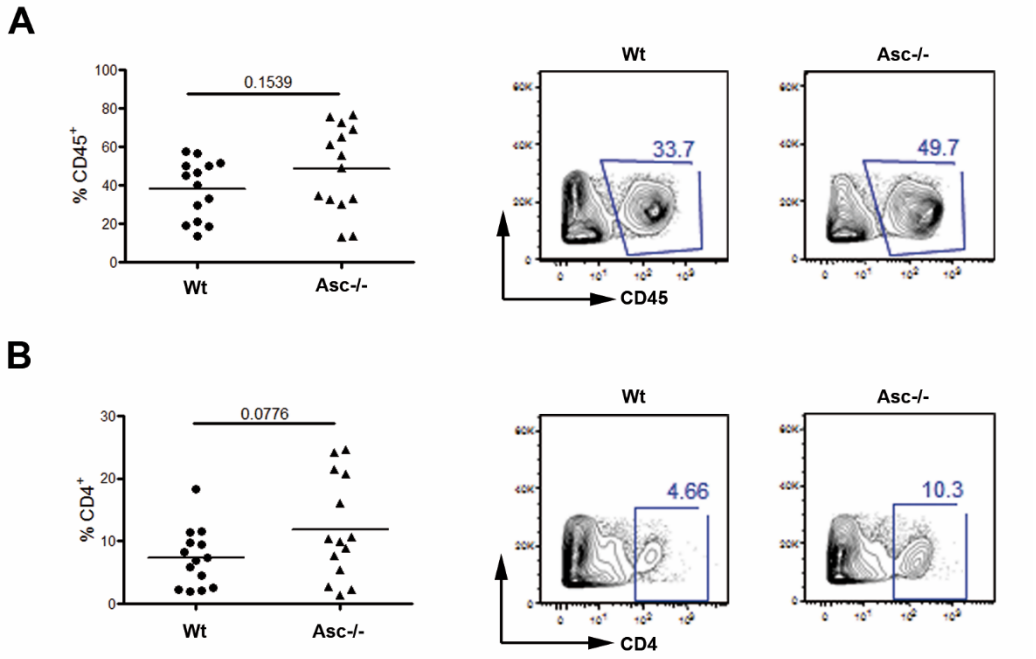

Figure 26: Infiltration of CD45⁺ and CD4⁺ cells into the stomach.

(A) Percentage of leukocytes (CD45⁺) cells infiltrating the gastric mucosa of wild type and Asc-deficient mice infected for 1 month with *H. pylori* PMSS1. Cells were pregated on live cells based on forward and side scatter. Horizontal lines indicate mean values. Results from two pooled experiments are shown (t-test). Representative FACS blots of CD45⁺ cells are shown.

(B) Percentage of CD4⁺ T cells infiltrating the gastric mucosa of wild type and Asc-deficient mice infected for 1 month with *H. pylori* PMSS1. Cells were pregated on live cells based on forward and side scatter. Horizontal lines indicate the mean. Results from two pooled experiments are shown (t-test). Representative FACS blots of CD4⁺ cells are shown.

Infection with *H. pylori* led to infiltration of CD45⁺ and CD4⁺ cells into the stomach. Infected Asc knockout mice showed slightly more CD4⁺ cells than wild type animals (Figure 26B).

IL-1β and IL-18 are known to contribute to induction of IL-17 and IFNγ responses, respectively. Both cytokines are important for clearing *H. pylori*, and further they contribute to inflammation. Hence, gastric expression of IL-17 and IFNγ was measured.

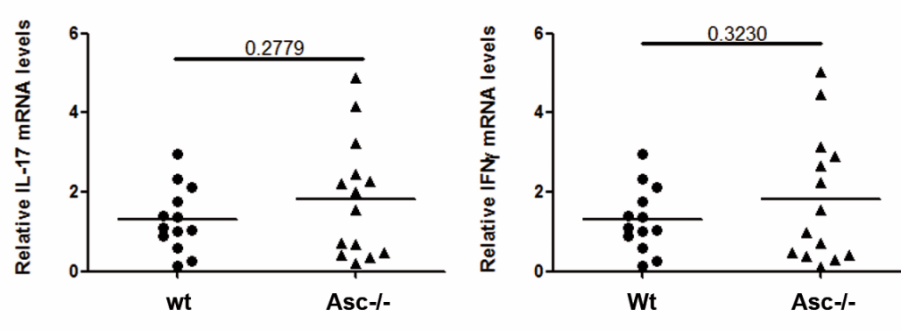

Figure 27: Gastric IL-17 and IFNγ expression induced upon *H. pylori* infection.

Relative IL-17 and IFNγ mRNA expression in the stomach of *H. pylori*–infected wild type and Asc-deficient mice infected with PMSS1 for 1 month. Results were normalized to GAPDH. Horizontal lines indicate mean values. Results from two pooled experiments are shown (t-test).

Upon infection with *H. pylori,* expression of IL-17 and IFNγ was induced, however, expression levels did not differ significantly between *H. pylori*-infected Asc-deficient and wild type animals (Figure 27).

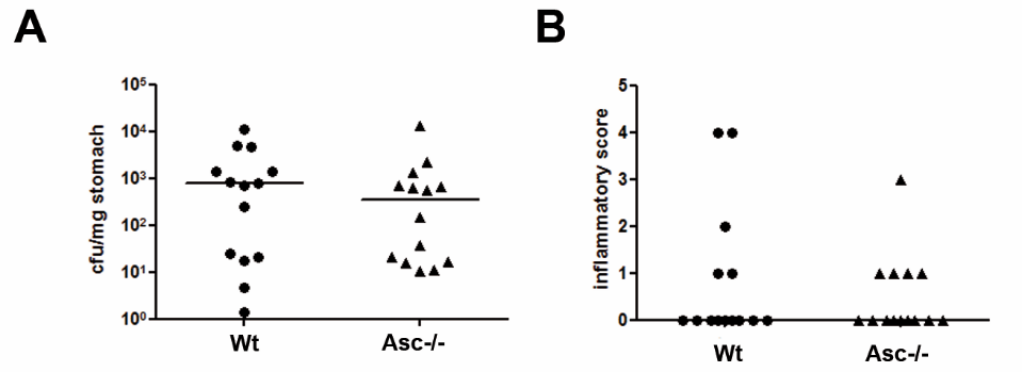

Figure 28: Asc-deficiency does not alter colonisation and inflammation.

(A) Colony forming units (cfu) per milligram stomach of wild type and Asc$^{-/-}$ mice infected 1 month with the *H. pylori* strain PMSS1. Results from two pooled experiments are shown. Horizontal lines indicate median values.

(B) Inflammatory score assessed in the gastric mucosa of wild type and Asc-deficient mice after 1 month of infection. Results from two pooled experiments are shown. Horizontal lines indicate median values.

No difference in bacterial burden between Asc-deficient and wild type mice was detected (Figure 28A), also, there were similar levels of gastric inflammation in Asc-deficient and -proficient animals (Figure 28B).

Altogether, these results indicate that ASC regulates immune responses, however these altered host responses did not result in changed bacterial colonisation levels.

3.2.2 NLRP3 inflammasome activation triggers host responses which contribute to clearing and inflammation

IL-1β and IL-18 secretion upon *H. pylori* infection was greatly reduced in absence of NLRP3 in BMDCs. Therefore, it was investigated whether *H. pylori*-induced NLRP3 inflammasome activation triggers gastric IL-1β and IL-18 production and whether this plays a role in the immune response toward *H. pylori* infection *in vivo*.

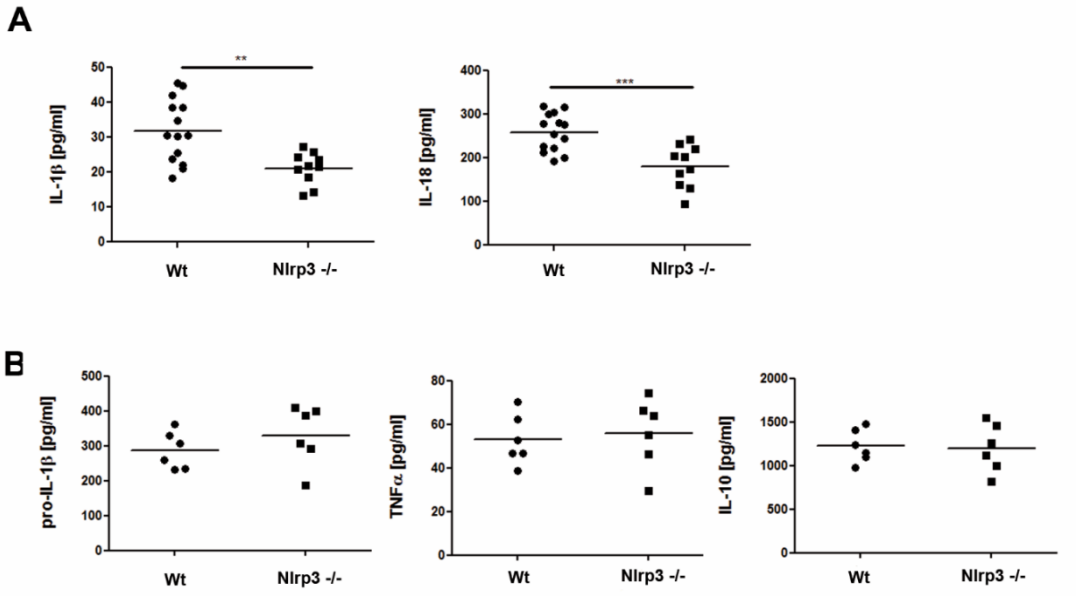

Figure 29: *H. pylori*-induced IL-1β and IL-18 production depends on the NLRP3 inflammasome *in vivo*.

(A) IL-1β and IL-18 protein levels detected in the gastric extracts of wild type and Nlrp3$^{-/-}$ mice infected for 1 month with the *H. pylori* strain PMSS1. Results from two experiments are shown. Horizontal lines indicate mean (**≤0.01, ***≤0.001, t-test).

(B) Pro-IL-1β, IL-10, and TNFα protein levels detected in the gastric extracts of wild type and Nlrp3-deficient mice infected for 1 month with the *H. pylori* strain PMSS1. Results from one experiment is shown. Horizontal lines indicate mean.

Compared to *H. pylori*-infected wild type mice, infected Nlrp3-deficient mice showed reduced levels of IL-1β and IL-18 in the stomach (Figure 29A). Thus, NLRP3 is crucial for *H. pylori*-induced inflammasome activation *in vivo*. In contrast, the accumulation of pro-IL-1β or the production of inflammasome-independent cytokines IL-10 and TNFα were not affected by the lack of NLRP3.

Cytokines contribute to recruitment and differentiation of immune cells. To analyse if the lack of IL-1β and IL-18 alters infiltration of immune cells, amounts of lymphocytes and $CD4^+$ T cells, the cell types which are considered to contribute to bacterial clearing but also to inflammation, were analysed.

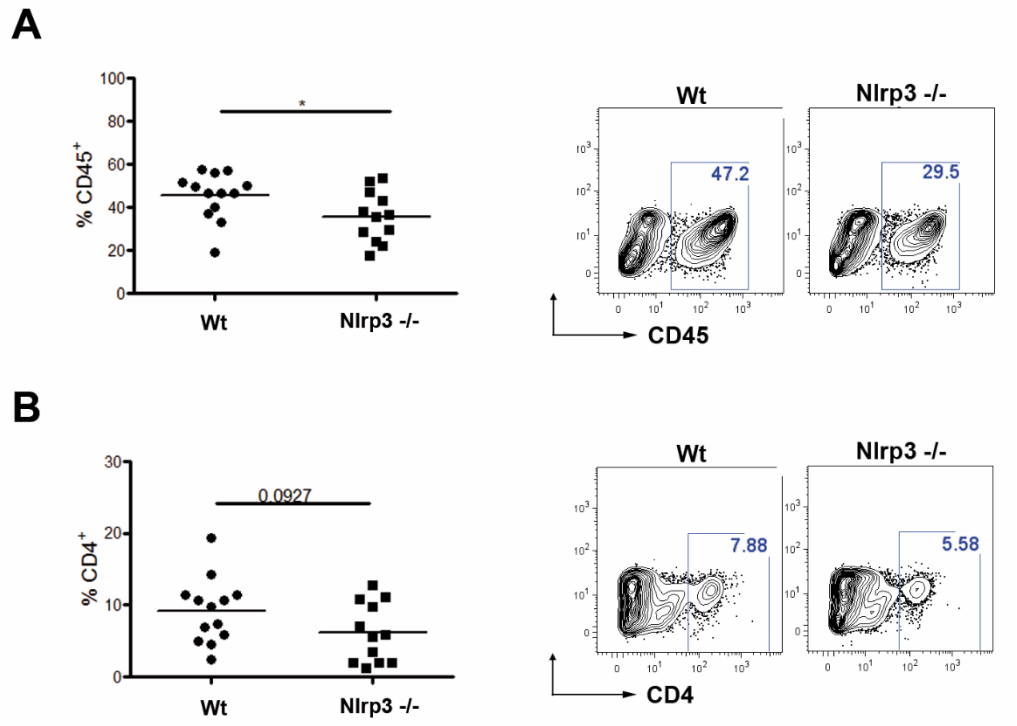

Figure 30: Nlrp3-deficient mice show lower infiltration of lymphocytes.

(A) Percentage of leukocytes ($CD45^+$) cells infiltrating the gastric mucosa of wild type and Nlrp3-deficient mice infected for 1 month with *H. pylori* PMSS1. Cells were pregated on live cells based on forward and side scatter. Horizontal lines indicate the mean. Results from two pooled experiments are shown (t-test). Representative FACS blots are shown of $CD45^+$ cells.

(B) Percentage of $CD4^+$ T cells infiltrating the gastric mucosa of wild type and Nlrp3-deficient mice infected for 1 month with *H. pylori* PMSS1. Cells were pregated on live cells based on forward and side scatter. Horizontal lines indicate the mean. Results from two pooled experiments are shown (t-test). Representative FACS blots are shown of $CD4^+$ cells.

Infection with *H. pylori* led to infiltration of $CD45^+$ and $CD4^+$ cells into the stomach, however lower infiltration of $CD45^+$ cells was observed in the stomach of NLRP3

knockout mice compared with wild type animals (Figure 30A). *H. pylori*-infected NLRP3 knockout mice also showed a tendency towards lower infiltration of $CD4^+$ cells (Figure 30B).

To assess the influence of the inflammasome in the adaptive immune response toward *H. pylori*, levels of IL-17 and IFNγ in the gastric mucosa were measured.

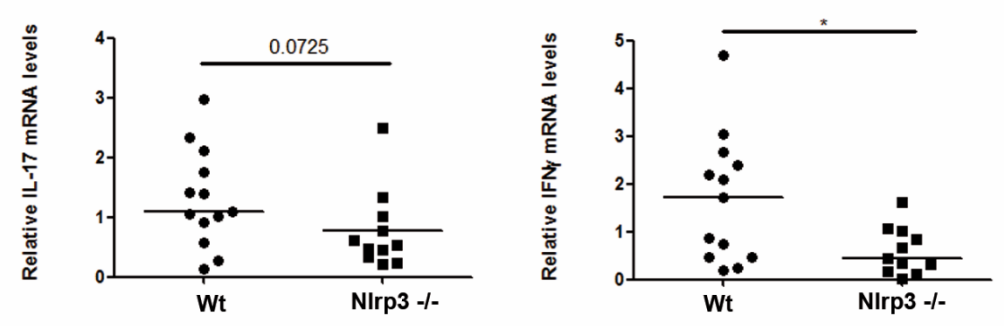

Figure 31: Nlrp3-deficient mice show lower gastric IL-17 and IFNγ expression.

Relative IL-17 and IFNγ mRNA expression in the stomach of *H. pylori*–infected wild type and Nlrp3-deficient mice. Results were normalized to GAPDH. Horizontal lines indicate the median. Results from two pooled experiments are shown (*≤0.05, Mann-Whitney U test).

Upon infection with *H. pylori*, gastric expression of IL-17 and IFNγ were induced. However, expression of both cytokines was reduced in *H. pylori*-infected Nlrp3-deficient mice compared to wild type animals (Figure 31).

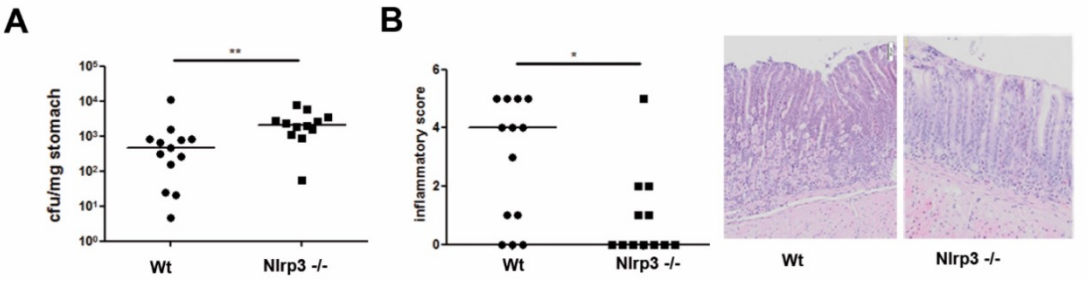

Figure 32: Nlrp3-deficient mice show impaired bacterial clearing.

(A) Colony forming units (cfu) per milligram stomach of wild type and $Nlrp3^{-/-}$ mice infected 1 month with the *H. pylori* strain PMSS1. Results from two pooled experiments are shown. Horizontal lines indicate median (**≤0.01, Mann-Whitney U test).

(B) Inflammatory score assessed in the gastric mucosa of wild type and Nlrp3-deficient mice after 1 month of infection. Results from two pooled experiments are shown. Horizontal lines indicate the median (*p≤0.05, Mann–Whitney U test). Representative micrographs of H&E-stained stomach sections are shown. Scale bar, 50 µm.

Nlrp3-deficient mice showed higher colonisation compared to wild type mice (Figure 32A). This lower bacterial load in wild type mice was accompanied by higher degree of inflammation compared to NLRP3 knockout mice (Figure 32B). This lower inflammatory score corresponded the results analysing infiltration of immune cells by flow cytometry (Figure 30).

Altogether, these results indicate that *H. pylori*-induced IL-1β and IL-18 induction during *H. pylori* infection depends on NLRP3. The NLRP3 inflammasome affects gastric *H. pylori* colonisation as well as the subsequent immune response and gastric pathology.

3.2.3 *H. pylori* evades clearance by activating NLRC4 inflammasome

We and others did not detect significant differences in *H. pylori*-induced IL-1β or IL-18 from BMDCs (172, 175). Thus, it was analysed if Nlrc4 dependent inflammasome activation indeed does not play a role in eliciting IL-1β production and following immune response toward *H. pylori in vivo*.

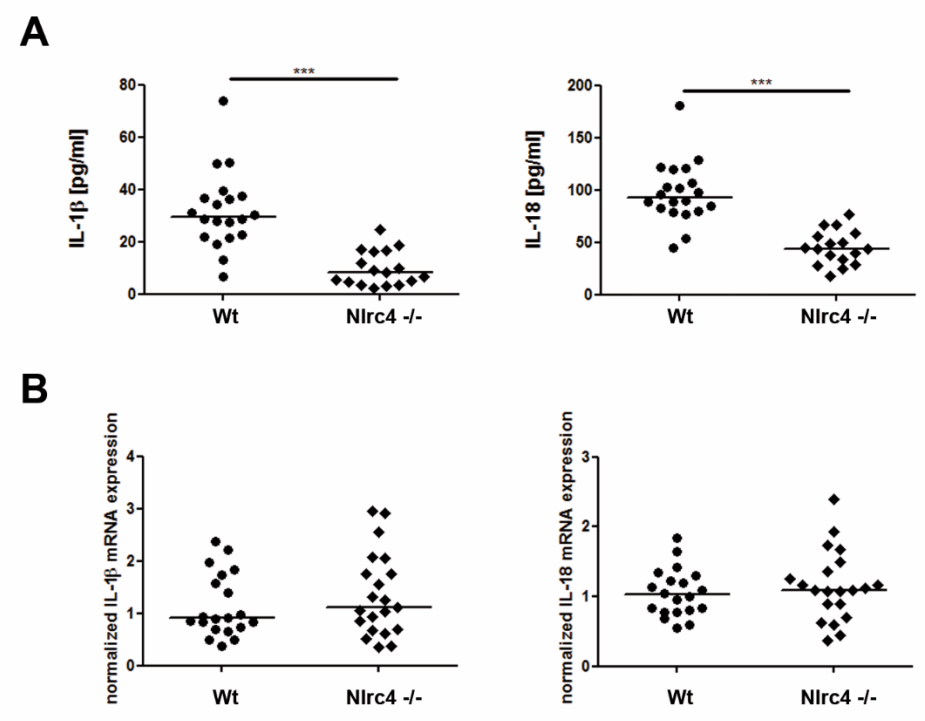

Figure 33: *In vivo*, IL-1β and IL-18 is not cleaved in Nlrc4 knockout mice

(A) IL-1β and IL-18 protein levels detected in the gastric extracts of wild type and Nlrc4$^{-/-}$ mice infected for 1 month with the *H. pylori* strain PMSS1. Results from three pooled experiment are shown. Horizontal lines indicate median values (***≤0.001, Mann Whitney U Test).

(B) IL-1β and IL-18 expression levels detected in the gastric extracts of wild type and Nlrc4$^{-/-}$ mice infected for 1 month with the *H. pylori* strain PMSS1. Results from three pooled experiment are shown. Horizontal lines indicate median values (*≤0.05, Mann Whitney U Test).

Surprisingly, in contrast to stimulation of Nlrc4-deficient DCs *in vitro*, infection with *H. pylori* induced neither mature IL-1β nor IL-18 in the stomach of NLRC4 knockout mice (Figure 33A). However, the expression of the pro-forms was induced upon infection in both genotypes and did not differ (Figure 33B).

Apart from immune cells, IL-1β and IL-18 can be produced by other cells such as gastric epithelial cells. Thus, it was tested if murine primary gastric epithelial cells secrete inflammasome-associated cytokines and if their secretion is affected by Nlrc4-deficiency.

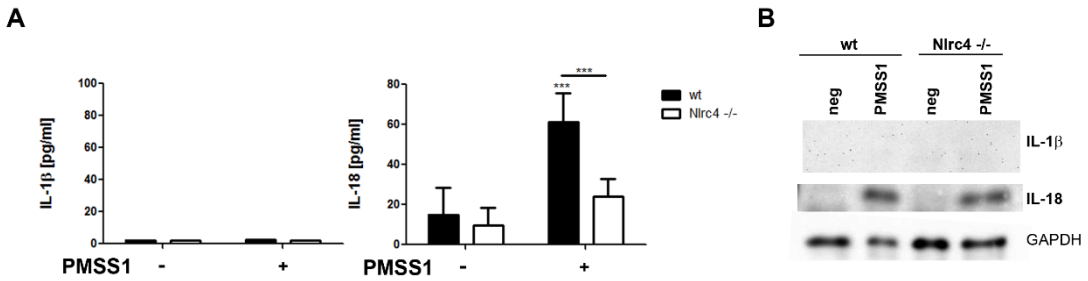

Figure 34: *H. pylori*-triggered IL-18 production from primary gastric epithelial cells depends on Nlrc4.

(A) Wildtype and Nlrc4$^{-/-}$ primary epithelial cells were infected o/n with *H. pylori* PMSS1 (MOI 50). Levels of IL-1β and IL-18 were analysed in the supernatant by specific ELISAs. Results are presented as mean±SD of six independent experiments. Unstimulated cells were compared to *H. pylori*-infected cells (asterisks shown on the top of the bars) ($p<0.0001$ ANOVA, ***≤0.001 Bonferroni-corrected for pairwise comparison).

(B) Wildtype and Nlrc4$^{-/-}$ primary epithelial cells were infected o/n with *H. pylori* PMSS1 (MOI 50). Protein levels of pro-IL-1β and pro-IL-18 were detected in cell lysate. GAPDH was measured as loading control. One representative western blot from the experiments shown in (A) is presented.

Infection of primary gastric epithelial cells with *H. pylori* did not result in production of IL-1β. However, expression of pro-IL-18 was induced upon infection in wild type and Nlrc4-deficient cells, but the secretion of IL-18 from gastric epithelial cells was decreased in absence of NLRC4 (Figure 34).

To analyse if the lack of gastric NLRC4 inflammasome activation results in altered bacterial clearing, colonisation levels and inflammation were analysed in NLRC4 KO mice.

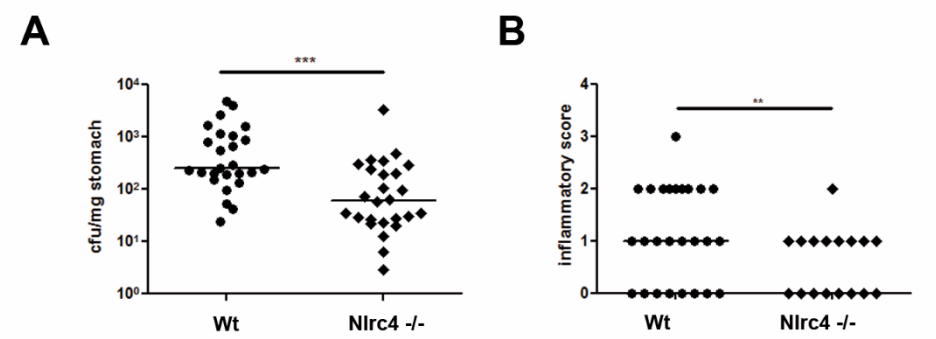

Figure 35:Nlrc4 impairs bacterial clearing but induces inflammation.

(A) Colony forming units (cfu) per milligram stomach of wild type and Nlrc4$^{-/-}$ mice infected 1 month with the *H. pylori* strain PMSS1. Results from four pooled experiments are shown. Horizontal lines indicate median values (***≤0.001, Mann-Whitney U test).

(B) Inflammatory score assessed in the gastric mucosa of wild type and Nlrc4-deficient mice after 1 month of infection. Results from four pooled experiments are shown. Horizontal lines indicate median values. (**≤0.01, Mann-Whitney U test).

Nlrc4-deficient mice showed lower colonisation compared to wild type mice (Figure 35A). Surprisingly, this was accompanied with lower gastric inflammation (Figure 35B).

To elucidate how this phenotype is coming off, immune responses were analysed in more detail. Since induction of innate immune cells is crucial for differentiation of adaptive immune responses, expression of cytokines linked to T cell differentiation but also to clearing and gastritis were examined. Further, also epithelial-derived chemokine production linked to inflammation was analysed. CXCL8 (IL-8) is the main chemokine produced by epithelial cells upon contact with *H. pylori* in humans. It is important for initial recruitment of immune cells. Mice lack a direct homologue of CXCL8, but the chemokines KC (CXCL1) and MIP-2 (CXCL2) are considered as functional orthologues of IL-8.

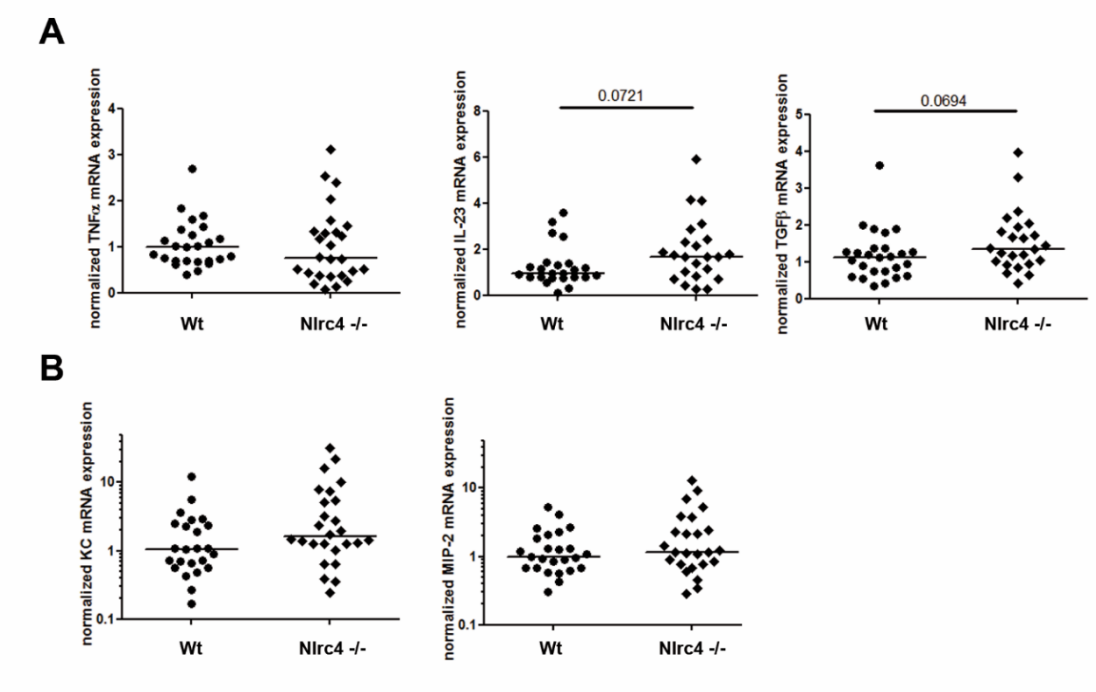

Figure 36: Gastric cytokine and chemokine expression.

(A) Relative TNFα, IL-23 and TGFβ mRNA expression in the stomach of *H. pylori*–infected wild type and Nlrc4-deficient mice. Results were normalized to GAPDH. Horizontal lines indicate the median. Results from four pooled experiments are shown (Mann Whitney U test).

(B) Relative KC and MIP-2 mRNA expression in the stomach mucosa of *H. pylori*–infected wild type and Nlrc4-deficient mice. Results were normalized to GAPDH. Horizontal lines indicate the median. Results from four pooled experiments are shown (Mann Whitney U test).

When analysing cytokine expression in the stomach, no significant differences in the expression of TNFα, IL-23 or TGFβ could be detected. However, there was a trend towards a higher expression of the T_H17 linked cytokines IL-23 and TGFβ in NLRC4 knockout mice (Figure 36A). These cytokines are mainly produced by innate immune cells such as DCs or macrophages, and trigger T cell responses. TGFβ is important for differentiation of T_H17 cells whereas IL-23 is important for the maintenance and activation of this T cell subset. In contrast, TNFα is produced by DCs, macrophages, NK cells but also T_H1 cells.

Although expression of the IL-8 orthologues was induced upon infection, there were no differences in KC and MIP-2 expression between *H. pylori*-infected Nlrc4-proficient and -deficient mice (Figure 36B).

To analyse if differential recruitment of immune cells to the stomach might be responsible for the phenotype of NLRC4 KO mice, composition and amounts of infiltrating immune cells were analysed.

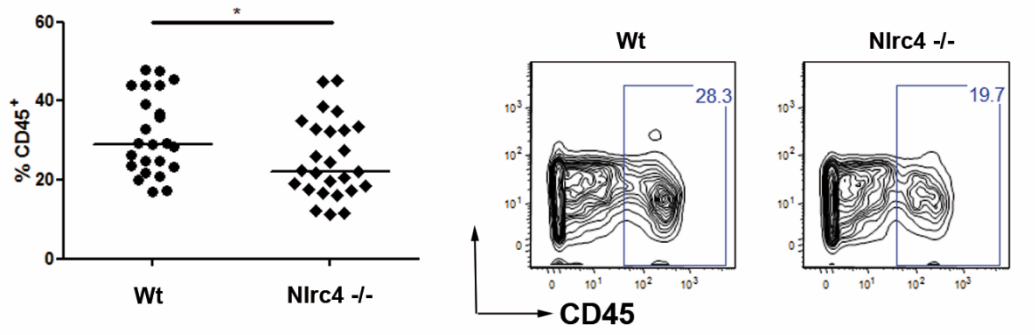

Figure 37: NLRC4 knockout mice show lower infiltration of hematopoietic cells.

Percentage of leukocytes ($CD45^+$) cells infiltrating the gastric mucosa of wild type and Nlrc4-deficient mice infected for 1 month with *H. pylori* PMSS1. Cells were pregated on live cells based on forward and side scatter and EMA staining-. Horizontal lines indicate the median. Results from four pooled experiments are shown (*≤0.05; t-test). Representative FACS blots are shown.

Infection with *H. pylori* led to infiltration of $CD45^+$ cells into the stomach, however, lower amounts of these cells were observed in the stomach of Nlrc4-deficient mice compared to wild type animals (Figure 37). These results fit with the histologic evaluation, were less inflammatory cells were present (Figure 35B).

CD45 is expressed on all hematopoietic cell, thus composition of these cells was characterised. Since T cell are the functional component of *H. pylori*-induced cellular immune response linked to bacterial clearing, infiltration of T cells was analysed.

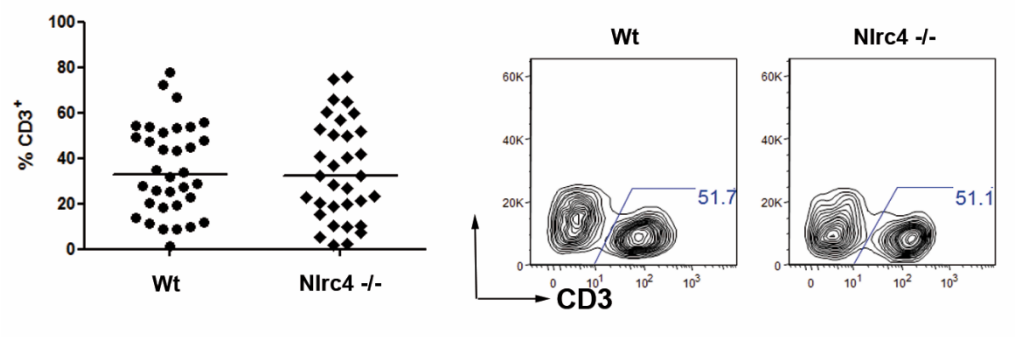

Figure 38: Nlrc4-deficiency does not alter total T cell levels.

Percentage of $CD3^+$ cells infiltrating the gastric mucosa of wild type and Nlrc4-deficient mice infected for 1 month with *H. pylori* PMSS1. Cells were pregated on live cells based on forward and side scatter and EMA staining, and pregated on $CD45^+$. Horizontal lines indicate the median. Results from four pooled experiments are shown.

Upon infection with *H. pylori*, T cells were recruited into the stomach, but there were no differences in T cell levels between *H. pylori*-infected wild type and NLRC4 knockout mice (Figure 38).

Since there was no difference in the whole amounts of T cells, it was investigated, if the composition of infiltrating T cell differs between the genotypes, and this might cause better clearing of the bacterium.

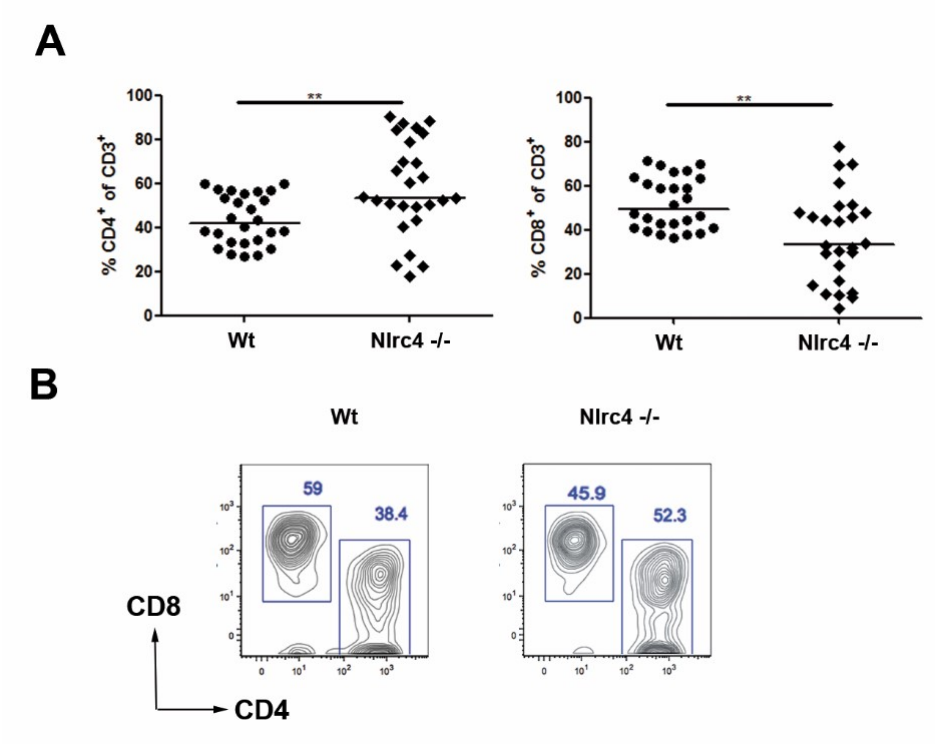

Figure 39: Nlrc4-deficient mice show higher CD4⁺ T cell responses.

Percentage of $CD4^+$ and $CD8^+$ T cells infiltrating the gastric mucosa of wild type and Nlrc4-deficient mice infected for 1 month with *H. pylori* PMSS1. Cells were pregated on live cells based on forward and side scatter and EMA staining, and pregated on $CD45^+$and $CD3^+$ cells. Horizontal lines indicate the median. Results from four pooled experiments are shown (**≤0.01, ***≤0.001; t-test). Representative FACS blots are shown.

Upon infection with *H. pylori*, $CD4^+$ and $CD8^+$ T cells were infiltrating the gastric mucosa, however, their ratio differed in Nlrc4-deficient and wild type mice. In Nlrc4-deficient animals a stronger infiltration of $CD4^+$ T cells was observed, whereas in wild type animals there were more $CD8^+$ T cells (Figure 39).

To characterise the T cell response further, expression of IL-17, IFNγ and IL-10 were analysed. These cytokines are typical for T_H17, T_H1 or T_{reg} cells, respectively. Further, also expression of FoxP3, the main transcription factor of regulatory T cells, was measured.

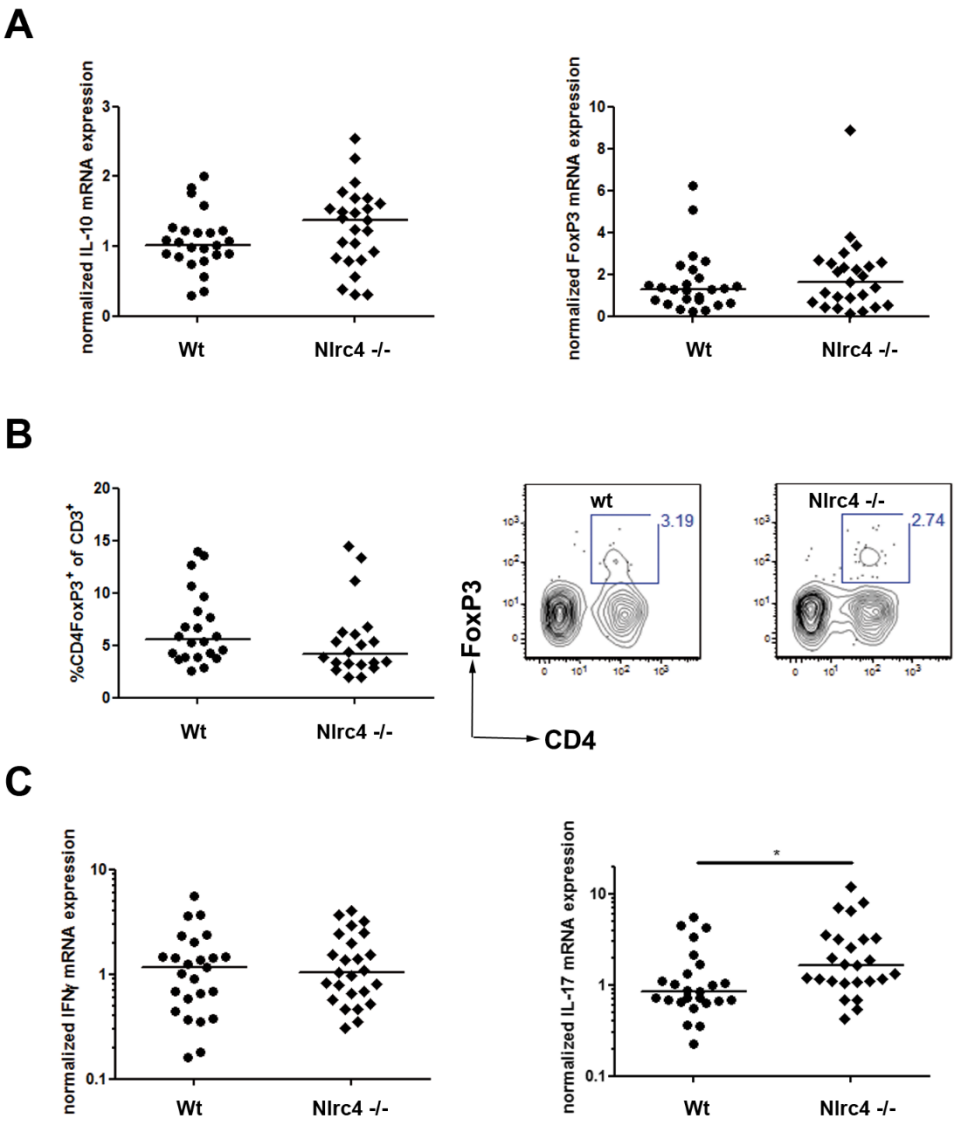

Figure 40: Nlrc4-deficient mice show higher IL-17 expression.

(A) Relative IL-10 and FoxP3 mRNA expression in the stomach mucosa of *H. pylori*–infected wild type and Nlrc4-deficient mice. Results were normalized to GAPDH. Horizontal lines indicate the median. Results from four pooled experiments are shown (Mann-Whitney U test).

(B) Percentage of regulatory T cells ($CD4^+FoxP3^+$) infiltrating the gastric mucosa of wild type and Nlrc4-deficient mice infected for 1 month with *H. pylori* PMSS1. Cells were pregated on live cells based on forward and side scatter and EMA staining, and gated on $CD45^+$ and $CD3^+$ cells. Horizontal lines indicate the median. Results from three pooled experiments are shown. Representative FACS blots are shown.

(C) Relative IL-17 and IFNγ mRNA expression in the stomach mucosa of *H. pylori*–infected wild type and Nlrc4-deficient mice. Results were normalized to GAPDH. Horizontal lines indicate the median. Results from four pooled experiments are shown (*≤0.05, Mann Whitney U test).

Upon infection with *H. pylori,* gastric expression of the T_{reg}-associated transcription factor Foxp3 and the regulatory/anti-inflammatory cytokine IL-10 were induced in both genotypes, but their expression was not significantly affected by the lack of NLRC4

(Figure 40A). Corresponding to the expression of FoxP3, there were the same amounts of T_{reg} cells in infected Nlrc4-deficient and wild type animals (Figure 40B). Expression of the inflammatory cytokines IFNγ and IL-17 was induced by *H. pylori*. While the expression of IFNγ did not differ between infected wild type and Nlrc4-deficient mice, IL-17 expression was higher in the knockout animals (Figure 40C). The higher $CD4^+$ levels in NLRC4 knockout mice might be T_H17 cell, and might be responsible for the higher IL-17 expression found in these mice, since T_H17 are considered as the main producer of IL-17 upon *H. pylori* infection.

Another cell type which can produce IL-17 in *H. pylori* infected mice are γδ-T cells.

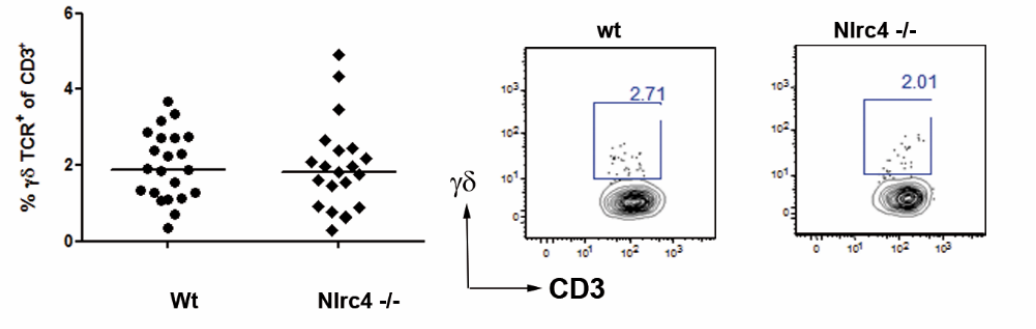

Figure 41: Nlrc4 knockout mice do not differ in levels of γδ-T cells.

Percentage of γδ-T cells infiltrating the gastric mucosa of wild type and Nlrc4-deficient mice infected for 1 month with *H. pylori* PMSS1. Cells were pregated on live cells based on forward and side scatter and EMA staining, and gated on $CD45^+$ and $CD3^+$ cells. Horizontal lines indicate the median. Results from three pooled experiments are shown. Representative FACS blots are shown.

γδ-T cells were infiltrating the stomach upon *H. pylori* infection but there was no difference between *H. pylori*-infected wild type and Nlrc4-deficient animals (Figure 41).

One other immune cell subset, which is associated with IL-17 but also with inflammation are neutrophils.

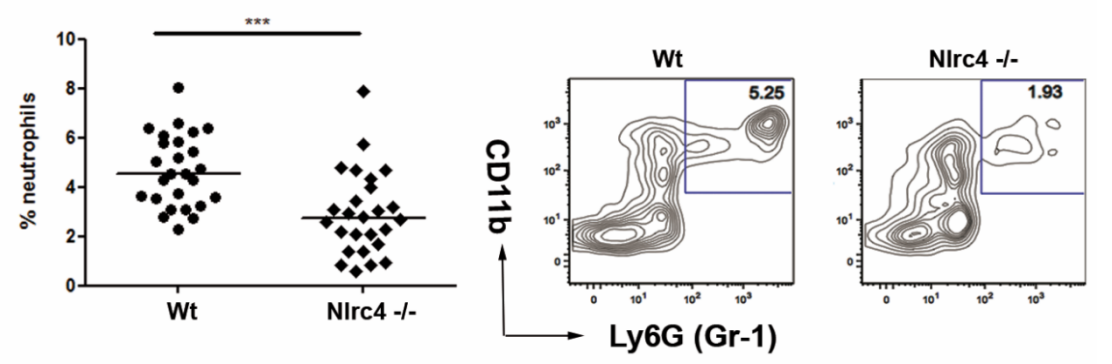

Figure 42: Nlrc4 knockout mice show lower infiltration of neutrophils.

Percentage of neutrophils ($Ly6G^+CD11b^+$) infiltrating the gastric mucosa of wild type and Nlrc4-deficient mice infected for 1 month with *H. pylori* PMSS1. Cells were pregated on live cells based on forward and side scatter and EMA staining, and pregated on $CD45^+$. Horizontal lines indicate the median. Results from four pooled experiments are shown (***≤0.001; Mann Whitney U test). Representative FACS blots are shown.

Upon infection neutrophils infiltrated the gastric mucosa. *H. pylori*-infected NLRC4 knockout animals showed much lower amounts of these cells compared to wild type mice (Figure 42).

A recent study linked high IL-17 levels in absence of IL-18 to induction of defensins, which fostered clearing of *P. aeruginosa* without inducing detrimental inflammation (351). Consequently, it was analysed if defensins are involved in better clearing of *H. pylori* in absence of NLRC4.

Studies analysing defensin production upon *H. pylori* infection were almost exclusively performed in human cells. The two defensins mainly linked to *H. pylori* infection are human β-defensin-1 (hDB-1) and hDB-2. Both are bactericidal against *H. pylori* (352, 353). mDefb-1 and mDefb-3 are considered as their functional orthologues in mice (354, 355). Till now, murine β-defensin-1 (mDefb-1) and mDefb-3 expression was not analysed during *H. pylori* infection. hDef-1 and hDef-2 are regulated in a CagPAI-dependent manner, thus mDefb-1 and mDefb-3 expression in mice infected with PMSS1 or SS1 was analysed.

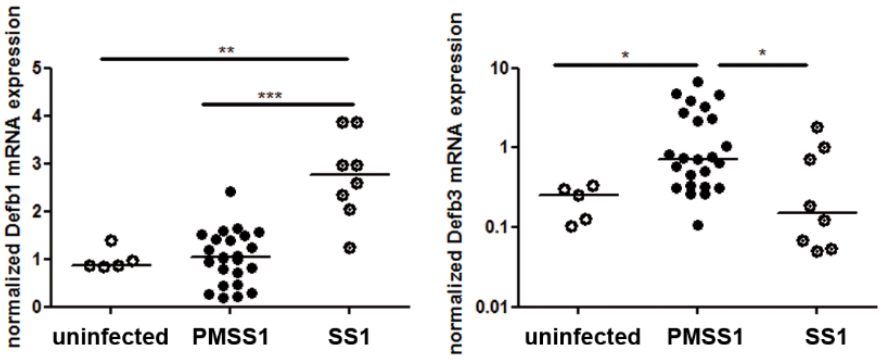

Figure 43: mDefb-1 and mDefb-3 expression is regulated in a CagPAI-dependent manner.

Relative mDefb-1 and mDefb-3 mRNA expression in the stomach mucosa of *H. pylori*–infected wild mice. Results were normalized to GAPDH. Horizontal lines indicate the median. Results from pooled experiments are shown (p=0.0005 (mDefb-1), p=0.0059 (mDefb-3), ANOVA Kruskal-Wallis test; *≤0.05, **≤0.01, ***≤0.001, Dunn's Multiple Comparison Test).

Infection with the CagPAI-proficient PMSS1 strain did not upregulate gastric expression of mDefb-1. However, an infection with the Cag-PAI-deficient SS1 strain resulted in higher expression levels compared to non-infected or PMSS1-infected animals (Figure 43). The expression of mDefb-3 was induced upon PMSS1 infection, in contrast infection with SS1 did not lead to mDefb-3 induction (Figure 43). Thus, mDefb-1 and mDefb-3 expression was regulated by *H. pylori* in a CagPAI-dependent manner. A similar expression was reported for the human orthologues.

Since NLRC4 activation depends on bacterial secretion systems, and defensin expression during *H. pylori* infection is regulated by the CagPAI, defensin expression in NLRC4 KO mice which were infected with PMSS1 or SS1 was investigated.

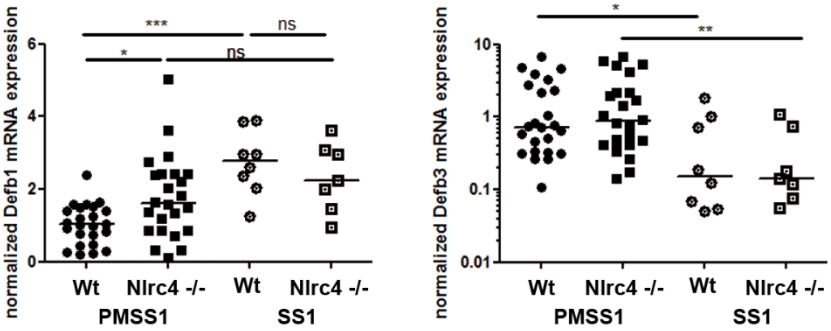

Figure 44: mDefb-1 upregulation in Nlrc4 knockout mice depends on functional CagPAI.

Relative mDefb-1 and mDefb-3 mRNA expression in the stomach mucosa of *H. pylori*–infected wild and Nlrc4 knockout mice. Mice were infected with PMSS1 or SS1 for 1 month as indicated. Results were normalized to GAPDH. Horizontal lines indicate the median. Results from pooled experiments are shown (ANOVA p=0.0002 (Defb-1), p=0.0066 (Defb-3); *≤0.05, Mann Whitney U test).

Higher expression of mDefb-1 in PMSS1-infected Nlrc4-deficient mice was detected. In contrast to infection with PMSS1 strain, mDefb-1 levels did not differ between wild type and Nlrc4-deficient mice infected with SS1 strain. Infection with SS1 resulted in similar mDefb-1 levels as seen in PMSS1-infected NLRC KO mice (Figure 44). Thus, the block of mDefb-1 induction in PMSS1-infected wild type mice was dependent on NLRC4 and CagPAI.

Expression of mDefb-3 did not differ between wild type and Nlrc4$^{-/-}$ animals that were infected with PMSS1. Also the expression levels of SS1-infected wild type and Nlrc4-deficient mice did not differ but the levels of SS1-infected animals were significant lower than the ones of PMSS1-infected animals (Figure 44). Thus, induction of mDefb-3 expression was not Nlrc4-dependent but CagPAI-dependent.

To analyse if this upregulation of mDefb-1 is specific for NLRC4 and not general for NLR KO mice, mDefb-1 expression was analysed in PMSS1-infected NLRP3 KO mice.

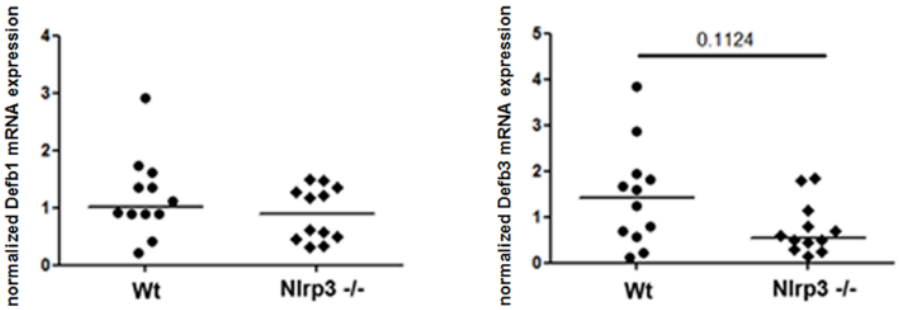

Figure 45: mDefb-1 upregulation is specific for Nlrc4 deficiency.

Relative mDefb-1 and mDefb-3 mRNA expression in the stomach mucosa of *H. pylori*–infected wild and Nlrp3 knockout mice. Mice were infected with the PMSS1 strain. Results were normalized to GAPDH. Horizontal lines indicate the median. Results from two pooled experiments are shown (Mann Whitney U test).

Absence of NLRP3 did not result in altered mDef-1 expression, and also mDef-3 expression was not affected by lack of NLRP3 (Figure 45).

In summary, NLRC4 inflammasome activation might be one factor by which *H. pylori* dampens host defences and enables its chronic colonisation of the stomach.

4 Discussion

Infection with *H. pylori* is one of the most prevalent bacterial infections worldwide. It is estimated that more than 50 % of the world's population harbours the bacterium, with infection rates being much higher in developing countries than in developed ones. Although *H. pylori* infection does not cause disease in most infected people, it is a major risk factor for peptic ulcer disease, ulcers of the stomach and duodenum. Further, long-term infection with *H. pylori* can cause gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Every year nearly one million new gastric cancer cases are diagnosed, making gastric cancer the fourth most common cancer globally. Patients with gastric cancer have a very bad prognosis, thus every year gastric cancer kills more than 700 000 people, making it the second most common cause of cancer-related deaths in the world (356).

Although an immune response against *H. pylori* is triggered, the bacterium is not eliminated. The coevolution of *H. pylori* and humans has driven *H. pylori* to develop evasion mechanisms by escaping and manipulating immune responses, making them ineffective in eliminating the bacterium. However, the long-term presence of this inflammatory milieu in the stomach may predispose gastric cells to become cancerous. Particularly the pro-inflammatory cytokine IL-1β has been extensively reported to play an essential role in *H. pylori* infection and *H. pylori*–associated gastric pathology. The importance of IL-1β in gastric cancer development is emphasized by the observation that polymorphisms in *IL-1B* gene that result in higher cytokine production increase the predisposition to gastric cancer in *H. pylori*-infected individuals (328). The important role of IL-1β was substantiated by studies in mice showing that gastric IL-1β overexpression induced spontaneous precancerous lesions and gastric cancer (326), whereas gastric tumour development was suppressed in IL-1β-deficient mice (357). IL-18 was also shown to be increased in *H. pylori*-infected patients. IL-18 has been linked to increased metastasis and immune escape of gastric tumour cells (358), and certain *IL-18* polymorphisms predispose individuals to the development of gastric cancer (325).

Several studies linked increased levels of IL-1β and IL-18 to *H. pylori*-induced pathologies, however, the involvement of the inflammasome was not studied until recently.

In the current study, it was shown that *H. pylori* indeed triggers caspase-1 cleavage, and that caspase-1 activity is essential for secretion of mature active IL-1β and IL-18 from different murine and human immune cells such as DCs, macrophages and neutrophils. In contrast, B, T and NK cells did not produce IL-1β upon *H. pylori* infection. *H. pylori*-triggered IL-1β and IL-18 secretion by innate immune cells was also observed in murine BMDCs, human macrophages and neutrophils in other studies (172, 175, 315, 359).

There are several inflammasome types known which are activated in innate immune cells upon stimulation with pathogens. The protein ASC is part of several inflammasome complexes. ASC was essential for *H. pylori*-induced caspase-1 activation and thus IL-1β/IL-18 secretion in this study. This is in accordance with two other studies showing that casapse-1 activation and IL-1β production were absent in Asc-deficient cells (172, 175). Furthermore, NLRP3 was identified as the inflammasome mainly activated in response to the *H. pylori*, whereas NLRC4 seemed not be involved in *H. pylori*-induced IL-1β and IL-18 secretion from BMDCs. Interestingly, a recent study reported a downregulation of Nlrc4 upon *H. pylori* infection in a human macrophage cell line (184). This might explain why there was no Nlrc4-dependent IL-1β production upon *H. pylori* infection in DCs. Transcription of Nlrc4 is regulated by the tumour suppressor p53 (360). *H. pylori* is able to inhibit p53 pathways by degradation of p53 (361). By the downregulation of Nlrc4 *H. pylori* avoids Nlrc4-dependent inflammasome activation, and thus absence of NLRC4 has no impact on *H. pylori*-induced IL-1β secretion *in vitro*. These results are in agreement with two other studies, which showed that BMDCs from Nlrc4 knockout mice secreted unaltered levels of IL-1β upon infection with *H. pylori*, whereas the lack of NLRP3 resulted in reduced levels of IL-1β and absence of capase-1 activation (172, 175). Further, IL-1β and IL-18 secretion was reduced in human THP-1 cells after treatment with NLRP3-specific siRNA (183). AIM2 and NLRP6 inflammasome activation was excluded in *H. pylori*-induced IL-1β secretion and caspase-1 activation from BMDCs (175).

Most of the studies analysing *H. pylori*-induced IL-1β production focused on how PRRs were involved in NLRP3-dependent IL-1β induction and secretion. BMDCs from TLR2 knockout mice showed less IL-1β secretion because the expression of Nlrp3 was reduced (172, 175). Further, TLR2 might also contribute to pro-IL-1β induction since IL-1β secretion was blocked in TLR2-deficient cells infected with *H. pylori*, but *H. pylori*-induced IL-1β was rescued in LPS prestimulated cells (172). These results argue that TLR2 is important for the priming step to induce Nlrp3 and pro-IL-1β expression. In contrast, in another study, stimulation of TLR2 knockout BMDCs with *H. pylori* led to unaltered pro-IL-1β mRNA levels (175), claiming that TLR2 stimulation only led to upregulation of Nlrp3 but not to pro-IL-1β. In human neutrophils, blocking of TLR2 did not alter IL-1β production at all (359). NOD2 might be also involved in *H. pylori*-induced IL-1β production, but this is controversial, since one study reported reduced IL-1β levels upon *H. pylori* infection due to lower pro-IL-1β and Nlrp3 expression in NOD2-deficient BMDCs (172) while another claimed that NOD2 is dispensable (175). These results indicate that NOD2 signalling might contribute to the priming step upon *H. pylori* infection, since *H. pylori*-induced IL-1β secretion was rescued in LPS prestimulated cells (172). Absence of NOD1, for which NFκB activation in epithelial cells was shown (55, 178), did not affect *H. pylori*-induced IL-1β secretion in DCs (172). Likewise, *H. pylori* induced IL-1β production was not altered in absence of TLR4, TLR5 and TLR9 (175). However, TLR4 signalling contributed to the priming step, since LPS from *H. pylori* induced TLR4-dependent expression of pro-IL-1β, and infection with a mutant lacking the LPS O-side chain resulted in lower IL-1β secretion due to less production of pro-IL-1β but not in altered caspase-1 cleavage. When cells were primed with LPS, *H. pylori*-induced IL-1β production triggered by this LPS mutant strain was rescued (175). The TLR4-dependent capacity to induce pro-IL-1β is in accordance with a study in human THP-1 macrophages. However, in THP-1 cells, the LPS of *H. pylori* was also able to induce caspase-1 cleavage (332). This is in contrast to murine macrophages, in which LPS of *H. pylori* could not induce caspase-1 activation and mature IL-1β secretion. Notably, even an inhibiting effect of *H. pylori* LPS was observed, since IL-1β secretion induced by other gram-negative bacteria was blocked in *H. pylori* LPS-prestimulated cells. This antagonistic effect of LPS from *H. pylori* was specific to the non-canonical inflammasome activation, but was not seen with the

canonical stimuli ATP or *P. aeruginosa* (294). Thus, it is not surprising that *H. pylori*-induced IL-1β secretion did not depend on the TRIF-IRF7-IFNaR signalling (175), which is crucial for non-canonical inflammasome activation.

The studies of Kim et al. (172) and Koch et al. (175) mainly analysed how *H. pylori* primes the NLRP3 inflammasome activation. In our study, however, we focused on the activation step of inflammasome activation and provided direct evidence that *H. pylori* provides the second signal inducing the assembly and activation of the inflammasome, and identified the main virulence factors and cellular mechanisms involved.

Because of the broad spectrum of stimuli, which are able to activate the NLRP3 inflammasome, NLRP3 has been considered to be a sensor of the disruption of host physiology. *H. pylori* possesses several virulence factors able to profoundly change cellular physiology. Therefore, it is not surprising that a number of molecular mechanisms involved in the *H. pylori*–mediated activation of the NLRP3 inflammasome were found. Common NLRP3 inflammasome–activating mechanisms include potassium efflux, lysosomal destabilization, and generation of ROS. Blocking any of these three stimuli in immune cells substantially reduced IL-1β secretion upon *H. pylori* infection. This is in accordance with studies in human neutrophils and THP-1 cells, where treatment with ROS scavenger NAC inhibited inflammasome-associated cytokine secretion (183), and block of potassium efflux by KCl or Glyburide, which inhibits ATP-sensitive potassium channels, resulted in lower IL-1β production (359). However, the ATP-gated ion channels P2X7R seemed not be involved in this potassium efflux effect (175).

The p58 subunit of *H. pylori* VacA was shown to induce potassium efflux from liposomes at acidic pH (362), whereas induction of ROS by VacA has been demonstrated in some studies (363-365). In addition, VacA was described to lower mitochondrial transmembrane potential (366), another mechanism by which the NLRP3 inflammasome can be activated (254). These observations suggest that VacA might be involved in inflammasome stimulation. Indeed, VacA-deficient bacteria were less efficient in inducing IL-1β release and caspase-1 cleavage, confirming an involvement of VacA in inflammasome activation. This observation is in contrast to

data from Kim et al. (172) who could not detect changes in IL-1β secretion after infecting cells with *H. pylori* strains deficient for VacA. One possible explanation for this discrepancy is our strategy of using LPS as priming signal to dissect the bacterial factor acting as second signal triggering the inflammasome, which was not respected in the previous study.

Although other virulence factors such as gGT can induce ROS production, no significant effects on IL-1β secretion were observed when using gGT-deficient bacteria. This is in accordance with data from Koch et al. (175). We speculate that in the absence of gGT other virulence factors such as VacA can elicit the cellular mechanisms responsible of inflammasome activation. A double mutant (ΔgGT VacA) could be used to proof this.

Further, the CagPAI of *H. pylori*, but not CagA, was important for IL-1β secretion. This observation is in line with the previous results by Kim et al. (172) but in contrast to the study from Koch et al. (175). A strain-dependent effect is not likely to be the explanation for this discrepancy, since Kim at al. showed CagPAI dependence in two different *H. pylori* strains and one of the strains - G27- was the strain Koch et al. were using for infecting BMDCs. T4SS-dependent IL-1β secretion was confirmed in the study in human PBMCs with the same G27 mutant strain. Further, in a third strain - PMSS1 - the requirement of functional T4SS in providing the activation signal for *H. pylori*-induced inflammasome assembly was confirmed. Many bacterial species have been described to activate primarily the NLRC4 inflammasome via T3SS and T4SS (367). Interestingly, the delivery of effector molecules through the T4SS has been recently shown to be required for NLRP3 inflammasome activation as well (292, 368), indicating that the presence of an active T4SS might represent an alternative mechanism of NLRP3 activation during bacterial infection. Stimulation with *Salmonella* SopE, which is injected via a T3SS into the host cell, works as a guanine nucleotide exchange factor for the Rho GTPase and activated caspase-1 (368). In contrast effector proteins such as ExoU and ExoS (*P. aeruginosa*) or YopE (*Y. enterocolitica*), which inhibit Rho GTPases blocked caspase-1 activation (227, 369, 370). For *H. pylori* it was shown that the Rho GTPases RacA and Cdc42 are activated in a CagPAI-dependent but CagA-independent manner in epithelial cells (371). Interestingly, also endocytosis of VacA involves activation of these small GTPases (372). Rho GTPases are involved in actin cytoskeleton

organization and phagocytosis as well as NADPH oxidase activation and ROS production (373), mechanisms linked to Nlrp3-dependent inflammasome activation. Apart from CagA, *H. pylori* peptidoglycan is also delivered into host cells through the T4SS. The particulate nature of peptidoglycan was found to be essential for activation of NLRP3 inflammasome and alteration of peptidoglycan by *Staphylococcus aureus* strongly reduces production of IL-1β in response to infection (374). Therefore, we speculate that *H. pylori* peptidoglycan injected into the host cell might be involved in the activation of the inflammasome. Further experiments are needed to substantiate this hypothesis.

It was interesting to note a markedly reduced IL-1β secretion when human PBMCs were infected with *H. pylori* strains lacking either of the adhesins BabA or SabA, suggesting that adhesion-mediated T4SS-dependent secretion of a factor independent of CagA induces activation of the inflammasome. The T4SS can also bind directly to the host cell (e. g. via integrins). Thus, the reduced adhesion to the host cell might also contribute to the decreased IL-1β secretion from cells infected with CagPAI-deficient strains. Thus, binding of the bacterium to the host cells represents a crucial event for inflammasome activation. This might also involve phagocytosis of *H. pylori*, which is delayed and reduced in adhesion-deficient strains.

One kinase which might be involved in NLRP3 inflammasome activation is Syk. Syk can either be important for the priming step by inducing NFκB downstream of different PRRs, or by inducing cellular responses such as cytoskeletal changes, ROS production or Ca^{2+} flux (375), all mechanisms linked with NLRP3 inflammasome activation. Indeed, *H. pylori* activates this kinase and blockage of Syk activity decreased *H. pylori*-induced IL-1β secretion. *H. pylori*-induced Syk signalling is involved in the priming step to induce expression of IL-1β but also is required for caspase-1 activation (Master thesis Theresia Fischer). Further studies are needed to analyse how this kinase is involved in *H. pylori*-induced IL-1β secretion. *H. pylori* binds to several receptors (e.g. TREM-1 (376), DC-SIGN (377, 378), CEACAM (47), integrin (379-381), which signal via Syk. Whether one of these receptors or another Syk-coupled receptor, for which an *H. pylori* binding was not shown before, are involved in *H. pylori*-induced inflammasome activation is object of current studies. We speculate that by binding to

one of these receptors *H. pylori* induces IL-1β expression but also triggers in a Syk-dependent manner downstream event such as ROS production, phagocytosis or ion fluxes, which are crucial for *H. pylori*-induced IL-1β secretion. Further experiments are needed to substantiate this hypothesis.

Having observed that *H. pylori* can activate the inflammasome in different innate immune cells, the involvement of the inflammasome in the immune response to the bacterium was analysed *in vivo*. Because the *in vitro* results indicated that NLRP3 was mainly responsible of regulating IL-1β secretion upon *H. pylori* infection, Nlrp3-deficient mice were infected with *H. pylori* strain PMSS1, which is proficient for all the virulence factors previously shown to be important for inflammasome activation. In contrast to Kim et al. (382), this study found that mice lacking NLRP3 were colonised at higher levels compared with wild-type animals. This higher bacterial colonisation was accompanied with lower inflammation and T cell infiltration. Moreover, also reduced levels of IL-17 and IFN-γ were detected in the gastric mucosa of Nlrp3-deficient mice upon infection with *H. pylori*. These results suggest that NLRP3 activation triggered by *H. pylori* in innate immune cells represents an important mechanism contributing to the inflammatory response toward the bacterium and are in line with previous observations by Hitzler et al. (315) showing that IL-1 receptor-deficient mice present less *H. felis*–associated pathology and show reduced T_H1 and T_H17 responses. This attenuated immune response might explain why IL-1R- or IL-1β-deficient mice were higher colonised during infection with a CagPAI-proficient *H. pylori* strain (172), and less metaplastic changes upon *H. pylori* infection were observed in PMSS1-infected IL-1R-deficient mice (329). When IL-1R knockout mice were infected with the CagPAI-deficient SS1 strain no differences in lymphocyte infiltration, gastric IFNγ and IL-17 expression, and bacterial burden were observed (315). This work as well as Kim et al. (172) clearly showed that functional CagPAI is crucial for full inflammasome activation in DCs. This was also confirmed *in vivo*, where infection with SS1 led to low or no gastric IL-1β protein production (383). Surprisingly, in a recent study from Koch et al. Nlrp3 knockout mice (B6.129S6-Nlrp3^{tm1Bhk}/J (384)) showed lower gastric colonisation due to higher IFNγ production upon PMSS1 infection (175). This phenotype recapitulates the phenotype of IL-18R knockout mice and was similar to the phenotype

of caspase-1 or IL-18 knockout mice in studies from the same group. These knockout mice showed lower colonisation, mainly due to higher IL-17 production (106, 315). It was postulated that these knockout mice control *Helicobacter* infections better because they failed to induce IL-18 or its signalling, which is crucial for the induction of T_{reg} cells. The absence of the regulatory responses facilitated stronger IL-17 and IFNγ responses, which cleared the infection but also induced pathology (106, 315). In contrast to the results from the studies from Hitzler et al. (315) and Oertli et al. (106), IL-18 deficient mice were colonised to a similar extend as were wild type animals in two other studies (127, 176). This difference in IL-18 KO mice was not due to the *H. pylori* strain used because, except from the study from Oertli et al. in which PMSS1 was used, the other three studies used the SS1 strain and the same IL-18 KO strain. In the studies from Hitzler et al. (SS1) and Oertli et al. (PMSS1), which were conducted from the same group, the IL-18 KO mice showed lower bacterial burden compared to wild type animals. Interestingly, the PMSS1-infected IL-$18^{-/-}$ animals displayed a slight greater reduction than SS1-infected ones. Thus, the phenotype seemed to be strengthened by inoculation with a CagPAI-proficient strain, probably due to differential IL-18 levels in PMSS1-infected wild type animals compared to SS1-infected ones. In contrast to the study from Hitzler at al., Kim at al. observed higher colonisation of caspase-1 knockout mice. These mice displayed similar colonisation levels than the IL-1R knockout mice from the same study (172, 315). These different results in caspase-$1^{-/-}$ mice might be due to the different *H. pylori* strains used for infection. As this study showed *in vitro*, caspase-1 activation was much lower in SS1-infected cells compared to cells infected with PMSS1.This might explain why in mice infected with the SS1 strain no differences in colonisation were detected in contrast to mice infected with a CagPAI-proficient strain.

NLRP3 mediated inflammasome activation might be an early event in *H. pylori* infection, responsible for eliciting the strong adaptive immune response to control the infection. Because of the different mechanisms developed by the bacterium to evade the host's immune system, the infection persists and the levels of IL-1β remain high. IL-1β has been shown to directly inhibit acid secretion from rat gastric parietal cells (385), which may contribute to hypoacidity and finally, together with TNF-α, to parietal cell

loss (386). Furthermore, polymorphisms inducing elevated levels of IL-1β have been associated with increased risk of gastric cancer. It has to be noted that the risk was restricted to the non-cardia subsite, suggesting that the distribution of the inflammation is important in terms of gastric cancer risk (387). Taken together, these events pave the way for the development of gastric atrophy, which is considered the initial step of gastric carcinogenesis according to the Correa hypothesis, which can under conditions of continuous inflammation eventually lead to the development of gastric cancer.

Surprisingly, when analysing Asc-deficient mice, no effects on bacterial burden were detected, although Asc KO mice do not produce mature IL-1β and IL-18. This is in agreement with a study of Benoit et al. (388). They also could not detect differences in colonisation or inflammation after two months of infection, despite absence of mature IL-1β and IL-18 in ASC KO mice. Only after 16 weeks there was slightly lower bacterial load accompanied with higher gastric IFNγ levels and moderate gastritis in wild type animals (388). In our study, ASC knockout mice in addition to their defect in producing mature IL-1β and IL-18, also showed lower levels in pro-IL-1β, IL-10 and TNFα. The expression of these cytokines is inflammasome independent but NFκB dependent. Depending on its expression level, cellular location and the presence of other proteins, Asc can be an activator of NFκB (389-391) or suppressor of this pathway (392-394). Thus, knockdown of Asc in THP-1 cells resulted in lower IL-1β, IL-6, IL-8, IL-10 and TNFα expression due to less NFκB activation (395). Further, Asc modulates MAPK activity and chemokine induction (395), STAT3, ISGF3, NF-AT and AP-1 (396), pathways involved in cytokine production. Probably, by attenuation of several host responses, the lack of inflammasome activation can be compensated and the bacterium is cleared.

In this study as well as in others (172, 175) *H. pylori*-triggered IL-1β secretion was not changed in Nlrc4-deficient BMDCs. However, *H. pylori* infected Nlrc4$^{-/-}$ mice failed to produce mature IL-1β and IL-18 in the stomach. This lack of IL-18 might be due to the inability of gastric epithelial cells from NLRC4 KO mice to secrete mature IL-18 despite expression of its pro-form. This is in accordance with studies using primary human gastric epithelial cells and murine biopsies, where mature IL-18 was induced upon

H. pylori infection (319, 388). Interestingly, no pro-IL-1β could be detected in *H. pylori*-infected murine primary gastric epithelial cells. This is in agreement with a study of Shigematsu et al. that only saw IL-1β staining in cells infiltrating the lamina propria but not in epithelial cells in biopsies from *H. pylori*-infected mice (357). In contrast, two other studies showed IL-1β staining in immune and epithelial cells in *H. pylori*-infected mice (331, 388). Induction of IL-18 expression upon *H. pylori* infection was shown in different gastric cell lines but secretion of mature IL-18 was not analysed (319). Also production of mature IL-1β was observed in gastric cell lines upon *H. pylori* infection in some studies (331) but not in others (397). It has to be noted that cell lines derived from gastric tumours or achieved by immortalisation might have features which healthy cells do not have. Interestingly, KATO III cells showed constitutive mature IL-18 and mature caspase-1 (330). KATO III cells originate from a gastric carcinoma, which has already metastasised (398), and IL-18 has been linked to increased metastasis and immune escape of gastric tumours (358).

Although Nlrc4-deficient mice failed to produce IL-1β and IL-18 in their stomach, they showed lower bacterial burden, which was not accompanied by inflammation. Analysing gastric cytokine expression and infiltration of immune cells, the main differences we could find were higher gastric IL-17 expression and more $CD4^+$T cells in NLRC4 KO mice. We conclude that in absence of NLRC4 more T_H17 cells infiltrated the stomach, since also expression of TGFβ and IL-23, cytokines that are crucial for induction and maintenance of T_H17, were increased in NLRC4 KO mice.

Further, in NLRC4 knockout mice less neutrophils could be detected. Neutrophils infiltrated stomach upon *H. pylori* infection. By the production of reactive oxygen intermediates neutrophils play an important role in gastritis (399-401). It has been shown that depletion of neutrophils in *Helicobacter*-infected mice decreased the severity of gastritis (402). Thus, the higher numbers of neutrophils in wild type mice might be responsible for the higher inflammation seen in these mice. It could be possible that some of these neutrophils are myeloid-derived suppressor cells (MDSCs). MDSCs are a morphological, phenotypic, and functional heterogenic cell subset. They are characterized by their myeloid origin, immature state, and by their potent ability to suppress immune responses. MDSCs are not present under healthy conditions or acute

bacterial infection but are found during chronic infection, inflammation, or cancer. MDSCs are usually characterized by expression of surface markers Gr-1$^+$ and CD11b$^+$. Gr-1 consists of the two components Ly6C and Ly6G. Ly6G is exclusively expressed on neutrophils, whereas Ly6C is highly expressed on monocytes. Thus, MDSCs can be divided into neutrophilic MDSCs (PMN-MDSCs, CD11b$^+$Ly6G$^+$Ly6C$^{low/int}$) and monocytic MDSCs (Mo-MDSCs, CD11b$^+$Ly6G$^-$Ly6C^{high}). Inflammatory monocytes and neutrophils are phenotypically almost identical to Mo-MDSCs and PMN-MDSCs, respectively. However, there are functional differences. Inflammatory monocytes and neutrophils cannot suppress T cell functions. In contrast to inflammatory monocytes, Mo-MDSCs upregulate arginase-1 and iNOS simultaneously. By this, peroxynitrites are produced which inhibit CD8$^+$ T cell functions (403, 404). PMN-MDSCs express high levels of arginase-1 and myeloperoxidase, and produce high levels of ROS. By this, PMN-MDSCs suppress several immune functions (405-407) but also induce inflammation. Further, MDSCs are less phagocytic than inflammatory macrophages or neutrophils. To test if these Ly6G$^+$CD11b$^+$ cells seen in *H. pylori*-infected mice are PMN-MDSCs further characterizations, such as expression of additional surface (e. g. CD11c and MHC-II are lower in MDSCs than in inflammatory cells) and functional markers (e. g. arginase-1, myeloperoxidase, IDO), are needed. However, the presence of MDSCs would explain the stronger gastritis and the limited bacterial clearing seen in *H. pylori*-infected wild type mice. Generation of MDSCs is triggered by IL-1β and IL-18 (326, 408). Interestingly, these cells were responsible for the induction of gastric inflammation in the transgenic mouse model, in which stomach-specific IL-1β production was sufficient for gastric cancer development (326).

Despite altered production of cytokines and recruitment of immune cells, also diverse production of antimicrobial peptides might contribute to better bacterial clearing observed in Nlrc4-deficient mice. mDef-3 expression was not affected by the absence of Nlrc4. Infection with PMSS1 led to induction of mDefb-3, whereas mDefb-3 levels of SS1 infected animals were similar to levels in naïve mice. This is in accordance with human studies reporting that hDB-2, the orthologue of mDefb-3, is induced upon infection with *H. pylori* (180, 352, 409-414) in a CagPAI-dependent manner (180, 414). In contrast to mDefb-3, there was a difference in mDefb-1 expression between wild

type and NLRC4 KO mice. In PMSS1-infected mice, higher expression of mDefb-1 was observed in absence of NLRC4. NLRC4 KO mice showed higher mDefb-1 expression than uninfected controls. In contrast, in wild type mice infection with PMSS1 did not lead to higher mDefb-1 expression compared to naïve animals. However, SS1-infected wild type and Nlrc4-deficient animals showed similar and higher expression levels than uninfected mice. Thus, mDefb-1 might be induced by *H. pylori*, but CagPAI-containing strains can block this induction. This inhibition of mDefb-1 was absent in NLRC4 KO mice. The regulation of the human orthologue hDB-1 by *H. pylori* is poorly analysed and there are conflicting results in literature. Most studies showed that hBD-1 is constitutively expressed in the stomach (415) and is not induced by *H. pylori in vivo* and *in vitro* in gastric cell lines (180, 352, 410-412). Other studies showed upregulation (413, 416) or downregulation (414) *in vivo* and *in vitro* upon *H. pylori* infection. This discrepancy is hard to explain since the studies used the same gastric cells lines (AGS and MKN7), similar infection doses, and in part also same *H. pylori* strains. Further, some authors confirmed their findings by different methods such as Q-PCR, ELISA and immunohistochemistry. The downregulation of hDB-1 seems to be regulated by the NOD1-NFκB pathway, since inhibiting or knockdown of NFκB abolished downregulation of hDB-1. Infection with *H. pylori* harbouring a functional CagPAI and able to produce peptidoglycan resulted in lower hBD-1 expression compared to infection with CagPAI-deficient strains or a mutant which attenuated peptidoglycan production (414). In a study that reported upregulation of hBD-1, this was Erk dependent (416). These findings corroborate in part with our *in vivo* results. We observed lower mDefb-1 levels in PMSS1-infected wild type animals than in SS1-infected mice, indicating that mDefb-1 might be downregulated in a CagPAI-dependent manner. Infection with SS1 strain resulted in mDefb-1 induction, suggesting that upregulation of mDefb-1 expression is CagPAI-independent.

H. pylori can downregulate Def-1 expression in gastric epithelium during infection. This might be one mechanism contributing to the persistence of the bacterium. Def-1 possesses bactericidal activity (352, 353), and it can increase bactericidal activity of other anti-microbial peptides such as hDB-2 and LL-37 (352, 353). Therefore, downregulation of Def-1 provides an additional effect preventing bacterial clearing.

Infection with CagPAI-positive strains blocked mDefb-1 upregulation. This lower mDefb-1 expression correlated with higher bacterial loads and severe inflammation in wild type mice. Negative correlation between hDB-1 levels and bacterial density and inflammation was also seen in human studies (414, 417). Downregulation of defensin-1 by the CagPAI might also contribute to the increased risk for gastric cancer in individuals infected with CagPAI^{+} strains. There are polymorphisms in hDB-1 (DEFB1) gene which are associated with *H. pylori*-induced gastric pathologies (416). Interestingly, other pathogens such as *Neisseria* and *Shigella* can downregulate β-defensin-1 and other antimicrobial peptides by a yet undetermined mechanisms (418, 419).

Since regulation of defensin-1 in general, in gastric epithelium and during *H. pylori* infection is hardly analysed, we can only speculate how *H. pylori* regulates the Def-1 expression. *P. aeruginosa* infected NLRC4 KO mice showed, similarly to *H. pylori* infected mice, no NLRC4-driven production of IL-18, which resulted in higher IL-17 production compared to wild type mice. This IL-17 induced different defensins, that cleared *P. aeruginosa* infection without inducing strong inflammation (351). In skin keratinocytes and bronchial epithelium IL-17 alone or synergistically with IL-22 triggered many antimicrobials such as beta-defensins and S100A9, S100A7, and S100A8 (420, 421). In gastric epithelial cells IL-17A and IL-22 synergistically induced expression of antimicrobials including defensin-1 (422). Since defensin-1 is regulated by NFκB, different factors such as cytokines, probably including IL-18, might regulate the expression.

NLRC4 activation by *H. pylori* prevents the induction of effective immune responses to clear the bacterium. Infiltration of MDSCs might cause inflammation and dampens effective host responses. Downregulation of mDefb-1 was specific in NLRC4 KO mice and was not seen in NLRP3 KO mice. This might be because β-defensins were mainly produced by the epithelium and NLRP3 is not present in gastric epithelial cells. We speculate that NLRP3 and NLRC4 are important during *H. pylori* infection in different cells. NLRC4 is constitutively expressed in macrophages, whereas NLRP3 has to be induced. Since *H. pylori* downregulates NLRC4 (184) but induces NLRP3 (181) in macrophages, IL-1β and IL-18 secretion from immune cells might be mainly Nlrp3-

dependent. However, in the stomach, additional secretion of IL-18 from gastric epithelial cells occur. In contrast to stratified non-keratinizing mucosa that covers the oral cavity and oesophagus, epithelium of ectocervix and vagina, and stratified transitional epithelial cells of the urinary tract, NLRP3 expression is absent in glandular epithelium of the alimentary, reproductive, and respiratory tract (423). Thus, since gastric epithelial cells do not express NLRP3, IL-18 secretion is Nlrc4-dependent in this cell type. Surprisingly, lack of one of the inflammasomes could not be compensated by induction of the other inflammasome since lack of either NLRP3 or NLRC4 was sufficient to block *H. pylori*-induced IL-1β/-18 secretion completely. Probably this is due to the fact that knockdown of one NLR can have profound effects on the expression profile of other NLRs (424) and the capacity of *H. pylori* regulating NLRs expression (181, 184). Interestingly, during gastric cancer NLRC4 is downregulated whereas NLRP3 is upregulated.

H. pylori activates the inflammasome in different cells, such as immune cells, as well as epithelial cells. Different inflammasome types, bacterial virulence factors, and molecular mechanisms regulate the *H. pylori*-induced IL-1β and IL-18 secretion. *In vivo H. pylori*-induced IL-1β and IL-18 production in the stomach depends on different inflammasome types. The activation of inflammasomes has an important role in the onset and establishment of *H. pylori* infection and in the subsequent inflammatory response of the host.

5 Graphical summary

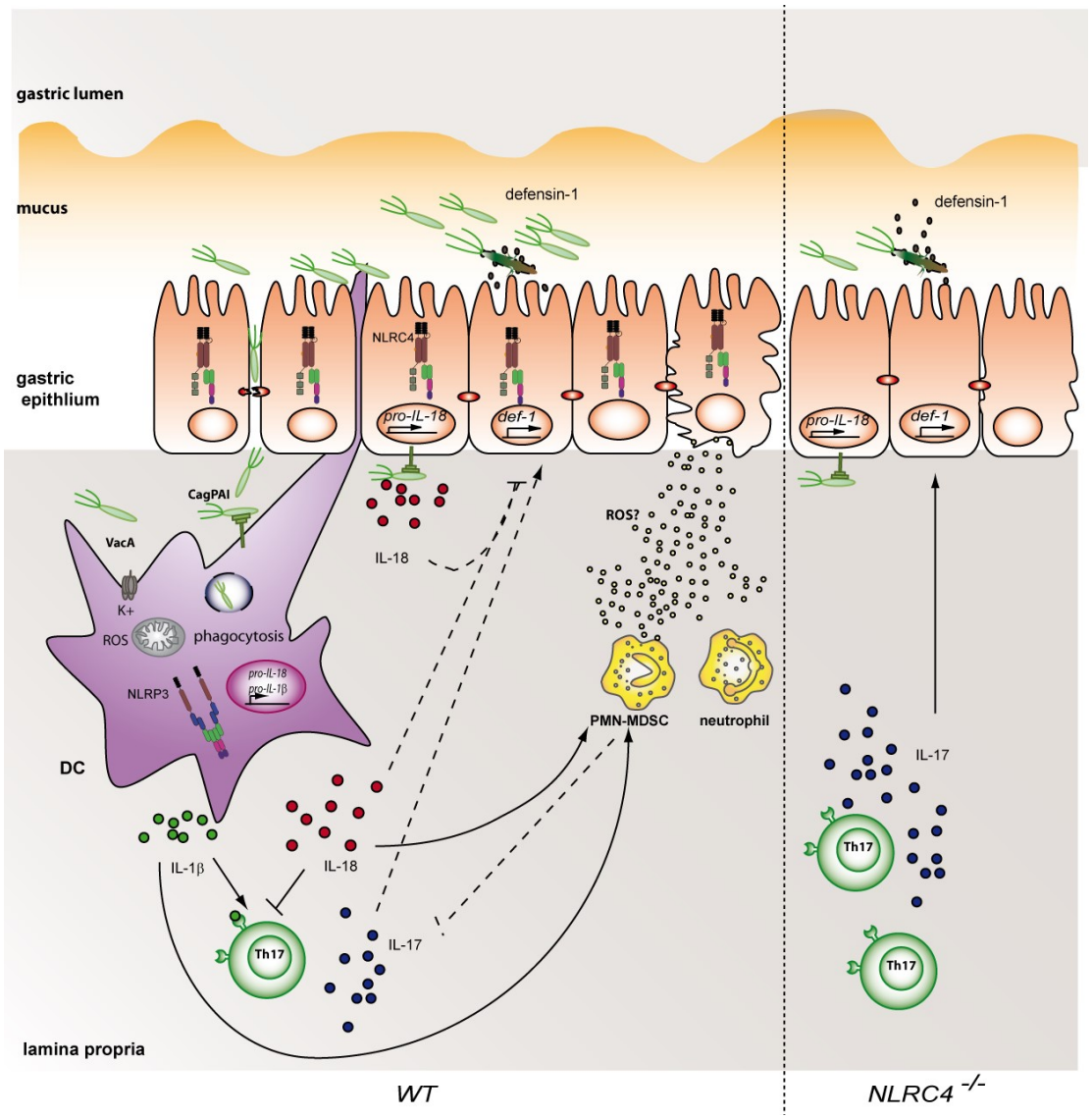

Figure 46: Role of NLRP3 and NLRC4 inflammasomes during *H. pylori* infection.

In DCs *H. pylori* triggered the expression of pro-IL-1β and pro-IL-18. In a NLRP3-dependent manner the inflammasome was activated and mature IL-1β and IL-18 were secreted. Potassium efflux, ROS production and phagocytosis were involved mechanisms in the *H. pylori*–mediated activation of the NLRP3 inflammasome. Further, *H. pylori*-induced inflammasome activation was dependent on the bacterial virulence factors CagPAI and VacA. *In vivo*, there was no production of mature L-1β and IL-18 in absence of NLRP3. IL-1β and IL-18 triggered T cell responses and inflammation, and by this bacterial clearing. In gastric epithelial cell, *H. pylori* activated the expression of pro-IL-18, which was cleaved by the NLRC4 inflammasome. *In vivo*, no mature IL-1β and IL-18 were produced in absence of NLRC4. However, higher levels of T_H17 cells and increased production of defensin-1 were seen in NLRC4 KO mice. This was accompanied with lower inflammation in these mice. Defensin-1 was induced by infection with the CagPAI-deficient *H. pylori* strain, in contrast there was no induction upon infection with the CagPAI-proficient strain in wild type mice. However, in absence of NLRC4 there was still upregulation of defensin-1 in mice infected with the CagPAI-proficient strain. Due to these results, we hypothesise that IL-18 blocks T_H17 differentiation and thus IL-17-dependent upregulation of β-defensin-1. IL-18 might also block β-defensin-1 induction directly. Due to lower production of β-defensin-1, which is bactericidal toward *H. pylori*, less bacteria are killed. Additional, IL-1β and IL-18 production might lead to the recruitment of MDSCs, which inhibit T cell responses but simultaneously their ROS production lead to inflammation.

6 References

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7 Publications

***Helicobacter pylori* gamma-glutamyl transpeptidase and vacuolating cytotoxin promote gastric persistence and immune tolerance**

Oertli, M., Noben, M., Engler, D. B., Semper, R. P., Reuter, S., Maxeiner, J., Gerhard, M., Taube, C., Muller, A.

Proceedings of the National Academy of Sciences of the United States of America 110, 3047-3052. 2013

***Helicobacter pylori*-Induced IL-1beta Secretion in Innate Immune Cells Is Regulated by the NLRP3 Inflammasome and Requires the Cag Pathogenicity Island**

Semper, R. P., Mejias-Luque, R., Gross, C., Anderl, F., Muller, A., Vieth, M., Busch, D.H., Prazeres da Costa, C., Ruland, J., Gross, O., Gerhard, M.

Journal of Immunology 193, 3566-3576, 2014

***Helicobacter pylori* γ-glutamyltranspeptidase induces tolerogenic human dendritic cells by activation of glutamate receptors**

Käbisch, R[1]., Semper, R. P.[1], Wüstner, S., Gerhard, M.[2], Mejias-Luque, R.[2]

[1]Equally contributing first-authors, [2]Equally contributing last-authors

Journal of Immunology 196, 4246-52, 2016;

***Helicobacter pylori* exploits the Nlrc4 inflammasome to dampen host defenses**

Semper, R. P., Vieth, M., Gerhard, M . Mejias-Luque, R

In preparation

8 Conference abstracts

Vaccination against *Helicobacter pylori* with gamma-glutamyltranspeptidase

F Anderl, K Goller, R Semper, C Bolz, B Kalali, RM Schmid, D. H. Busch, M Gerhard

Zeitschrift für Gastroenterologie 2009; 47(10)

Evaluation of different routes and adjuvants for immunization against *H. pylori*

R. Semper, F. Anderl, K. Goller, C. Bolz, D. H. Busch, M. Gerhard

Helicobacter 2010;15(4):314-402

Gamma-glutamyltranspeptidase as promising vaccine candidate to treat *H. pylori* infection

F. Anderl, K. Goller, R. Semper, C. Bolz, B. Kalali, R. Schmid, D. H. Busch, M. Gerhard

Helicobacter. 2010;15(4):314-402

Therapeutic efficacy of a *Helicobacter pylori* vaccine dependent on antibodies and T cells

F. Anderl, R. Semper, K. Goller, C. Bolz, B. Kalali, D. H. Busch, M. Gerhard

Helicobacter 2011;16, Issue Supplement s1:1–152

***Helicobacter pylori* activated the NLRP3 inflammasome**

R. Semper, M. Oertli, D. Engler, F. Anderl, M. Ritter, C. Prazeres Da Costa, J. Ruland, A. Mueller, M. Gerhard

International Journal of Medical Microbiology 2012;302, Supplement 1(3-155).

9 Presentations

Involvement of the inflammasome in infection with *Helicobacter pylori*

Oral presentation at “8th *Helicobacter* Workshop”, DGHM; Herrsching/Ammersee, November 18-20, 2011

***Helicobacter pylori* activated the NLRP3 inflammasome**

Poster presentation at the 64th Annual Meeting of the German Society for Hygiene and Microbiology (DGHM), Hamburg, September 30 - October 03, 2012; Abstract published in International Journal of Medical Microbiology. 2012;302, Supplement 1(3-155).

Involvement of the inflammasome in infection with *Helicobacter pylori*

Oral presentation at “11th International Workshop on Pathogenesis and Host Response in *Helicobacter* Infections”, European Study Group on Pathogenesis and Immunology in *Helicobacter* Infections (ESGPIHI), Helsingør, July 2-5, 2014.

10 Declaration

Hiermit erkläre ich, Raphaela Patricia Semper, dass ich die bei der Fakultät für Medizin eingereichte Dissertation mit dem Titel „The diverse roles of NLRP3 and NLRC4 inflammasomes during Helicobacter pylori infection“ im Institut für medizinische Mikrobiologie, Immunologie und Hygiene unter der Anleitung und Betreuung durch Prof. Markus Gerhard ohne sonstige Hilfe erstellt und bei der Abfassung nur die angegebenen Hilfsmittel benutzt habe. Teile der Arbeiten wurden bereits im The Journal of Immunology unter dem Titel “*Helicobacter pylori*-induced IL-1β secretion in innate immune cells is regulated by the NLRP3 inflammasome and requires the Cag Pathogenicity Island"(338) veröffentlicht.

München, März 2016

11 Acknowledgment