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Effect of *Helicobacter pylori* infection on dendritic cell maturation, signalling and subsequent adaptive immune response

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Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microliter
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Allophycocyanin
APS	Adenosine 5´ phosphosulfate
ATP	Adenosine triphosphate
BabA	Blood group antigen-binding adhesin
B cell	B lymphocyte
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine Serum Albumin
^{13}C/ ^{14}C	Carbon-13/ Carbon-14
Cag	Cytotoxin associated gene
CD	Cluster of differentiation
CpG	Cytosine-phosphatidyl-Guanine
CO_2	Carbon dioxide
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
E. coli	*Escherichia coli*

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked Immunosorbent Assay
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FcR	Fc receptor
FCS	Fetal calf serum
FlaA/B	flagellin subunit A/B
FliC	*Salmonella* enteritidis flagella filament protein
g	Gram
gGT	gamma-Glutamyltranspeptidase
Gln	Glutamine
GM-CSF	granulocyte–macrophage colony-stimulating factor
h	Hour
HCl	Hydrogen chloride
HLA-DR	MHC class II cell surface receptor
H. pylori	*Helicobacter pylori*
H_2O	Water
H_2SO_4	Sulphuric acid
H_3PO_4	Phosphoric acid
HRP	Horseradish peroxidase
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin

IRF	Interferon regulatory factor
JAK	Janus kinases
Kb	kilo base
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
L	Litre
LB	Lucia broth
LPS	Lipopolysaccharide
mA	Milliampere
MALT	Mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
Mg^{2+}	Magnesium
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cell
MOI	Multiplicity of infection
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre
mM	Millimolar
MyD88	Myeloid differentiation primary response gene (88)
N_2	Nitrogen
NaCl	Sodium chloride

Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Disodium phosphate
NaOH	Sodium hydroxide
Nap	Neutrophil-activating protein
NBT	Nitro blue tetrazolium chloride
NK cell	Natural killer cell
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NH_3	Ammonia
ng	Nanogram
O_2	Oxygen
OD	Optical density
PAI	Pathogenicity island
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pen/Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
PRR	Pattern-recognition receptor
pH	Pondus Hydrogenii
RNA	Ribonucleic acid
rRNA	ribosomal RNA

rpm	Rounds per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
T cell	T lymphocyte
TEMED	Tetramethyldiamine
TGF-β	Transforming growth factor beta
Th cell	T helper cell
TIR	Toll/Interleukin-1 receptor
TIRAP	toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAM	TRIF–related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
Tris	Tris(hydroxymethyl)aminomethane
UreA/B/...	urease subunit A/B…
VacA	vacuolating cytotoxin A
v/v	Volume per volume
w/v	Weight per volume
WB	Western blot
WHO	World health organization
x g	fold of gravitation

Introduction

1. Introduction

1.1. Discovery of *Helicobacter pylori*

Several years before the first description of an „unidentified curved bacilli on gastric epithelium in active chronic gastritis“ by Robin Warren and Barry Marshall in 1983, the physician Georg Ernst Konjetzny suggested an infectious agent as cause of gastric ulcer development (1). However, the *in vitro* cultivation of the pathogen and its detailed description was unsuccessful. Warren and Marshall were able to isolate the bacterium from a gastric biopsy and cultivate it (2). The 3rd Koch's postulate was fulfilled by a self-experiment of Marshall who swallowed the pathogen and developed a gastritis that was confirmed by biopsy. Years later, the pathogen was characterised by 16S ribosomal ribonucleic acid (rRNA) analysis and classified as *Helicobacter pylori* (*H. pylori*) - a representative of a new genus (*helico* [*gr.*]: spiral shaped) (3).

1.2. Epidemiologyof *H. pylori*

H. pylori is a gram negative, microaerophil bacterium that selectively colonises the human stomach. It is assumed that the inhabitation of the human stomach began 60,000 years ago, even before humans started to spread over the world (4). Today *H. pylori* colonisation is one of the most prevalent infections worldwide, almost 3 billion people are infected (5), however the prevalence is different. While its distribution in industrialised countries is about 20-50 %, in developing countries up to 90% of the population are affected (6).

H. pylori infection mainly occurs in early childhood, since the pathogen can be transmitted from mother to child (6). In addition, genetically identical strains isolated from members within the same family suggest an oral-oral or faecal-oral bacterial transmission (7).

1.3. *H. pylori*-induced pathology

Gastric colonisation with *H. pylori* induces histologic gastritis in all infected individuals, since it always leads to an infiltration of neutrophilic and mononuclear cells into the gastric mucosa (8). However, in the majority of patients *H. pylori* does not cause clinical symptoms and the infection can persist without further problems (9). A proportion (10-20%) of infected patients develops gastric hyperacidity and peptic ulcers, which can be cured by antibiotic treatment. However, a smaller percentage (0.1-4%) of infected patients develops distal gastric adenocarcinoma, depending on the circumstances of the infection and the individual immune response towards the bacterium (9). The clinical progress of the disease during chronic infection depends on the anatomical location of the *H. pylori*-induced gastritis. Colonisation of the antrum causes an increased production of acid that damages the proximal sections of the duodenum (10). Consequently, acid disrupted duodenal cells are replaced by gastric epithelial cells (gastric metaplasia). A sustained *H. pylori* colonisation and a subsequent expansion of the inflammation until the small intestine can provoke erosions that are described as preliminary stage of duodenal ulcer (11).

Colonisation of the corpus can develop into an atrophic gastritis (12). Destroyed glands of the gastric mucosa resemble the mucosa of the intestine. This intestinal metaplasia is considered to be an early stage in the development of gastric adenocarcinoma (13). Since the risk for adenocarcinoma is increased by a factor of six upon *H. pylori* infection, the pathogen was classified as class-I carcinogen by the WHO in 1994.

Furthermore, *H. pylori* is known to be involved in the development of mucosa-associated lymphoid tissue (MALT) lymphoma (14), a malignant transformation of chronically stimulated lymphocytes.

Although *H. pylori* infection is the main cause of duodenal and gastric ulceration and a major risk factor for gastric adenocarcinoma and lymphoma, *H. pylori*-positive patients have only a limited risk of developing ulcer disease and gastric cancer (15) (16). Importantly, the development of these diseases is influenced by the virulence of the infecting *H. pylori* strain, the genetic susceptibility of the host and environmental co-factors.

1.4. Virulence factors of *H. pylori*

Although infection with *H. pylori* almost always results in chronic active gastritis, most infected patients develop no other complications and are free of any obvious clinical symptoms. This suggests that some *H. pylori* strains may be more virulent than others.
Investigations of the course of disease identified strains associated with increased pathology. The cytotoxin associated gene A (CagA) and the vacuolating cytotoxin A (VacA) play a major role for the pathogenesis of the *H. pylori* infection.

1.4.1. The cag Pathogenicity Island

The cytotoxin associated antigen A (CagA), which is translocated via a type IV-secretion system into eukaryotic target cells is encoded by the *cagA* gene which is located in a 40 kb gene region with the features of a pathogenicity island (PAI) (17) (18). The *H. pylori* PAI was originally named cytotoxin-associated gene (cag), since it was initially associated with the expression of VacA. Later it was shown that both virulence factors are independent of each other, even though *cag*-negative strains often do not express VacA (19). Interestingly, biopsies from patients with severe gastric diseases including chronic active gastritis, peptic ulcer disease, MALT lymphoma and gastric cancer exhibit CagA-positive *H. pylori* in more than 90 % of cases, indicating a direct correlation between the presence of the *cagA* gene with those diseases (20).
Besides *cagA*, the cag PAI encodes a type IV secretion system (17) (18). It forms a complex channel structure through the bacterial membranes in order to transport proteins into the cytoplasm of the host (21). Once inside the cell, CagA becomes phosphorylated on a tyrosine residue (EPIYA motif) by a host cell Src-kinase (22) which triggers morphological changes in the actin cytoskeleton, described as hummingbird phenotype (23), and leads to impaired binding of tight junctions and the acquisition of a scattering phenotype. It was also demonstrated that *cag*-positive strains influence gastric epithelial cell proliferation, reduce cell viability and attenuate apoptosis. These effects might possibly explain the increased risk of gastric cancer (24) however, since a single point mutation in the cag-PAI could abolish CagA translocation, the presence of the *cagA* gene alone is not equivalent to virulence.

1.4.2. VacA

VacA is a cytotoxin secreted by *H. pylori* that has damaging effects on epithelial cells, since it forms an anion-selective voltage-gated ion channel, leading to the outflow of bicarbonate and organic anions (25) (26).Although all isolated *H. pylori* strains have a *vacA* gene, not all show a vacuolating activity, suggesting heterogeneity of the *vacA* gene. Indeed, different allelic variants in the *vacA* sequence in two regions of the gene have been described:
the s allele (located in the region encoding the N-terminal signal region) which may occur as the alleles s1 or s2 type, and the m allele (located at the middle of the *vacA* gene) which is present as m1 orm2 type (27). The presence of *vacA* s1 genotype is a prerequisite for the secretion of an active cytotoxin, whereas *vacA* s2 strains have a defective signal sequence (28). *H. pylori* genotypes with strong vacuolating activity (s1m1 *vacA*) are usually associated with the presence of the *cagA* gene. Moreover, *CagA*/s1m1*vacA*-positive strains correlate with severe disease and the occurrence of ulcers or gastric cancer (27). Because of this clinical relevance they are called type I strains. In contrast to this, type II strains have only a weak vacuolating activity and do not possess a *cag*-pathogenicity island (29) (30) (31).
The expression of VacA and the consequent damage of epithelial cells in the human gastric mucosa could potentially offer a survival advantage to *H. pylori,* once colonisation is established. It is argued that VacA-induced epithelial injury might decrease the integrity of the mucosa (32). The subsequent efflux of nutrients might favour the growth of *H. pylori*. Similarly formation of anion-selective channels in the apical membranes of gastric epithelial cells may also promote *H. pylori* growth in the gastric mucosa (26). The consequent release of hydrogen carbonate from cells is hypothesised to neutralise gastric acidity (33).

1.4.3. Urease

The pH of the human stomach is around 1.4. A key factor for the survival of *H. pylori* in the acidic lumen of the stomach is the production of urease. This enzyme catalyses the hydrolysis of urea that occurs in serum, saliva and gastric juice to CO_2 and NH_3. Ammonia is protonated and thus buffers the periplasm and the immediate environment of *H. pylori,* leading to the formation of a neutral pH microenvironment (34). Furthermore, ammonia, a favoured nitrogen source, is incorporated into proteins and other nitrogenous compounds in *H. pylori* by a single pathway (35): NH_3 + Glutamate + ATP $\rightarrow$ Glutamine + ADP. Furthermore, glutamine serves as nitrogen donor for other nitrogenous compounds including alanine,

glycine, serine, histidine, tryptophan, AMP, carbamoyl-phosphate, and glucosamine 6-phosphate (36).
In total, seven genes are needed for the synthesis of an active urease (37). The two structural subunits are encoded by *ureA* and *ureB,* whose expression is sufficient to produce an assembled apoenzyme (38). For synthesis of a catalytically active urease the accessory genes *ureI, ureE, ureF, ureG,* and *ureH* must also be expressed (37). Previously, UreI has been postulated to form a urea-specific pore in the cytoplasmic membrane of the bacteria that opens at low pH and closes at high pH, thus regulating urea availability to cytoplasmic urease (34). Importantly, this pore is controlled by external pH via a shift in periplasmic pH (39).

1.4.4. Flagella

A unipolar bundle of two to six sheathed flagella enable the bacterium to move through the viscous mucus to the epithelial layer (40). The low pH of its habitat and the constant shedding of the mucus layer represent a continuous threat for *H. pylori* that has to move towards the epithelial cells in order to avoid being washed out by the mucus flow or killed by the stomach acid. For that reason, it has been postulated that the bacterium has the ability to swim actively towards the mucus layer following chemotactic compounds, such as amino acids (41), mucin (42), urea, sodium bicarbonate and sodium chloride (43). As the rotation of the flagellum motor was shown to depend on a proton motive force, it has been suggested that the urease-driven hydrolysis of intracellular urea may supply this proton motive force and that chemotactic movement toward urea may provide the substrate for the hydrolysis (44).
Moreover, the motility of the bacteria also influences the inflammatory response, since it could be shown that non-motile, coccoid bacteria that occur in a stress situation, such as extended stationary phase or nutrient limitation, cause lower IL-8 secretion by gastric epithelial cells compared to motile, bacillary *H. pylori* (45) This leads to an infiltration of neutrophils and monocytes into the gastric mucosa and causes inflammation and mucosal damage (46) (47) (48).

1.4.5. Adhesins

Since the gastric epithelium and mucus are in continuous turnover and peristalsis ensures constant movement of food, the binding of *H. pylori* to epithelial cells is essential for the survival of the bacteria. Upon infection, the bacterial adhesion occurs first to mucin, which

covers the epithelium and generally prevents microbial colonisation. *H. pylori* has the ability to bind to mucin via the neutrophil-activating protein Nap (49) (50), which causes degradation of mucin and leads to decreased mucosal viscosity. Consequently, the majority of *H. pylori* survive in the gastric mucus and migrate to the epithelial cell surface (51). *H. pylori* adheres to the less acidic gastric antrum region through outer membrane proteins, such as the blood group antigen binding adhesin (BabA), the sialic acid binding adhesin (SabA), the adherence-associated lipoprotein (AlpA and AlpB) or the outer membrane inflammatory protein (OipA).
The broad expression of outer membrane proteins ensures the pathogen not only to adhere to its host, but also enables the translocation of CagA.
To date, BabA-Lewis b is the best-characterised adhesin-receptor interaction in *H. pylori* (52) (53) (54). Although almost all *H. pylori* strains harbour BabA, they differ in the expression levels. Notably, BabA-positive *H. pylori* are not only associated with higher colonisation than BabA-negative bacteria, but also with stronger gastric injury (55). This might be explained by the response of epithelial cells to bacterial binding, since endocrine cells release the hormone gastrin, which consequently stimulates parietal cells in the corpus region to hypersecrete acid (56) (57). This increased acid production may lead to gastric metaplasia and duodenal ulcers. Furthermore, improved bacterial adhesion and colonisation was shown to induce increased IL-8 secretion by epithelial cells, causing a stronger inflammatory response and mucosal destruction (58).

1.4.6. γ-Glutamyltranspeptidase

γ-Glutamyltranspeptidase (gGT) is an enzyme exhibiting hydrolysis activity with very high affinities for glutamine and glutathione (59). It is constitutively expressed by all *H. pylori* strains, but has only been found in a few other bacteria (60). Although its exact role in bacteria is unknown, gGT has been hypothesized to play a role in the transport of amino acids (59). It is also speculated that the gGT-mediated depletion of glutamine and glutathione, which are important nutrients for the maintenance of healthy gastrointestinal tissue, account for the damage of mammalian cells (59).On the other hand, it has been shown that gGT also leads to the production of reactive oxygen species (ROS) (61) by primary gastric epithelial cells, resulting in oxidative DNA damage of the host cells (62). Furthermore, *H. pylori* gGT has been described as a virulence factor inhibiting T cell proliferation *in vitro* (63).

1.5. Host immune response to *H. pylori*

Generally, the innate and the adaptive immune system contribute to the protection of the host. In a first line of defence, cells of the innate system recognise pathogens and respond to them in a non-specific way. But unlike the adaptive immune system, innate responses do not grant permanent or protective immunity against the pathogen.

Remarkably, *H. pylori* can persistently colonise the stomach for the lifetime of the host despite activation of innate and an adaptive immune responses.

1.5.1. Innate immune response to *H. pylori*

Upon infection, *H. pylori* penetrates the viscous mucus layer and proliferates near the surface of epithelial cells. In addition, bacterial lipopolysaccharide (LPS) (64) and numerous chemotactic components released by *H. pylori* permeate damaged epithelial cells and cause a substantial immune activation. This is manifested by continuous epithelial cell cytokine signalling and gastric mucosal infiltration by neutrophils (65), macrophages (66), and dendritic cells (DCs) (67).

1.5.1.1. Neutrophil granulocytes

During the acute phase of inflammation neutrophils are one of the first cells that migrate towards the site of inflammation, following chemical signals such as IL-8. Once there, neutrophils traverse the epithelium in large numbers and encounter bacteria in the mucus layer (68). Upon ingestion of opsonised bacteria, neutrophils generate phagosomes, into which reactive oxygen species and hydrolytic enzymes are secreted. During this process, the enzyme NADPH oxidase becomes activated (69). *H. pylori,* however, is able to disrupt NADPH oxidase targeting. Consequently, superoxide anions are released into the extracellular milieu and do not accumulate inside phagosomes (69), leading to the escape of phagocytic killing. Moreover, *H. pylori* neutrophil-activating protein (NAP) further stimulates the production of oxygen radicals from neutrophils and facilitates adhesion of neutrophils to endothelial cells (70). Since superoxide anions accumulate in the extracellular space and not inside phagosomes, bacteria and neutrophils act in concert to damage the gastric mucosa (69).

1.5.1.2. Macrophages

Macrophages are leukocytes with the ability to phagocyte opsonised pathogens. However, opsonisation in the stomach is inefficient since low pH and mucins prevent antibody binding (71) (72). Nevertheless, *H. pylori* has been shown to be internalised by macrophages (68), but it avoids phagocytic killing by preventing phagosome maturation (73). Instead, *H. pylori* induces the formation of megasomes, which enable bacterial survival (74).
Beside phagocytosing bacteria, macrophages also initiate the adaptive immune response through antigen presentation and the secretion of cytokines that direct lymphocyte recruitment and differentiation (75). However, in contrast to dendritic cells (DCs), macrophages have only a limited capability to activate naïve T cells (76).
Historically, macrophages have been classified into two main groups designated M1 and M2. M1 macrophages typically participate in the early immune response to invading pathogens and support T helper cell type 1 (Th1) immunity. They are induced by Interferon (IFN)-γ and microbial products (77). In contrast, M2 macrophages are involved during the resolution of inflammation and promotion of Th2 immunity and are induced by Th2- or anti-inflammatory cytokines and growth factors (e.g. IL-4, IL-10 and Transforming growth factor-β (TGF-β)) (77) (78).
During *H. pylori* infection, macrophages are recruited to the gastric mucosa, where they contribute to the production of cytokines and chemokines. In *H. pylori*-induced atrophic gastritis enhanced numbers of M1 macrophages are present. However, they fail to upregulate co-stimulatory molecules after *H. pylori* infection (79), which may lead to insufficient T cell activation.

1.5.1.3. Dendritic cells

DCs are antigen-presenting cells (APCs), which play a critical role in the activation and regulation of the adaptive immune response. Moreover, they are the most efficient presenters of exogenous peptides to T lymphocytes as they express the highest number of MHC class II molecules per cell (80).
Generally, DCs are derived from hematopoietic bone marrow progenitor cells and evolve from lymphoid or myeloid precursors (81). In humans, myeloid DCs are considered as conventional DCs, they differ from lymphoid DCs by their ability to efficiently phagocyte pathogens (82). In contrast, lymphoid DCs, which are referred to as plasmacytoid DCs (pDCs), have the capacity to produce large amounts of type I interferons upon exposure to

viruses as well as bacterial components, such as Cytosine-phosphatidyl-Guanine (CpG) oligonucleotides. This leads to the induction of plasma cell differentiation (83), which in turn produce antibodies against invading pathogens, such as viruses. Moreover, triggered pDCs may also differentiate into mature DCs that are able to induce the differentiation of $CD4^+$ T cells (82).

There are several indications for the involvement of gastric DCs in the pathogenesis of *H. pylori* gastritis. Primarily, gastric myeloid DCs have been identified in human patients infected with *H. pylori* (67). Furthermore, DCs were shown to be recruited to the gastric mucosa in experimental *Helicobacter* infection where they open tight junctions between epithelial cells, send dendrites outside the epithelium and directly sample bacteria, whereby the integrity of the epithelial barrier is preserved (84). In addition, DCs were also observed in the lamina propria and epithelium of heavily infected and inflamed mucosa, where DC luminal endings directly contact *H. pylori* (85).

1.5.1.3.1. TLR-mediated antigen recognition

The differences in reactivity to diverse microbial products between both DC subtypes might also be explained by the varying expression of pattern-recognition receptors (PRR). Generally, of all cells of the immune system, DCs express the broadest repertoire of PRRs, including the Nucleotide Oligomerisation Domain receptors (NLRs), the Toll-like receptors (TLRs), and several C-type lectins, such as DC-specific intercellular-adhesion-molecule-grabbing non-integrin (CD209) or Dectin-1 (86). The best described class of PRRs is the TLR-family, consisting of 10 different receptors in humans. Each of them is responsible for the detection of specific pathogen-associated molecular patterns (PAMPs), including LPS(TLR-4) (87), bacterial lipoproteins and lipoteichoic acids (the heterodimers TLR-1/-2 (88) or TLR-2/-6 (89)), flagellin (TLR-5) (90), unmethylated CpG motifs of bacterial and viral deoxyribonucleic acid (DNA) (TLR9) (91), double-stranded ribonucleic acid (RNA) (TLR-3) (92) and single-stranded viral RNA (TLR-7/-8) (93). However, myeloid and plasmacytoid DCs differ in their expression of TLRs. While all TLRs except TLR-7 and TLR-9 were found on conventional DCs, plasmacytoid DCs only expressed TLR-7 and TLR-9 (94).

Individual TLRs trigger specific biological responses. For example, TLR-3 and TLR-4 generate both, type I interferon and inflammatory cytokine responses, whereas cell surface TLR-1/-2, TLR-2/-6 and TLR-5 induce mainly inflammatory cytokines (95). These

differences are explained by the presence of the toll-interleukin 1 receptor (TIR) domain–containing adaptor molecules, including Myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF–related adaptor molecule (TRAM), which are recruited by individual TLRs and activate distinct signalling pathways (95). MyD88 is universally used by all TLRs except TLR-3, and activates the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokine expression (90). In contrast, TRIF is utilised by TLR-3 and TLR-4 and induces alternative pathways that lead to activation of the interferon regulatory factor 3 (IRF3) and NFκB and the consequent induction of type I interferons and inflammatory cytokines. TRAM and TIRAP function as sorting adaptors that recruit TRIF to TLR-4 and MyD88 to TLR-2 and TLR-4, respectively.

The negative regulation of TLR-induced responses is important for suppressing inflammation and deleterious immune responses. So far, many negative regulators that suppress TLR signalling pathways have been identified. These include splice variants for adaptors or their related proteins (96) (97), ubiquitin ligases (98) (99), deubiquitinases (100), transcriptional regulators and microRNAs (101) (102). Some negative regulators are upregulated by TLR signalling and modulate the TLR responses in negative feedback mechanisms; others are constitutively present and control primary TLR signalling. In addition to this TLR signal-associated negative regulation, anti-inflammatory factors produced indirectly during immune responses can also regulate TLR signalling. Potent anti-inflammatory factors include TGF-β and IL-10. The latter negatively regulates inflammation through a mechanism that blocks the expression of pro-inflammatory genes encoding cytokines, such as Tumor-Necrosis Factor (TNF)-α, IL-12p40, IL-1α, IL-1β and IL-6, chemokines, cell-surface molecules and other molecules required for the full activation of the innate and adaptive immune responses. Here, DCs function as an important source of IL-10 themselves.

The signalling pathway used by the IL-10 receptor to generate the anti-inflammatory response requires signal transducer and activator of transcription 3 (STAT3). Upon binding of IL-10 to its receptor, both IL-10R subunits are brought together, leading to the phosphorylation of constitutively bound Janus tyrosine kinases (JAK). Consequently, inactive cytosolic STAT3 binds to these newly appearing phosphotyrosine residues and immediately becomes phosphorylated by the JAKs. Subsequently, STAT molecules form dimers that dissociate from the receptor and are translocated to the nucleus, where they bind to DNA sequences of the promoter regions of cytokine-responsive genes and activate gene transcription.

Although it has been shown that TLR-signalling plays a critical role in the recognition of *H. pylori* by human gastric epithelial cells (103) (104), there is little information about the TLR-mediated recognition of *H. pylori* by human DCs. However in murine DCs, the involvement of TLR-2 and -4 in the recognition of live *H. pylori* and TLR-9 for the detection of bacterial DNA has been shown. Importantly, in the absence of TLR-signalling, the anti-inflammatory cytokine release was drastically reduced (105). Nevertheless, there is no clear evidence whether TLR-signalling during *H. pylori* infection is responsible for the subsequent immune response. Initial data also suggests that IRAK-M, a novel member of the interleukin-1 receptor-associated kinase (IRAK) family (106) and known to negatively regulate TLR-signalling (107), is involved in the modulation of IL-10 expression after *H. pylori* re-stimulation of LPS-tolerised murine DCs (108).

1.5.1.3.2. DC maturation

DCs are capable to evolve from immature, antigen-capturing cells to mature, antigen-presenting, T cell-priming cells. During this process DCs undergo a number of functional changes.

Initially, DCs convert antigens into immunogens that are presented by MHC molecules on the cell surface (109). Consequently, DCs lose the ability for further antigen internalisation. Furthermore, antigen internalisation can subsequently trigger their migration from peripheral tissues to lymphoid organs, where they interact with lymphocytes and can additionally cause their maturation (110). This comprises the production of cytokines and chemokines, but also the expression of co-stimulatory molecules needed for the initiation of an adaptive immune response. Notably, only one DC is sufficient to stimulate several thousand T cells (111). For optimal T cell activation, DCs provide naïve T cells with three signals (112). The first signal is the antigen-specific signal received as a result of binding of the T cell receptor to the MHC-presented peptide. The second signal is provided by the co-stimulatory molecules CD80 and CD86, which work in tandem to prime naïve T cells via CD28 (113), leading to T cell proliferation, differentiation and survival. An insufficient or absent co-stimulation may lead to T cell anergy or the development of immune tolerance (114). Furthermore, DCs secrete huge amounts of cytokines which are needed for lymphocyte attraction and the regulation of T cell polarisation (115).

DCs are able to activate both, $CD4^+$ and $CD8^+$ T cells. Therefore, antigens are presented differently. $CD8^+$cytotoxic T cells are stimulated by MHC class I-presented antigens, mainly

reflecting cytosolic antigens (116). Upon degradation by the proteasome, peptides enter the endoplasmic reticulum, where they bind to newly synthesised MHC class I molecules, which are then transported to the cell surface (117). $CD4^+$ T helper cells become stimulated upon antigen-presentation via MHC class II molecules (118). Therefore, captured antigens are lysed in the endosomes and subsequently enter a vesicle containing MHC class II, where they bind and are transported to the cell surface (119). The subsequently induced T cell response can vary, depending on the specific DC lineage (myeloid or lymphoid), the maturation stage of the DCs as well as by secreted cytokines (115).

1.5.1.3.3. Cytokinesinfluencing T cell polarisation

Cytokines, such as interleukins and interferons, are small cell-signalling proteins that are secreted by numerous cells. They play an important role in immunity, since they either stimulate the producing cells themselves or activate other cells of the immune system.
Upon *H. pylori* infection, DCs were shown to produce several cytokines, which are known to induce a characteristic T cell response.

IL-12 is a principal mediator of the early innate immune responses to intracellular pathogens and a key inducer of cellular immunity to these microbes (120). The two principal sources of IL-12 are activated macrophages and DCs (121). Structurally, IL-12 is a heterodimer that consists of a 35-kDa subunit (p35) and a 40-kDa subunit (p40). Interestingly, the p40 subunit of this cytokine is shared with IL-23 (122). The primary biological role of IL-12 is to initiate a series of responses involving macrophages, NK cells and T lymphocytes. Moreover, IL-12 is a potent stimulator of the Th1 pathway; furthermore, this cytokine induces IFN-γ production in NK cells and enhances the cytolytic functions of activated NK cells and cytotoxic $CD8^+$ T cells. In *H. pylori*-infected human gastric mucosa IL-12 has been demonstrated to drive the Th1 response (120).

IL-6 is produced by DCs and other cells in response to microbial infections and to other cytokines, especially to IL-1 and TNF (123). Its receptor has a two-subunit structure (124); one of the subunits binds IL-6 and the other (called gp130) transduces the signal by activating the JAK-STAT signalling pathway (125). Beside IL-1β and IL-23, IL-6 also plays an important role in human Th17 differentiation. This cytokine is thought to be implicated in the pathogenesis of autoimmune diseases by promoting chronic inflammation (126). Notably,

there are several reports on the association of Th17 cells with a *H. pylori* infection (127) (128).

IL-10 is a polypeptide with four α-helical domains that binds to a cytokine receptor and activates the JAK-STAT signalling pathway (129). This cytokine is a major regulator of activated macrophages and DCs. It is mainly produced by activated DCs themselves and thus serves as a negative feedback control (130). By inhibiting immune cell responses through limiting pro-inflammatory cytokine secretion and down-regulating the expression of MHC class II molecules and co-stimulatory molecules (131), IL-10 not only influences innate immunity, but also affects adaptive immune responses, such as Th differentiation (132).
Upon *H. pylori* infection increased levels of IL-10 have been found in the mucosa of infected individuals (133), leading to the expansion of regulatory T cells (T_{regs}) (84).

1.5.2. Adaptive immune response to *H. pylori*

Secreted cytokines attract cells of the adaptive immune system. Consequently, there is a gradual accumulation of chronic inflammatory cells in the gastric mucosa over the following weeks, including T and B lymphocytes that respond to bacterial stimuli by the production of cytokines and specific antibodies. However, despite a strong activation of adaptive immune cells upon *H. pylori* infection, the host immune system mainly fails to eliminate the bacterium, leading to lifelong persistence of the pathogen.

1.5.2.1. B cells

B cells are lymphocytes that express a specialised B cell receptor (BCR), allowing the cell to bind to a specific antigen. Upon antigen recognition and full activation, either T cell dependent or independent, B cells differentiate into memory B cells and plasma cells (134). The latter secrete large amounts of antibodies, which bind to pathogens and target them for removal by phagocytes and activate the complement system (135).
Once *H. pylori* infection is established, the number of B cells and levels of antibodies are increased in the gastric fluid (136) (137). However, gastric antibodies may be ineffective at limiting the infection and antibody-bound pathogens are weakly killed by enhanced phagocytosis due to the small number of lymphocytes in the lumen. Hence, the predominant

B cell response in mucosal tissues has adapted to produce secretory IgA that can be transported into the lumen to provide an offside defence by blocking microbial attachment or neutralising toxins (137). Nonetheless, mucosal antibodies may not be sufficient or even necessary for the protection of the host. Several studies showed that in the absence of IgA, the frequency of *H. pylori* infection is consistent (138) (139). Furthermore, the ability to reinfect patients with *H. pylori* shortly after antibiotic-treatment, despite the presence of antibodies generated to the initial infection, may be evidence that the antibody response does not play a meaningful role in reducing *H. pylori* colonisation (140) (141). Moreover, studies in mice have shown that immunity to *Helicobacter* can be induced in the absence of B cells (139).

1.5.2.2. T cells

Upon antigen-presentation through APCs T cells become activated and differentiate (142). While $CD4^+$ T helper cells secret cytokines, which attract several other immune cells (143) and $CD8^+$ cytotoxic T cells destroy infected cells directly (144), Tregs are known to actively suppress activation of the immune system and prevent pathological self-reactivity (145). Generally, naïve $CD4^+$ T cells can differentiate into Th1, Th2 and Th17 effector cells or T_{regs}, which differ in the secreted cytokines and in their function (146). While Th1 cells secrete mainly IFN-γ and regulate cellular immunity in response to intracellular bacteria, Th2 cells produce IL-4, IL-5, and IL-13 and mediate humoral responses against extracellular pathogens, parasites and toxins (146). In contrast, Th17 cells are involved in host defence against extracellular pathogens by mediating the recruitment of neutrophils and macrophages to infected tissues through IL-17 release and seem to play a key role in autoimmune diseases (146).

Upon *H. pylori* infection $CD4^+$ T cells are increased in infected gastric lamina propria (147). Cell responses were examined by intracellular staining for IFN-γ, IL-4 and IL-17 and revealed a predominant Th1 phenotype (148). However, IFN-γ secretion alone was insufficient to induce gastritis (147). Indeed, Th17 cells are involved in the inflammation of the stomach and influence bacterial colonisation (149).

Nevertheless, this T cell response cannot successfully clear the infection. This deficiency might be explained by immune evasion mechanisms developed by *H. pylori* (see paragraph 1.6), leading to attenuated immune responses.

Beside Th1 and Th17 polarised T cells, recent studies showed that *H. pylori*-infected individuals, as well as patients with gastric adenocarcinoma, have higher levels of

$CD4^+CD25^{high}$ T cells (150). These cells have the phenotype of T_{regs} since they express Foxp3, a transcription factor needed for the development and function of regulatory T cells. In contrast, mucosal $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^-$ cells express low levels of Foxp3 and are present in individuals with asymptomatic *H. pylori* infections, but also in duodenal ulcer patients (150). Preliminary indications for the role of T_{regs} in immune suppression were provided by mouse experiments in which $CD25^+$ cells were depleted. Several weeks after *H. pylori* infection mice with depleted $CD25^+$ cells developed a severe gastritis with enhanced cytokine expression and increased numbers of mucosal T cells, B cells and macrophages. Furthermore, this amplified gastric inflammatory response was accompanied by reduced bacterial loads (151). Consequently, it is argued that T_{regs} may suppress mucosal immune responses, leading to the persistence of *H. pylori* infections. In parallel, the permanent Th1-induced cell-mediated immunity against *H. pylor*i leads to the damage of epithelial cells and ulcerogenesis (152).

1.5.2.2.1. Cytokinesinfluencing*H. pylori*-induced adaptive immune response

Cytokines are not only mediators and regulators of innate immunity but also influence adaptive immune responses. They are mainly produced by activated $CD4^+$ T cells in response to specific antigens. Some of these cytokines act primarily on other lymphocyte populations, regulating their growth and differentiation; others affect mostly non-lymphoid effector cells, such as macrophages.

IL-2 is a major growth factor for antigen-activated T lymphocytes which is responsible for clonal T cell expansion after antigen recognition (153). In particular, IL-2 stimulates the growth and differentiation of B lymphocytes (154), activates NK cells (155), and potentiates apoptotic death of antigen-activated T lymphocytes (156). IL-2 is produced by activated T lymphocytes, mostly $CD4^+$ T cells and, in smaller amounts by $CD8^+$ T cells (157). Activation of T cells by antigen and co-stimulatory molecules on the surface of APCs is the necessary prerequisite for the production of IL-2 (158). However, *H. pylori* can inhibit lymphocyte proliferation by interfering with IL-2-mediated signalling (159).

IFN-γ is the principal activator of macrophages and the major inducer of Th1 cells (120) and is produced by NK cells (160), $CD4^+$ Th1 cells and $CD8^+$ cells (161). T cells produce IFN-γ in response to antigen recognition and its production is enhanced by IL-12p70 (162).

Furthermore, IFN-γ activates macrophages (163) and neutrophils (164), enhances the expression of MHC class I and class II molecules (165) and co-stimulatory molecules on antigen-presenting cells (166), promotes the differentiation of naïve $CD4^+$ T cells to the Th1 subset (167), inhibits the proliferation of Th2 cells (168) and enhances the cytolytic activity of NK cells (169). Upon *H. pylori* infection epithelial cells secrete IL-8 and attract lymphocytes (58). T cells polarise upon contact with APCs, mainly DCs, to IFN-γ -producing Th1 cells and cause cellular immunity (146), which in turn is involved in the damage of the epithelium.

IL-17 is a cytokine mainly produced by Th17 cells, which recruits neutrophils to the site of inflammation, contributing to tissue damage (170). The differentiation factors TGF-β and IL-6 and the growth and stabilisation factor IL-23 are involved in the development of IL-17A-producing Th17 cells (171). The participation of TGF-β in the differentiation of Th17 cells places the Th17 lineage in close relationship with T_{regs}, as TGF-β also induces differentiation of naïve T cells into $Foxp3^+$ T_{regs}. Consequently, both, Th17 and T_{regs}, have been described to reduce immunopathology in *H. pylori* infection by suppressing Th1 differentiation (172) (173), even at the expense of a higher *H. pylori* load in the gastric mucosa (174) (173).

TGF-β is able to control proliferation and cellular differentiation of many cells, including inhibitory effectsonTh1 cells (175). Additionally, TGF-β is believed to be important in the differentiation of both, T_{regs} and Th17 cells (171) (176).
During *H. pylori* infection gastric epithelial cells have been described to produce TGF-β, causing a reduction of $CD4^+$ T cell proliferation and the development of T_{regs} (177).

1.6. Immune evasion of *H. pylori*

Efficient colonisation of the human stomach by *H. pylori* is made possible inter alia by bacterial urease production that is required for acid resistance (34), expression of mucolytic molecules to evade the destruction by mucins (49) (50) or by flagellar motility that allows penetration to the mucus (40). Although damaged epithelium releases several chemokines that attract immune cells (58), they mainly fail to antagonise the infection. Consequently, *H. pylori* must have developed immune evasion mechanisms to overcome the host immune response.
Like all Gram-negative bacteria *H. pylori* has PAMPs such as LPS, CpG motifs, flagellin or methylated DNA, however, the *H. pylori*-specific PAMPs are almost immunologically inert. Compared to enterobacterial LPS, *H. pylori* LPS showed a 500-fold lower toxicity, 1000-fold

decreased mitogenicity and pyrogenicity (178). Additionally, *H. pylori* LPS is characterised by blood group structures similar to those commonly occurring in the gastric mucosa (179). Furthermore, *H. pylori* flagellin, consisting of FlaA and FlaB, demonstrated a 1000-times lower TLR-5-dependent immune stimulation than the *Salmonella typhimurium* flagella protein FliC *in vitro* (180) (181). Moreover, bacterial DNA contains unmethylated CpG motifs, which are a ligand of TLR-9, and described as an innate immune system stimulants (182). *H. pylori* DNA is subject to strain-specific methylation, however methylated *H. pylori* DNA seems to have only little immunostimulatory properties (183) (9).

It was previously described that *H. pylori* infection causes cellular immune response, in which the activation of phagocytes are involved (66). *H. pylori,* however, evades this immune defence by several mechanisms. Firstly, *H. pylori* is able to prevent nitric oxide (NO) production and secretion in macrophages, neutrophils and epithelial cells by generating the enzyme arginase, leading to a deficient killing of extracellular bacteria (184). Secondly, the pathogen can actively block its own uptake in a Cag-PAI-dependent manner (185). Thirdly, if phagocytised, type I *H.* pylori strains can survive intracellular within macrophages by phagosome fusion (megasome formation) (186).

H. pylori has evolved to evade not only the innate, but also the adaptive immune response. It has been shown that *H. pylori* VacA can inhibit antigen presentation (187) and T cell proliferation (159). Furthermore, inhibitory effects on T cell proliferation have also been described to be induced by *H. pylori* gGT (63).

1.7. Purpose of this study

H. pylori has co-existed with its host for thousands of years. Today, with a prevalence of about 50%, *H. pylori* infection is one of the most common chronic bacterial infections (5). Even though most infected individuals do not show any symptoms of the infection, they are still at higher risk than uninfected individuals to develop gastric diseases such as peptic ulcer disease and gastric adenocarcinoma (15). Moreover, *H. pylori* is linked to the suppression of allergic airway disease (188). Interestingly, *H. pylori*-mediated protection from asthma was shown to be dependent on the induction of T_{regs} (189).

A special role in the differentiation of T_{regs} is awarded to the DCs. Upon exposure to *H. pylori in vitro* and *in vivo* murine DCs have been shown to skew the T cell response from a Th1/Th17 response toward T_{reg} differentiation (84) (190). So far, however, there is disagreement about the means of tolerance induction through DCs. On the one hand, T_{reg} skewing was independent of *H. pylori* VacA and CagA, but dependent on TGF-β and IL-10 (84). In contrast, CagA-overexpressing murine DCs were shown to induce IL-10 producing T cells and impaired Th1 response (191). In addition, also gGT and VacA were described to induce tolerogenic DCs by influencing DC maturation and cytokine response (192).

During this thesis the role of human DCs for the induction of *H. pylori*-specific T cell response will be evaluated. In particular, special attention will be paid to mechanisms which involved in this process.

Materials

2. Materials

2.1. Bacterial strains

Helicobacter pylori G27	Baltrus et al, Journal of Bacteriology, 2009
Helicobacter pylori G27 ΔCagA	Kan^R, Figura et al, Helicobacter, 2004
Helicobacter pylori G27 ΔBabA	Kan^R, Borén
Helicobacter pylori G27 ΔgGT	Kan^R, Schmees et al, Gastroenterology, 2007
Helicobacter pylori G27 ΔUreA/B	Kan^R, transformation from 2808 ΔUreA/B
Helicobacter pylori G27 ΔVacA	Kan^R, Oertli et al, PNAS, 2013
Helicobacter pylori 2808	Rad et al, The Journal of Immunology, 2002
Helicobacter pylori 2808 ΔUreA/B	Kan^R
Helicobacter pylori 60190	Cover et al, Infection & Immunity, 1990
Helicobacter pylori 60190 ΔVacA	Kan^R, Bebb et al, Infection & Immunity, 2003
*Helicobacter pylori*PMSS1	Arnold et al, Gastroenterology, 2011
Helicobacter pylori SS1	Jeong et al, Antimicrob Agents Chemother., 2000
Helicobacter pylori P1	Moese et al, Infection & Immunity, 2004
Helicobacter pylori J99	Alm et al, Nature, 1999
Escherichia coli (*E. coli*) K12	kindly provided by Prof. Miethke, MIH, München, Germany

2.2. Cells

Peripheral blood mononuclear cells	(PBMCs), received from blood of healthy donors or buffy coats
Monocyte-derived DCs	isolated from PBMCs with the Monocyte-Isolation Kit (MiltenyiBiotec)
$CD4^+$ T cells	isolated from allogenic PBMCs with the $CD4^+$ T cell Isolation Kit (MiltenyiBiotec)

2.3. Reagentsfor cell culture

RPMI 1640 + L-Glutamine (Gln)	(Invitrogen GIBCO)
Fetal Bovine Calf Serum (FCS) Gold	(PAA)
X-VIVO 15	(Lonza)
Penicillin (Pen)	(Invitrogen GIBCO)
Streptomycin (Strep)	(Invitrogen GIBCO)
Recombinant human GM-CSF	(MiltenyiBiotec)
Recombinant human IL-4	(MiltenyiBiotec)
Infection medium	RPMI+ Gln, 10% heat-inactivated FCS Gold
Medium for generation of MoDCs	Infection medium,1% Pen/Strep, each 20 ng/ml recombinant human GM-CSF and IL-4
PBS	(Invitrogen GIBCO)
Accutase	(Sigma-Aldrich)
Glycerol	(Roth)
Cell culture plates	(BD)
L-Glutamine	(Sigma-Aldrich)
Anti-human IL-6 (6708)	(R&D)
Anti-human IL-10 (23738)	(R&D)
Anti-human TLR-2 (TL2.1)	(Imgenex)
Anti-human TLR-4 (HTA125)	(Imgenex)
Anti-human TLR-5 (Q2G4)	(Imgenex)
Recombinant gGT	(Schmees et al, Gastroenterology, 2007; C. Bolz)
LPS, ultrapure	(Sigma)
Pam3Cys	(InvivoGen)
Flagellin	(InvivoGen)
Recombinant human IL-6	(MiltenyiBiotec)
Recombinant human IL-10	(eBioscience)

2.4. Reagentsfor bacterial culture

Luria-Bertani (LB)-Medium	20 g/l Lennox-L-Broth (Gibco), autoclaved
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, 50 ml inactivated horse serum, 0.4 g potassium nitrate, Dent-Supplement
Horse serum	(Invitrogen)
Dent-Supplement	Vancomycin, Amphotericin B, Cefsulodin, Sodium, Trimethoprimlactat (Oxoid)
Freezing medium for *H. pylori*	BHI-medium, 20% Glycerol, 20% FCS
Freezing medium for *E. coli*	LB-medium, 20% Glycerol
Kanamycin	50 µg/ml (Sigma-Aldrich)

2.5. Reagents for cell isolation

MACS buffer	PBS + 0.5% BSA, 2 mM EDTA
PBS	(Invitrogen GIBCO)
Biocholl	(Biochrom)
MACS separation columns (LS)	(MiltenyiBiotec)

2.6. Reagents for SDS-PAGE

1x SDS sample buffer	62.5mMTris-HCl pH 6.8, 50 mM DTT, 2% (w/v) SDS, 10% (v/v) Glycerol, 0.01% (w/v) bromphenol blue
Acrylamide solution	40% (w/v) acrylamide / Bis 19:1 (Ambion)
Stacking gel	125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% APS, 0.1% TEMED
Separating gel	375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS, 0.04-0.06% TEMED
Electrophoresis buffer	25 mM Tris-HCl, 250 mM Glycin, 0.1% (w/v) SDS, pH 8.3
Transfer buffer	25 mM Tris-HCl, 190 mM Glycin, 0.01% (w/v) SDS, 20% Ethanol
Prestained Low Range Protein Molecular Weight Marker	(MBI Fermentas)

2.7. Reagentsfor Western blot

Blocking buffer	TBST + 5% BSA or TBST + 5% milk powder
TBS	150 mM NaCl, 20 mM Tris-HCl, pH 7.5
TBST (Washing buffer)	TBS, 0.1% Tween 20 (AppliChem)
Milk powder	(Roth)
Ponceau solution	0.5% Ponceau (Sigma), 1% acetic acid
Nitrocellulose membrane	(Protran)
Anti-human STAT3 (124H6)	(Cell Signaling)
Anti-human (p)-STAT3 (Tyr705) (D3A7)	(Cell Signaling)
Anti-human β-Actin	(Sigma-Aldrich)
Anti-human β-Catenin	(BD)
Anti-human (p)-IkB-α (Ser32/36) (5A5)	(Cell Signaling)
Anti-*H. pylori* CagA	(Roger Vogelmann, Mannheim)
Anti-pTyr (4G10)	(Millipore)
Anti-mouse Horseradish peroxidase (HRP)	(Promega)
Anti-rabbit HRP	(Promega)
Pierce ECL-solution	(Thermo Scientific)

2.8. Reagentsfor ELISA

10x PBS	80g NaCl + 2g KCl, 14.4g Na_2HPO_4 + 2.4g KH_2PO_4 (pH 7.4)
Washing buffer	1x PBS; 0.05% Tween 20
Assay solution, Coating /Blocking buffer, Substrate, Avidin-HRP	Ready-Set Go Kit (eBioscience)
Stop solution	1 M H_3PO_4
ELISA plates (96-well, flat bottom)	9018 Corning Costar (Corning)
ELISA reader	Sunrise (Tecan)
Anti-human IL-12p70 Ready-Set Go Kit	(eBioscience)
Anti-human IL-6 Ready-Set Go Kit	(eBioscience)
Anti-human IL-10 Ready-Set Go Kit	(eBioscience)
Anti-human IL-23 Ready-Set Go Kit	(eBioscience)
Anti-human IL-17A Ready-Set Go Kit	(eBioscience)

Anti-human IL-IFN-γ Ready-Set Go Kit	(eBioscience)
Anti-human IL-2 Ready-Set Go Kit	(eBioscience)
Anti-human TGF-β Ready-Set Go Kit	(eBioscience)

2.9. Reagentsfor flow cytometry

FACS buffer	1x PBS + 1% Bovine Serum Albumin (BSA)
BSA	(AppliChem)
Ethidiummonoazide bromide (EMA)	(MoBiTec)
Paraformaldehyde	4% PFA (Roth),
FACS	CyAN ADP (DakoCytomation)
Anti-human CD14 PE (61D3)	(eBioscience)
Anti-human CD45 PE (2D1)	(eBioscience)
Anti-human CD11c eFluor 450 (LN3)	(eBioscience)
Anti-human HLA-DR eFluor 450	(eBioscience)
Anti-human CD83 (APC)	(eBioscience)
Anti-human CD86 PE (IT2.2)	(eBioscience)
Anti-human CD80 FITC (2D10.4)	(eBioscience)
Anti-human Lewis b	kindly provided from Dr. C. de Bolós (IMIM-Hospital del Mar, Barcelona, Spain)
Anti-human CD4 eFluor 450 (OKT4)	(eBioscience)
Anti-human CD25 PE (BC96)	(eBioscience)
Anti-human Foxp3 APC (236A/E7)	(eBioscience)
Foxp3 staining buffer set	(eBioscience) fixation/ permeabilisation solution, permeabilisation buffer

2.10. Reagents for PCR

Green Taq	(Promega)
Gene Ruler TM 1kb DNA Ladder	(MBI Fermentas)

Methods

3. Methods

3.1. Cultivation of bacteria

3.1.1. Cultivation of *H. pylori*

H. pylori wild type strain G27 is a laboratory strain that expresses an intact Cag pathogenicity island and produces VacA.
Frozen cryo aliquots were thawed immediately in a water bath at 37°C and centrifuged (2 min, 1.000 x g) in order to remove the glycerol-containing freezing medium. The pellet was resuspended in warm BHI-dent + 10% FCS, plated out on a WC-dent plate and finally cultivated for 2 - 3 days at 37°C under microaerophil conditions (10% CO_2, 5% O_2, 85% N_2). G27 isogenic mutant strains G27ΔCagA, G27ΔBabA G27ΔVacA G27ΔgGT and G27ΔUreA/B were grown on WC-dent plates containing 50µg/ml of kanamycin for selection under the same conditions. Then *H. pylori* was transferred on a new plate for another day to increase its vitality. Bacteria were harvested, resuspended in warm BHI medium and directly used for experiments. The multiplicity of infection (MOI) was determined by measurement of the optical density (OD_{600}). Therefore, dilutions of the main culture were incubated on WC-dent plates. Colonies were counted after 2 days and the amount of vital bacteria per ml was calculated. Here ~2x 10^8 *H. pylori*/ ml were determined at OD_{600} = 1.0.
For preparation of aliquots bacteria were harvested from a WC-dent plate and resuspended in BHI-dent + 20% FCS + 20% glycerol. Afterwards, aliquots were immediately frozen in liquid nitrogen. The storage was at -80°C.

3.1.2. Cultivation of *E. coli*

E. coli K12 is an extenuated strain that is unable to colonise the human intestine. *E. coli* K12 was cultivated in LB-medium under shaking conditions (90-150 rpm) at 37°C. 1 OD unit (1 ml/OD_{600} = 1) was diluted in 30 ml medium and shaked for further 3h or until the OD_{600} =1 was reached. Around 7,4x 10^8 *E. coli*/ml were determined at OD_{600} = 1.0. For subsequent stimulation of MoDCs *E. coli* was fixed with 4% PFA in order to prevent overgrowing of the cells.

3.1.3. PFA fixation of bacteria

Bacteria were harvested, centrifuged (2min, 10.000 rpm) and the pellet was resuspended in 4% paraformaldehyde and incubated for at least 4h or overnight at 4°C. Afterwards, fixed bacteria were washed with PBS. After final resuspension in PBS, the optical density was measured at OD_{600} and the bacteria were stored at 4°C.

3.2. Cultivation of Monocyte-derived DCs

3.2.1. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from *H. pylori* negative donors by density gradient centrifugation with Biocoll (Biochrom, Germany). Therefore, blood was diluted in the same volume of PBS (w/o Ca^{2+}, Mg^{2+}), and Biocoll solution was overlayed with the same volume of PBS-cell-mixture and centrifuged for25 min at room temperature (450 x g, without break). Cells from the interphase containing mononuclear cells were harvested and washed at least 4 times with PBS (7 min, 300 x g, 4°C).

3.2.2. Isolation of monocytes

Human monocytes were isolated from PBMCs by depletion of non-monocytes with the Monocyte Isolation Kit II (Miltenyi Biotech). Therefore, the cell number was determined and the cells were washed with cold MACS buffer (PBS (pH 7.2) + 0.5% BSA + 2 mM EDTA). After centrifugation (7 min, 300 x g) up to 10^7 cells were resuspended in 30 µl MACS buffer, 10 µl FcR-block and 10 µl biotin-antibody cocktail containing biotin-conjugated monoclonal antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and Glycophorin A. Upon incubation for 10 min at 4°C, 30 µl buffer and 20 µl anti-biotin microbeads were added per 10^7 cells and incubated for further 15 min at 4°C. By washing with PBS excessive beads were removed. Finally the pellet was resuspended in 500 µl MACS buffer per 10^8 cells and transferred to a column in the magnet that retained all cells except monocytes that were collected in the flow-through. The purity and identification of the obtained cells was determined by flow cytometry. $CD14^+/CD45^+$ cells were defined as monocytes and used for generation of dendritic cells.

3.2.3. Generation of Monocyte-derived DCs

The cultivation of Monocyte-derived DC (MoDCs) is difficult because they are very sensitive and differentiate very fast.
Immature MoDCs grow in suspension and have a round shape. With increasing maturation, DCs develop dendrites and adhere to the surface of the cell culture dish.

Protocol 1

Monocytes were cultivated in X-VIVO 15 Medium (+1% Pen/Strep, 20 ng/ml recombinant GM-CSF, 20 ng/ml recombinant IL-4) that was developed for the cultivation of hematopoietic cells and can be used without FCS. Further 2 times GM-CSF and IL-4 were added to the cells. After 6-days of incubation, the expression of co-stimulatory molecules and activation markers on MoDCs was measured. $CD14^-$/ $HLA\text{-}DR^+$/ $CD83^-$/ $CD86^-$ cells were defined as immature MoDCs.

Protocol 2

Monocytes were cultivated in RPMI medium with glutamine, 10% heat-inactivated FCS Gold, 1% Pen/Strep, 20 ng/ml recombinant GM-CSF and 20 ng/ml recombinant IL-4. The next day cells were supplemented with further 20ng/ml GM-CSF and IL-4. After 3 days, fresh medium including cytokines was added. At day 6, cells were harvested and analysed by flow cytometry.

3.3. Infection and stimulation of MoDCs

0.5x 10^5 immature MoDCs were incubated in 100µl medium per well infection medium in a 96-well flat-bottom plate with *H. pylori* at MOI 5 for 1h, 2h, 3h, 4h, 5h, 24h, 48h, 72h and 96h. MoDCs were also stimulated with PFA-fixed *H. pylori* and PFA-fixed *E. coli* K12 at MOI 5.
Upon incubation, supernatants were harvested, centrifuged (5 min, 300x g) and used for determination of released cytokines by ELISA. Adherent cells were detached by addition of Accutase and analysed for expression of co-stimulatory molecules and activation markers by flow cytometry. $CD11c^+$/ $HLA\text{-}DR^+$/ $CD83^+$/ $CD86^+$ cells were defined as mature MoDCs.

In addition, immature MoDCs were also cultivated with *H. pylori* at different MOI (MOI 2, MOI 5, MOI 10, MOI 20 and MOI 50). After 24 h, maturation of DCs as well as the cytokine release was compared.

In order to prove the effect of different *H. pylori* strains and mutants on the maturation of MoDCs, immature cells were incubated for 24 h with the *H. pylori* wild type strains G27, P1, 60190, 2808, PM-SS1, J99 or different G27 mutant strains at MOI 5. Again, DC maturation and cytokine response was analysed.

3.4. Cultivationof CD4$^+$ T cells

By using the human CD4$^+$ T cell Isolation Kit II (MiltenyiBiotec) all non-CD4$^+$ cells were directly labelled and magnetically separated from PBMCs.

Therefore PBMCs were counted and centrifuged (7 min, 300 x g), up to 10^7 cells were resuspended in 40 µl MACS buffer and 10 µl biotin-antibody cocktail containing biotin-conjugated monoclonal anti-human antibodies against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and CD235a. After 10 minutes incubation at 4°C, further MACS buffer (30 µl per 10^7 cells) and anti-biotin microbeads (20 µl per 10^7 cells) were added and incubated for 15 min. After washing (300x g, 7 min, 4°C) the pellet was resuspended in 500 µl MACS buffer per 10^8 cells and transferred to columns, retaining all non-CD4$^+$ cells. The flow-through was collected and resuspended in RPMI / 10% FCS Gold and directly used for co-culture experiments. The purity of the harvested cells was determined by FACS (anti-CD45 and anti-CD4).

3.5. MoDC / CD4$^+$ T cellco-culture

0.5x 10^5 immature DCs were incubated in 100µl medium per well infection medium in a 96-well flat-bottom plate with *H. pylori* or PFA-fixed *E. coli* at MOI 5 overnight. Afterwards, the double amount of allogenic CD4$^+$ T cells was added to the infected MoDCs without changing the medium. After 72 h of co-cultivation, T cell proliferation as well as the expression of Foxp3 was analysed, as previously described (192). In parallel, supernatants were harvested to determine cytokine release by ELISA.

3.6. Neutralisation of Toll-like receptors

The pre-stimulation of MoDCs with neutralising antibodies prevents the binding of antigens to respective surface receptors.

0.5x 10^5 immature MoDCs were incubated in 100µl medium per well infection medium in a 96-well flat-bottom plate with 5 µg/ml anti-human TLR-2; 5 µg/ml anti-human TLR-4 or 5 µg/ml anti-human TLR-5 for 1h at 37°C. Afterwards *H. pylori* was added at MOI 5 for further 24 h.

To control the neutralisation efficiency, TLR-specific agonists were used for DCs stimulation in parallel. Therefore, DCs pre-incubated with anti-TLR antibodies were stimulated with 10 ng/ml LPS to stimulate TLR-4, 1 µg/ml Pam3Cysto activate TLR-2, or 10ng/ml Flagellin to stimulate TLR-5. Following incubation, supernatants were harvested and the amount of released cytokines was determined by ELISA.

3.7. Neutralisation of cytokines

During *H. pylori* infection of MoDCs neutralising antibodies inhibit the ability of cytokine to stimulate cells.

For IL-6 and IL-10 neutralisation experiments, 0.5x 10^5 immature MoDCs were incubated in 100µl medium per well infection medium in a 96-well plate with5 µg/ml anti-human IL-6 or 5 µg/ml anti-human IL-10 neutralising antibodies for 1 h. Subsequently, *H. pylori* was added at MOI 5 for further 24 h.

To control the neutralisation efficiency, recombinant proteins were used for DCs stimulation in parallel. Thus, immature DCs were incubated with 90 ng/ml recombinant human IL-6 or 10 ng/ml recombinant human IL-10 plus the neutralising antibodies as described above.

3.8. Flow Cytometry

Flow cytometry is a method used for analysing cells which pass a shaft of light with high speed. Thereby, several cell shapes and structures are emitted that define the characteristic of the cell. When large cells pass the shaft of light in a flat angle (FS = Forward Scatter) they scatter more light than smaller cells. The perpendicular refraction of light (SS = Sideward Scatter) gives information about the granularity of the cell. In parallel, fluorescence dyes can

be measured. With the help of fluorescence labelled antibodies a further characterisation of the cells can be done. During this work, the data were acquired with CyAn and analysed by FlowJo software.

3.8.1. Staining of surface antigens

For discrimination between live and dead, cells were stained with EMA (1:1000) for 30 min on ice. EMA is a fluorescence dye that covalently binds to nucleic acids after photolysis. Since living cells are relatively impermeable for EMA, the dye selectively marks the DNA of dead cells. After washing (5 min, 300 x g), cells were resuspended in cold FACS buffer. Fluorescence-labelled antibodies recognizing CD14, CD45, CD11c, HLA-DR, CD83, CD86, CD80, CD4, CD25 and CD127 were added following manufacturer's instructions for 30 min at 4°C in the dark. After incubation, the cells were washed and either resuspended in FACS buffer + 1% PFA for FACS analysis or prepared for staining of intracellular antigens.

3.8.2. Staining of intracellular antigens

Intracellular antigen staining was used to detect the expression of Foxp3 in $CD4^+$ T cells. Following the staining of surface antigens, washed cells were resuspended in Foxp3 fixation/permeabilisation solution following manufacturer's instructions for 30 min at 4°C in the dark. After washing (5 min, 300 x g) cells were stained in 1x permeabilisation buffer with the recommended amount of Foxp3 antibody. After 30-minute incubation in the dark at room temperature cells were washed, resuspended in FACS buffer and analysed by flow cytometry.

3.9. ELISA

ELISA is a method used for the detection of proteins, usually antibodies or cytokines, in a liquid sample. For determination of cytokine secretions of MoDCs upon *H. pylori* infection, culture supernatants were harvested and cytokine concentrations were assessed by sandwich ELISA according to the manufacturer's instructions. Therefore ELISA plates were coated over night at 4°C with the appropriate capture antibody. After 5-times washing with washing buffer free binding sites on the plate were blocked with assay solution for 1 h at room temperature. Excessive protein was removed by washing. Afterwards diluted standard and

samples were added and incubated over night at 4°C. Following washing, the biotin-conjugated detection antibody was added for 1 h at room temperature. When unbound antibodies were removed, Avidin-HRP was incubated for 30 min. After carefully washing, substrate was added and the chemical reaction was stopped after 15 min by 1M H_3PO_4. The plate was measured in the ELISA reader at 450nm.

3.10. Western blot

Western blot is a technique used to detect specific proteins by transferring (blotting) them to a carrier membrane. Cells were harvested at different times post infection and lysed in SDS 1x sample buffer. Equal amounts of lysate were loaded on 8% SDS-PAGE gels and separated proteins were transferred to a nitrocellulose membrane at 230 mA for 2 h. After blocking with buffer primary antibodies were incubated over night at 4°C according to manufacturer’s instructions. After 3-times washing with TBST (10 min), the HRP-labelled secondary antibody was incubated in TBST + 5% milk powder for 1 h at room temperature. After washing, bands were detected by ECL-solution.

3.11. Proliferation Assay

Proliferation of $CD4^+$ T cells upon co-cultivation with MoDCs was determined by the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). This assay is based on the quantification of ATP in the cell culture supernatant after induction of cell lysis that correlates with the presence of metabolic active cells. Therefore, 3.000 immature MoDCs were incubated in 100µl medium per well infection medium in a 96-well flat-bottom plate with *H. pylori* or PFA-fixed *E. coli* at MOI 5 overnight. Afterwards, 6.000 allogenic $CD4^+$ T cells were co-incubated for 72 h without changing the medium. Afterwards, plate was equilibrated for 30 min at room temperature before adding the same volume of substrate. By careful resuspension, lysis of the cells was induced and chemiluminescence was determined. The emitted light was proportional to the amount of ATP that was measured by a luciferase dependent oxygenation of luciferin in the presence on Mg^{2+}, ATP and O_2.

3.12. Isolation of *H. pylori* DNA

For the isolation of genomic DNA of *H. pylori* the DNeasy Blood & Tissue Kit (Qiagen) was used following manufacturer's instructions. *H. pylori* was harvested and lysed using proteinase K. Afterwards, buffered lysate was loaded onto a spin column. During centrifugation, DNA selectively bound to a membrane as contaminants pass through. Remaining contaminants and enzyme inhibitors were removed in two following wash steps and DNA was then eluted in water. The amount of purified DNA was determined as ratios of the absorbance 260/280 using the spectrophotometer.

3.13. Transformation of *H. pylori*

Wild type *H. pylori* of the donor strain (mutant) as well as the recipient strain (wild type) were cultured on appropriate WC-dent plates (with or without antibiotics). Next, DNA of the *H. pylori* donor strain was extracted (DNeasy Blood & Tissue Kit, Qiagen). The wild type strain was resuspended in BHI + 20 % FCS and mixed with 2 µg of mutant DNA. Following incubation for 10 min at 37 °C, mixture was applicated on a WC-dent plate. Colonies were plated out on antibiotic-containing WC-dent plates. Transformation success was tested by PCR.

3.14. Polymerase chain reaction

The principle of polymerase chain reaction (PCR) is based on the enzymatic amplification of a DNA segment using two oligonucleotide primers that are bound to opposite complementary strands of DNA. During this thesis, the method was used to proof *H. pylori* strains for *cagA* expression.

The cyclic amplification reaction was carried out with the following steps: The initiation step provided the denaturation of the template DNA by heating to 94°C. In 35 recurring cycles, the DNA was first divided in individual strands to which the primers hybridise upon lowering the temperature.

PCR mix:

cagA forward Primer	10 µM (TTG ACC AAC AAC CAC AAA CCG AAG)
cagA reverse Primer	10 µM (CTT CCC TTA ATT GCG AGA TTC C)
Green Taq	7.5 µl
H_2O	4.5 µl
DNA	1 µl (50ng)

3.15. Statistics

Data were presented as mean ± SD. For statistical analysis of flow cytometry data ANOVA with Dunnett's multiple comparison test was used. ELISA data were evaluated using the paired Student t test, since donors strongly differed in the release of cytokines. P values less than 0.05 were considered significant.

Results

4. Results

4.1. Generation of immature MoDCs

In the human blood only 0.3 – 0.9% of leukocytes are DCs, while around 10% are monocytes. *In vivo*, monocytes can move quickly to sites of infection in the tissues and differentiate into macrophages and DCs (193). This characteristic can be recapitulated *in vitro* to generate MoDCs.

Thus, PBMCs were first enriched from blood by separating erythrocytes and plasma during density gradient centrifugation. Afterwards monocytes were isolated from PBMCs by magnetic cell labelling. The purity of enriched monocytes was determined by the expression of CD45 and CD14, which are characteristic markers for leukocytes and monocytes, respectively. High levels of CD45/CD14 double-positive cells (>80% of the cells) were obtained after monocyte enrichment (Figure 1). To generate MoDCs, monocytes were subsequently stimulated with the cytokines GM-CSF and IL-4. The latter is used to inhibit the outgrowth of macrophages (194).

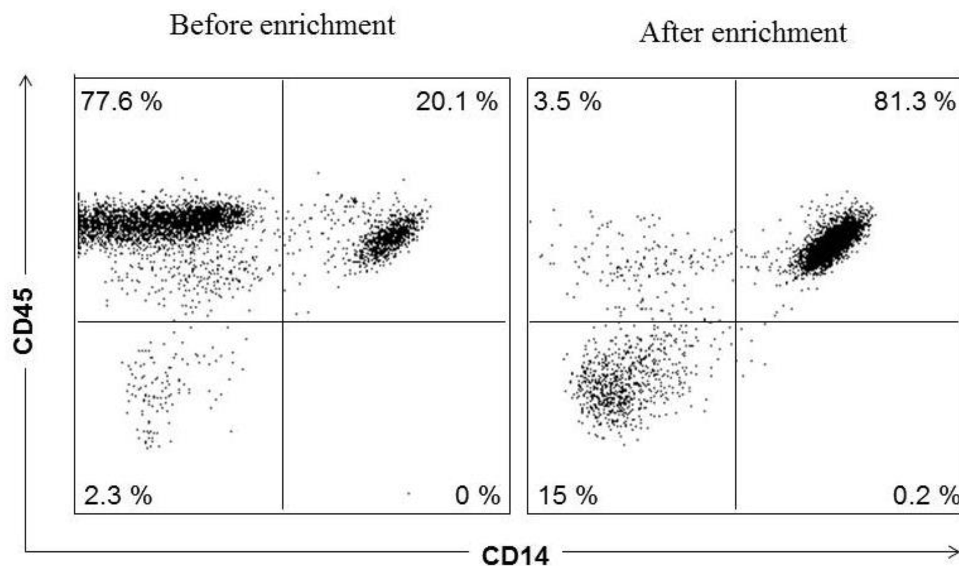

Figure 1: Purity determination of isolated human monocytes

Upon enrichment, purity of monocytes was determined by analysing CD45/CD14 double-positive cells by flow cytometry. Dotplot image shows one representative experiment of n=5.

Compared to monocytes and macrophages, MoDCs lose the ability to express CD14 (195). In order to monitor the differentiation of monocytes to MoDCs after cytokine treatment, the expression of CD14 was analysed at different time points. Furthermore, the presence of CD11c, a highly expressed protein in human DCs, was also determined. This marker was used for the discrimination of MoDCs in further experiments.

As shown in Figure 2A and C, the expression of CD14 markedly decreased up to 90% after 6 days of culture, indicating that the majority of cells were MoDCs. On the other hand, cytokine treatment did not affect the expression of CD11c (Figure 2B).

These results showed that high and pure amounts of MoDCs were generated from monocytes in the presence of GM-CSF and IL-4 and that complete differentiation was achieved after 6 days of treatment. Consequently, MoDC generation always followed 6-day incubation with GM-CSF and IL-4.

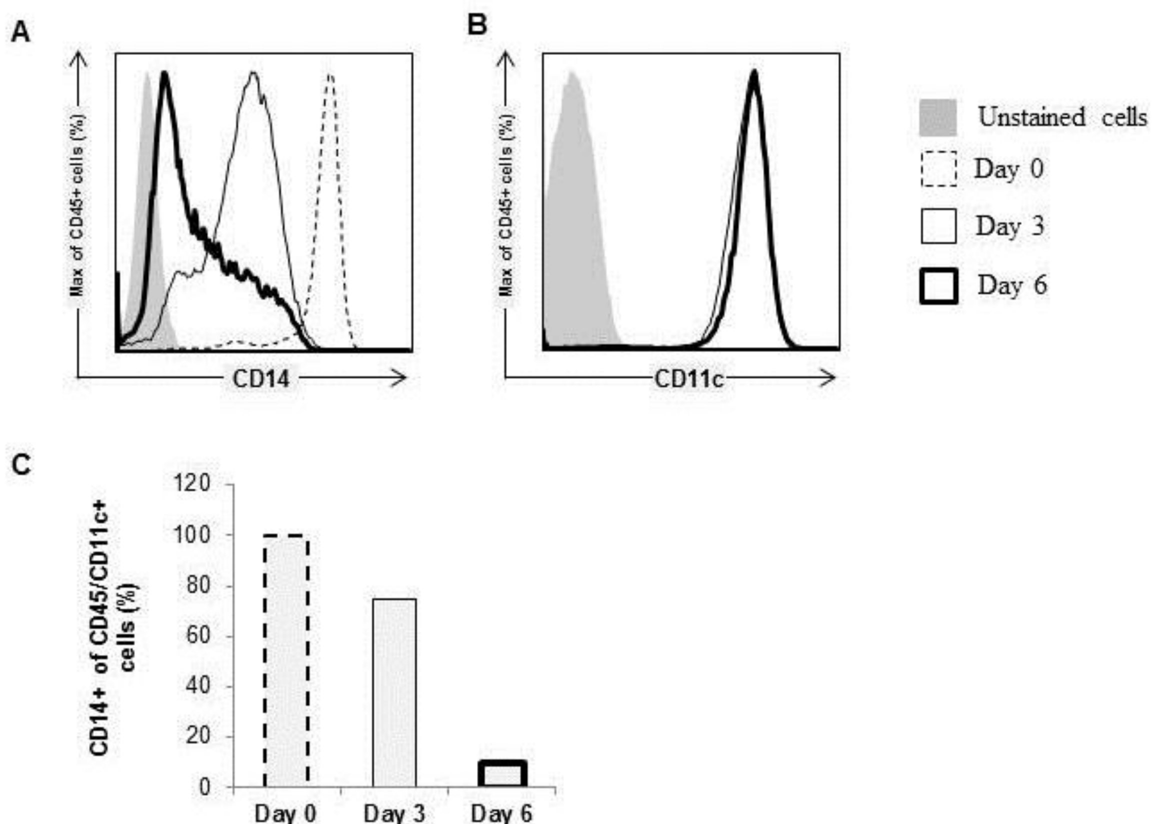

Figure 2: Expression of CD14 and CD11c during generation of MoDCs

Expression of CD14 in CD45$^+$ cells was determined by flow cytometry after isolation, at day 3 and day 6. (A+B) Histograms show one representative experiment of n=3. (C) Data in bar graphs are presented as mean of 2 independent experiments.

Different media for MoDC generation have been published, but it was not stated if or how the maturation status of MoDCs was affected by these media. Since the analysis of *H. pylori* on DC maturation was one of the main objectives of this work, it became indispensable to prove the influence of the medium. Consequently, immature MoDCs generated with different media were analysed for their expression of the co-stimulatory molecule CD86 and the activation marker CD83, which are hallmarks of DC maturation (196) (197).

When MoDCs were generated using X-VIVO 15medium without FCS (Figure 3A), the majority of cells exhibited an elongated shape. Furthermore, unstimulated MoDCs expressed high levels of the co-stimulatory molecule CD86, indicating that the cells were already pre-activated. By generating MoDCs in RPMI in the presence of endotoxin-low FCS (FCS Gold), the cells presented a rounder shape, typical for non-activated DCs, and expressed only low amounts of co-stimulatory molecules (Figure 3B), indicating that they were immature.

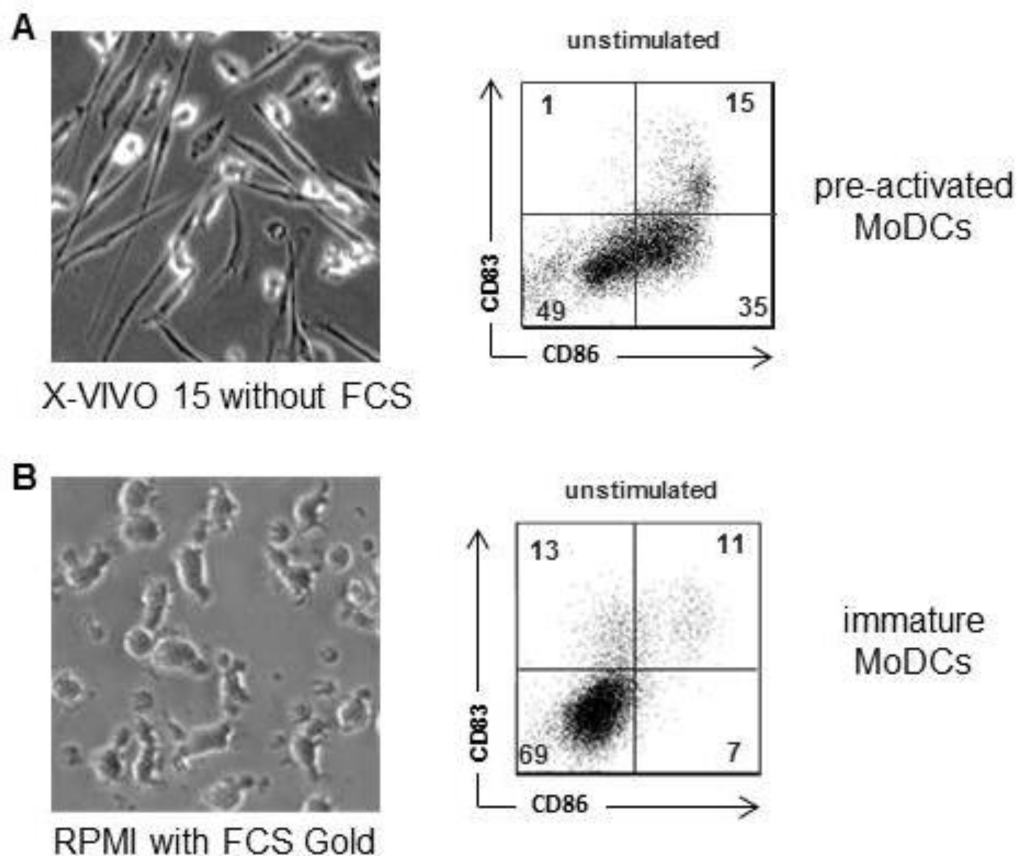

Figure 3: Morphology and maturation of MoDCs induced by different media

Monocytes were incubated in (A) X-VIVO15 without FCS or (B) RPMI with FCS Gold. Morphological changes of MoDCs were microscopically observed (20x) after 6 days. Maturation of MoDCs was monitored by the expression of CD86 and CD83 by flow cytometry. Dotplot and microscopic image show one representative experiment of n=3.

To evaluate if the different media used for the generation of immature MoDCs could influence the response of DCs to infection, both types of generated MoDCs were cultured with either *H. pylori* or *E. coli* at MOI 5 for 24h under aerobic conditions. The experimental setting deemed it necessary to fix *E. coli* with PFA, since it would quickly overgrow the cells. In contrast, *H. pylori* did not kill the cells under aerobic conditions; hence it was not necessary to fix it. Upon stimulation of MoDCs with *H. pylori* or *E. coli,* maturation and cytokine release of the cells was compared.

MoDCs generated in X-VIVO 15 medium expressed already high levels of CD83 and CD86, indicating that the cells were pre-activated. Stimulation of these cells with *H. pylori* G27 led to significant upregulation of CD86 and CD83. However, no differences between *H. pylori* and *E. coli*-stimulated MoDCs were detected (Figure 4A).

On the other hand, MoDCs generated with RPMI and FCS Gold were characterised by low CD83 and CD86 expression, suggesting that they were immature. These cells showed a strong induction of the expression of CD83 and CD86 in response to PFA-fixed *E. coli*. However, the observed levels induced by *H. pylori* were significantly lower, indicating only a partial maturation of MoDCs in response to *H.* pylori (Figure 4B).

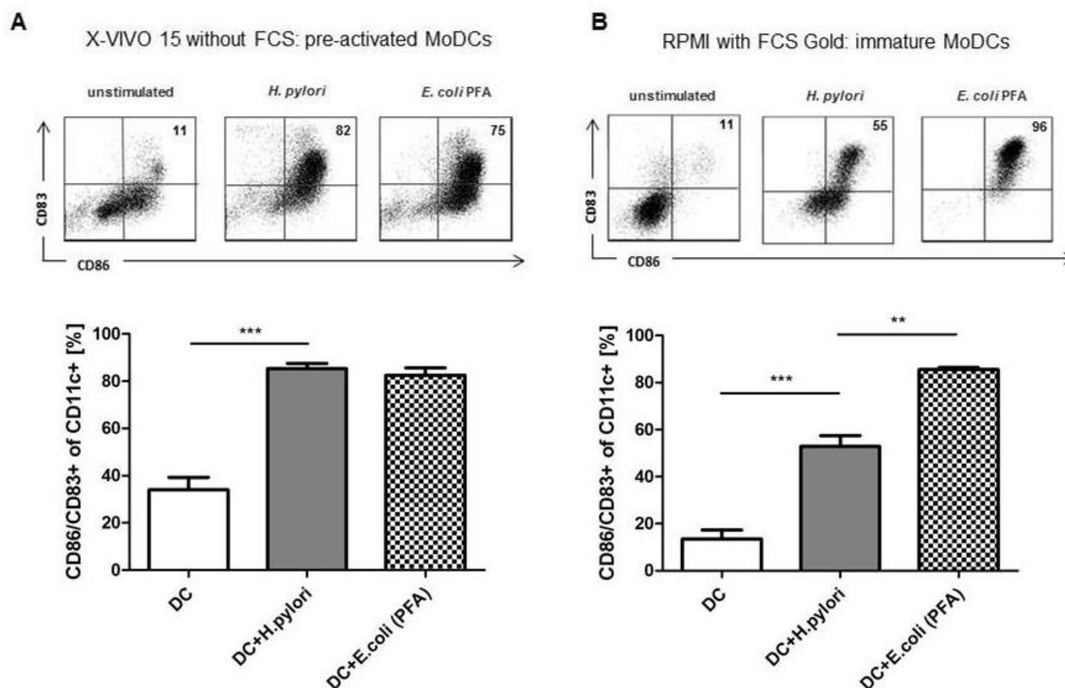

Figure 4: Maturation of pre-activated and immature MoDCs after bacterial stimulation

MoDCs were generated in (A) X-VIVO 15 without FCS or (B) in RPMI / FCS Gold and stimulated with *H. pylori* G27 or PFA-fixed *E. coli* K12 at MOI 5 for 24 h. Maturation of $CD11c^+$ MoDCs was assessed by analysing the amount of CD86/CD83 double-positive cells by flow cytometry. Dotplot image illustrates one representative experiment. Data in bar graphs are presented as mean ± S.D. of 4independent experiments. (ANOVA: ** $p \leq 0.005$; *** $p \leq 0.001$)

In response to bacterial stimulation MoDCs secrete different cytokines. One critical pro-inflammatory cytokine is IL-12p70, known to be a potent stimulator for the Th1 response that is observed during *H. pylori* (120) and *E. coli* (198) infections. On the other hand, also anti-inflammatory cytokines, such as IL-10, reported to dampen the immune response, are secreted. Consequently, the release of these two representative cytokines upon challenge with *H. pylori* and *E. coli* depending on the generation of MoDCs was compared.

As shown in Figure 5, pre-activated and immature MoDCs secreted similar amounts of IL-12p70 in response to *H. pylori*, whereas the levels of IL-10 were significantly higher in immature MoDCs. In contrast, immature *E. coli*-primed MoDCs produced higher levels of IL-10 and IL-12p70 compared to pre-activated MoDCs. These data indicated that the initial maturation status of MoDCs strongly influenced the cytokine response upon infection. Interestingly, immature MoDCs induced a stronger anti-inflammatory cytokine response, while pre-activated cells favour pro-inflammatory cytokine secretion, as shown by the different IL-10 / IL-12p70 ratio (Figure 6).

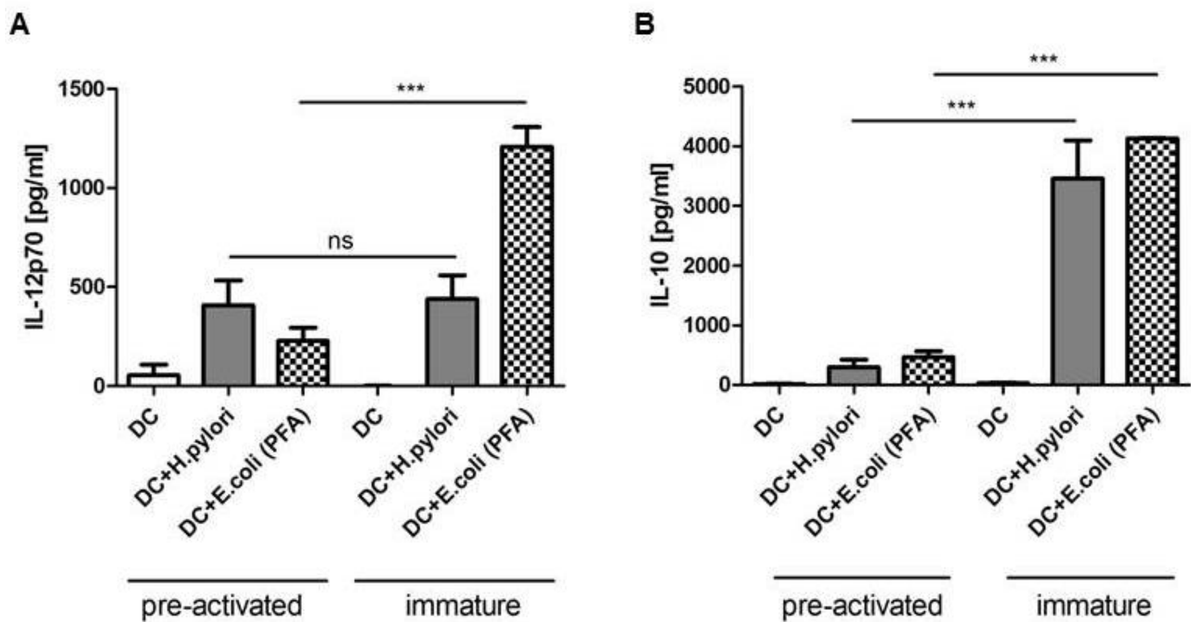

Figure 5: Cytokine response of pre-activated and immature MoDCs upon bacterial infection

Release of (A) IL-12p70 and (B) IL-10 in response to *pylori* G27 or PFA-fixed *E. coli* K12 at MOI 5 was compared in pre-activated and immature MoDCs after 24 h. Cytokine levels were determined by ELISA. Data in bar graphs are presented as mean ± S.D. of 3 independent experiments.
(ANOVA: *** $p \leq 0.001$; ns: not significant)

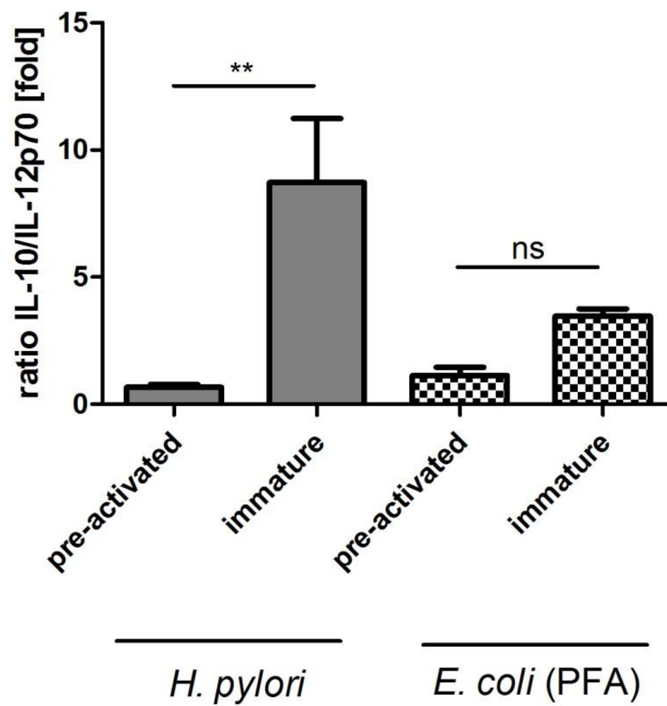

Figure 6: Ratio of IL-10/IL-12p70 in pre-activated and immature MoDCs induced by *H. pylori* and *E. coli*

Cytokine ratio of IL-10 / IL-12p70 in response to *H. pylori* G27 and PFA-fixed *E. coli* K12 in pre-activated or immature MoDCs was calculated after 24h infection at MOI 5.Data in bar graphs are presented as mean ± S.D. of 3 independent experiments. (ANOVA: ** $p \leq 0.005$; ns: not significant)

These results showed that the use of pre-activated MoDCs influenced the maturation and cytokine production in response to bacteria, which might lead to wrong data. Consequently, the use of immature MoDCs for infection experiments was very important.

4.2. *H. pylori* induces semi-maturation of MoDCs

A first aim of this study was to evaluate the effect of *H. pylori* on the maturation of MoDCs. For that reason, cells were infected with *H. pylori* at MOI 5 for 24 h. PFA-fixed *E. coli* was used as control. In order to verify if live *H. pylori* differentially influenced DC maturation compared to dead bacteria, immature MoDCs were also incubated with PFA-fixed *H. pylori*. The effect of *H. pylori* on the maturation of MoDCs was assessed by analysing the expression of CD80, CD86, CD83 and the MHC class II receptor HLA-DR by flow cytometry. Compared to *E. coli* that strongly induced the expression of CD80, CD86 and CD83 in MoDCs, *H. pylori*-challenged MoDCs were only partially activated, as shown by lower levels of these markers (Figure 7). In addition, the expression of MHC class II receptors was significantly lower in response to *H. pylori*. However, DC semi-maturation was only observed upon infection with live *H. pylori*, since PFA-fixed *Helicobacter* induced significantly increased expression of CD80, CD86, CD83 and MHC class II molecules, suggesting an active suppression of DC maturation.

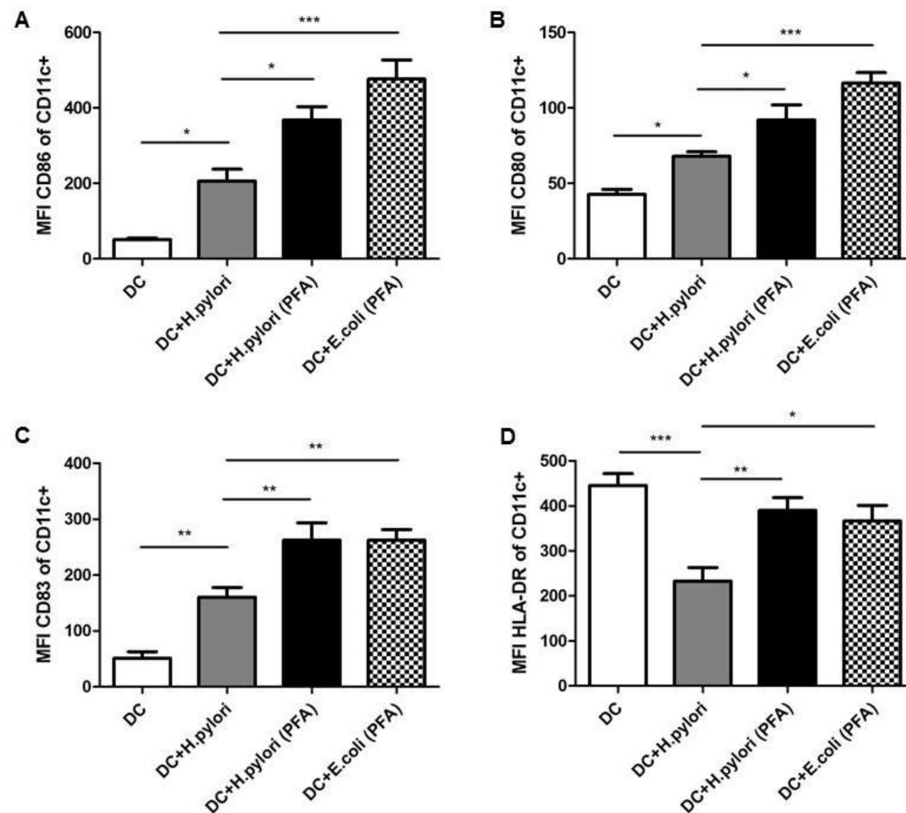

Figure 7: Expression of maturation markers on MoDCs upon *H. pylori* infection

Maturation of MoDCs upon stimulation with vital or PFA-fixed *H. pylori* G27 or *E. coli* K12 was determined by the expression of (A) CD86, (B) CD80, (C) CD83 and (D) MHC class II by flow cytometry. MFI: mean fluorescence intensity. Data in bar graphs are presented as mean ± S.D. of 10 independent experiments. (ANOVA: * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$)

In order to determine whether live *H. pylori* was actively dampening DC maturation, cells were co-incubated with live and PFA-fixed *H. pylori* at the same time. Lower DC maturation was detected when compared to dead bacteria alone, suggesting that bacterial virulence factors actively induce semi-maturation of DCs thereby preventing full DC activation triggered by PFA-fixed *H. pylori* (Figure 8). Comparable results were obtained when live *H. pylori* was added in combination to PFA-fixed *E. coli*.

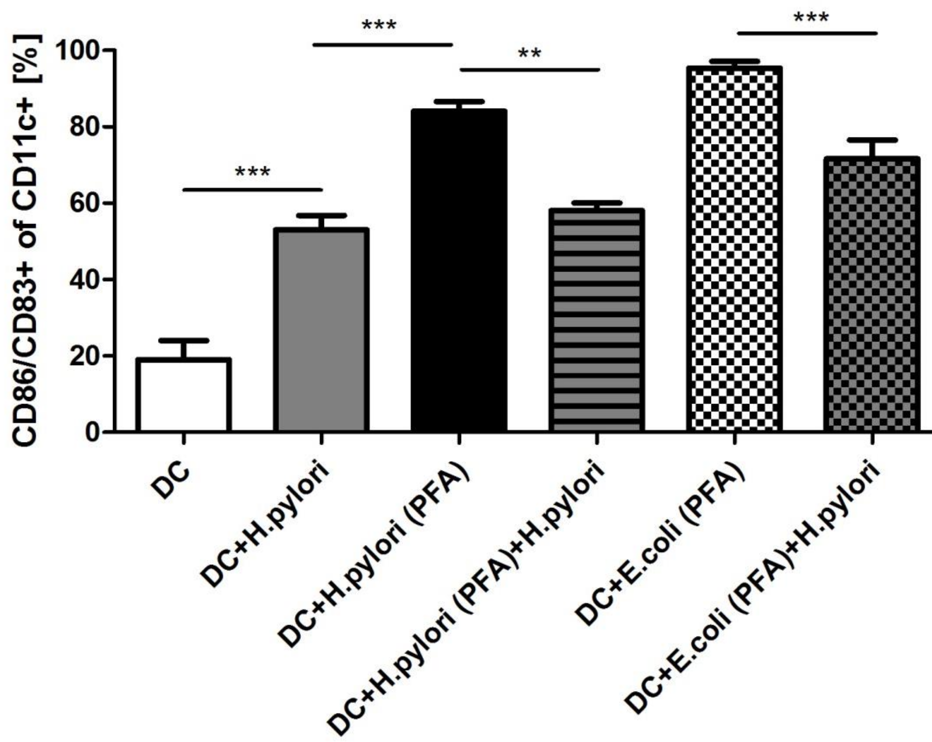

Figure 8: Maturation of MoDCs upon simultaneous stimulation with PFA-fixed bacteria and *H. pylori*

Immature MoDCs were primed with live and PFA-fixed *H. pylori* G27 or with live *H. pylori* and PFA-fixed *E. coli* simultaneously. Maturation was evaluated by measuring the amount of CD86/ CD83 double-positive cells after 24 h of stimulation. Data are presented as mean ± S.D. of 8 independent experiments.
(ANOVA: ** $p \leq 0.005$; *** $p \leq 0.001$)

4.3. *H. pylori*-induced DC semi-maturation is not time-, dose- or strain-dependent

Stimulation of immature MoDCs with the *H. pylori* strain G27 at MOI 5 caused markedly lower expression of CD86 and CD83 after 24 h compared to PFA-fixed *H. pylori* or *E. coli*. To analyse if a longer infection will influence the maturation of MoDCs, immature cells were primed with *H. pylori* G27 for 24 h, 48 h and 72 h at MOI 5.

Longer *H. pylori* stimulation time did not lead to enhanced expression of CD86 and CD83 on MoDCs (Figure 9), indicating that DC maturation was not delayed.

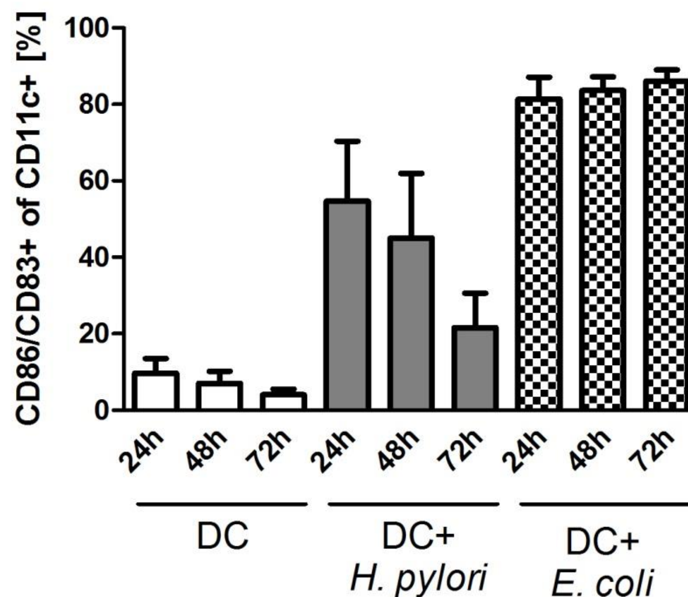

Figure 9: Time-dependent induction of DC maturation

Maturation of MoDCs upon stimulation with *H. pylori* G27 or *E. coli* K12 was determined after 24 h, 48 h and 72 h by the amount of CD86/CD83 double-positive cells by flow cytometry. Data are presented as mean of 3 independent experiments.

However, it remained still unclear whether *H. pylori* could survive the aerobic environment during infection, since dead bacteria could influence the maturation of MoDCs. Therefore, *H. pylori* survival was analysed after incubation under aerobic conditions for different times by re-cultivation in a microaerobic environment.

Bacterial colony formation on a WC-dent plate was observed at all time points, even after

96 h incubation of *H. pylori* under aerobic conditions, suggesting that *H. pylori* was able to survive aerobic incubation for up to 96 h (Figure 10).

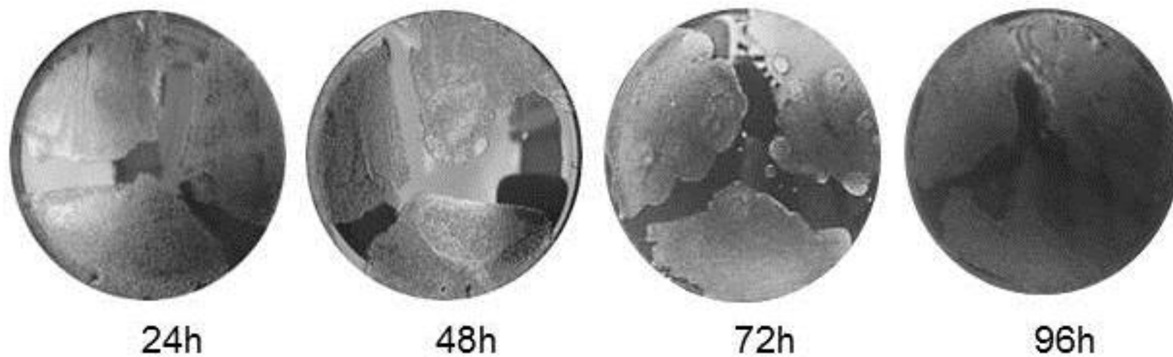

Figure 10: Re-cultivation of *H. pylori* after incubation under aerobic conditions

H. pylori G27 was resuspended in RPMI with FCS Gold and incubated for up to 96 h under aerobic conditions. Afterwards the bacterium was plated on WC-dent plates for 2 days and bacterial growth was controlled. Pictures show WC-dent plates of one representative experiment.

Additionally, it was investigated whether the bacterial dose influenced the maturation of MoDCs. Therefore, immature MoDCs were incubated with different MOIs of *Helicobacter* (MOI 2 to MOI 50). First, it could be observed that high bacterial load (MOI 50) led to cell death, shown by very low numbers of cells that were gated through the Forward- (FS) and Sideward scatter (SS), indicating that MoDCs have lost cell size and granularity. Consequently, MOI 50 was not used for further analysis (Figure 11).

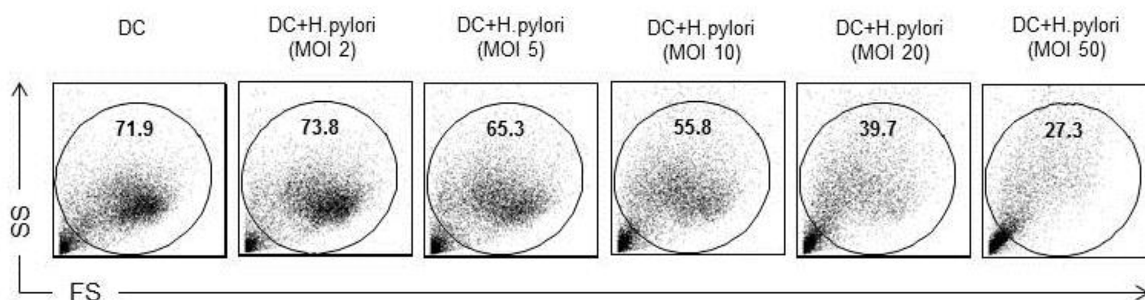

Figure 11: Cell size and granularity of MoDCs upon *H. pylori* infection at different MOI

Immature MoDCs were primed with increasing amounts of *H. pylori* G27 for 24 h. Size and granularity of MoDCs was verified by flow cytometry. Dotplot shows one representative experiment of n=2.

Moreover, the challenge of immature MoDCs with increasing amounts of *H. pylori* did not result in notably changes in the expression of CD86 and CD83 (Figure 12), indicating that bacterial dose was not the main reason of *H. pylori*-mediated DC semi-maturation.

Since incubation of MoDCs with *H. pylori* at MOI 5 caused low cell death, but induced the expression of maturation markers, MOI 5 was used for all further infections.

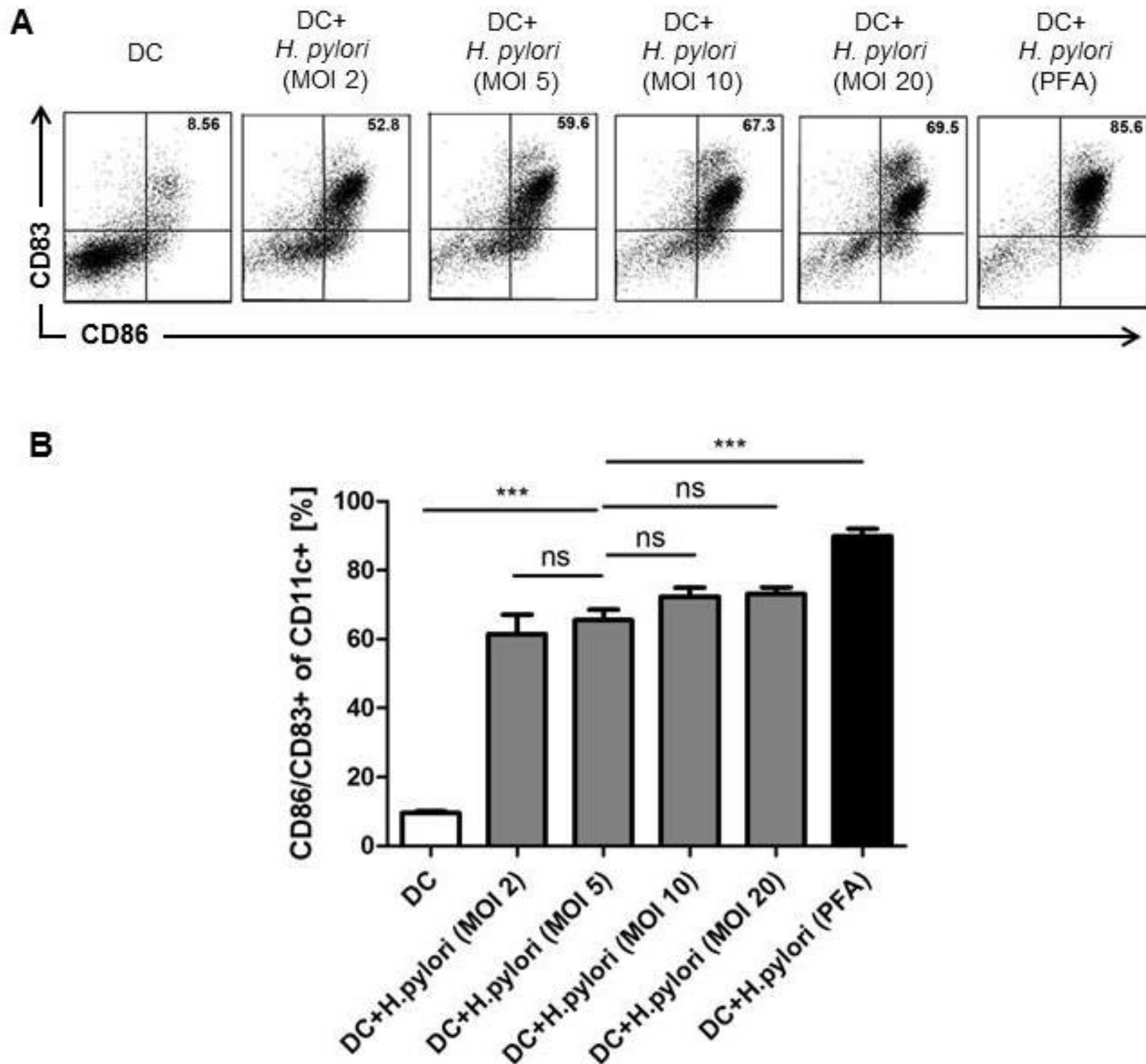

Figure 12: Maturation of MoDCs after *H. pylori* infection at different MOI

Maturation of MoDCs upon stimulation with increasing amounts of *H. pylori* G27 was determined by the expression of CD86 and CD83. (A) Dotplot of CD86/CD83 double-positive cells from one representative experiment. (B) Amount of CD86/CD83 double-positive cells was analysed by flow cytometry. Data are presented as mean ± S.D. of 3 independent experiments. (ANOVA: *** $p \leq 0.001$; ns: not significant)

To rule out that the observed effect on MoDCs was not exclusive to G27 *H. pylori* strain, immature MoDCs were also co-incubated with the type I strains J99 or 60190 and the type II strains P1 or PMSS1 at MOI 5 for 24 h and the maturation of MoDCs was compared.
As shown in Figure, all wild type strains induced similar expression of CD86 and CD83 that was significantly lower than those induced by PFA-fixed *H. pylori.* These results demonstrated that *H. pylori*-induced DC semi-maturation was not strain dependent.

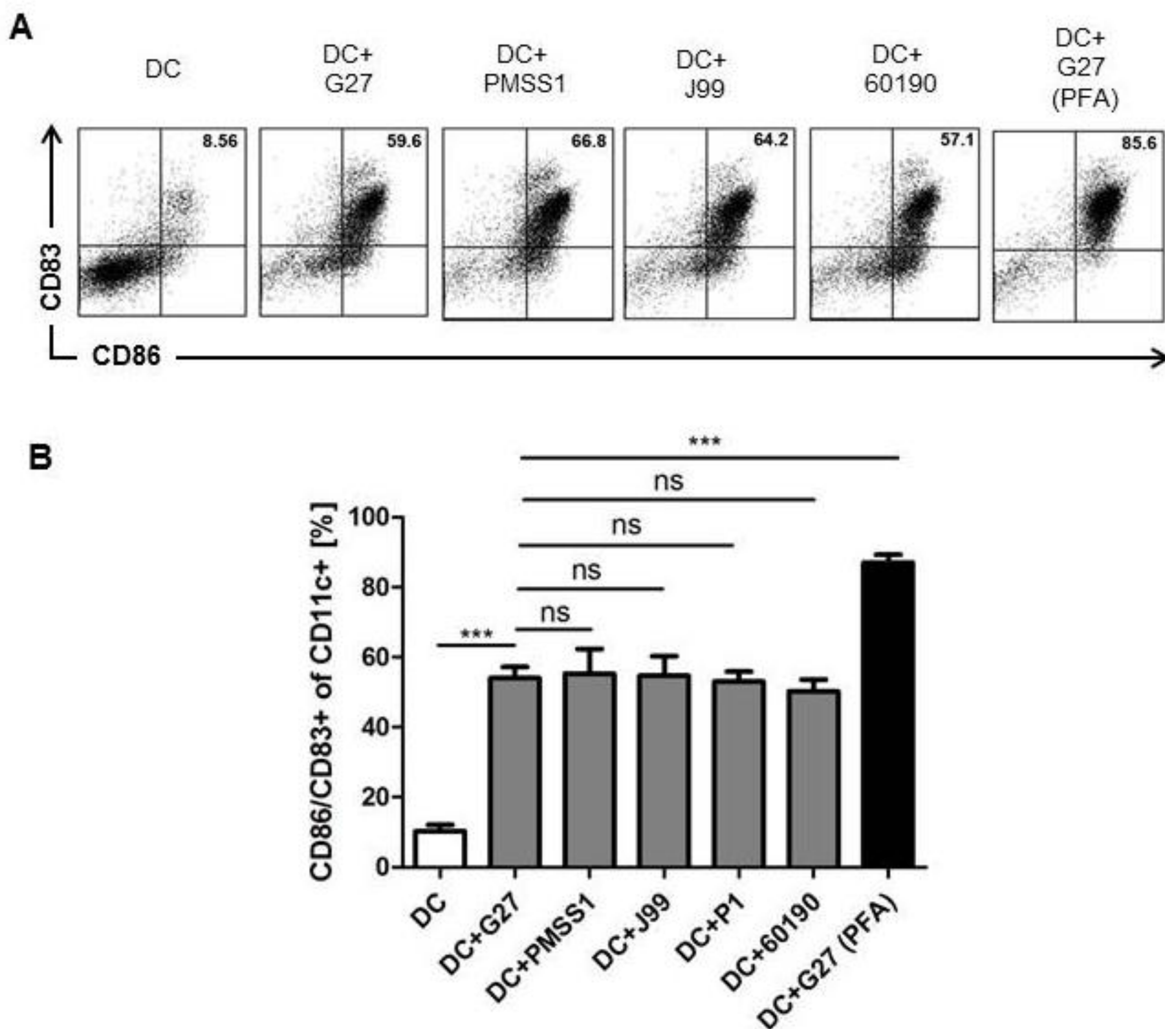

Figure 13: Maturation of MoDCs in response to different *H. pylori* strains

Maturation of MoDCs upon stimulation with *H. pylori* wild type strains G27, PMSS1, J99, P1 and J99was determined by the expression of CD86 and CD83. (A) Dotplot of CD86/CD83 double-positive cells from one representative experiment. (B) Amount of CD86/CD83 double-positive cells was analysed by flow cytometry. Data are presented as mean ± S.D. of 3 independent experiments. (ANOVA: *** $p \leq 0.001$; ns: not significant)

4.4. *H. pylori* induces an anti-inflammatory cytokine response in MoDCs

In *H. pylori*-infected individuals increased numbers of Th1 and Th17 cells, as well as an expansion of T_{regs} were observed (148) (150). Th1 and Th17 cells develop upon contact with DCs in the presence of IL-12p70 (120) and IL-6 (199), respectively, while IL-10 and TGF-β are known to induce T_{regs} (200) (201). Thus, in addition to the analysis of DC maturation, the effect of *H. pylori* on the cytokine release was also investigated.

Lower levels of the pro-inflammatory cytokines IL-12p70 and IL-6 were detected when MoDCs were co-incubated with live *H. pylori* compared to PFA-fixed *Helicobacter* or *E. coli* (Figure 14). In contrast, the release of anti-inflammatory IL-10 was significantly higher in response to live bacteria, indicating that *H. pylori* favoured an anti-inflammatory cytokine response. Notably, no TGF-β was measurable in all supernatants.

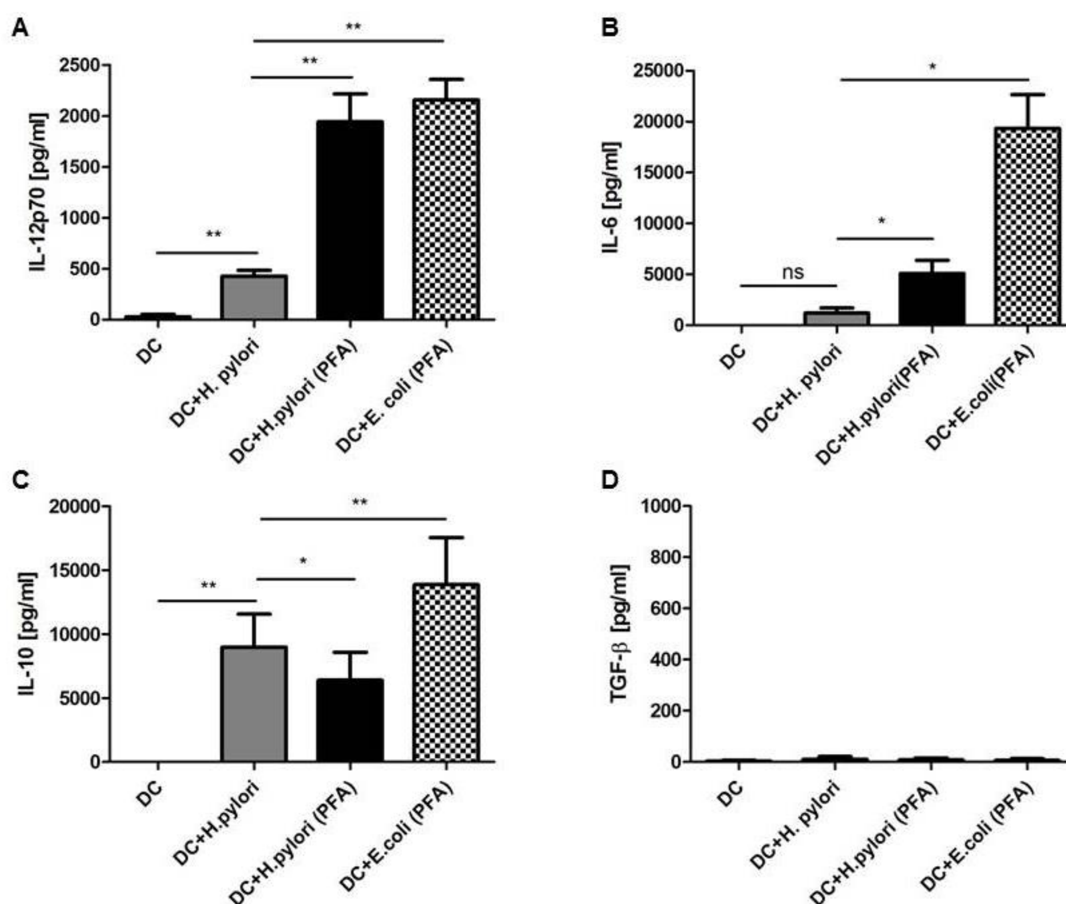

Figure 14: ***H. pylori*****-induced cytokine secretion by MoDCs**

Release of (A) IL-12p70, (B) IL-6,(C) IL-10 and (D) TGF-β by MoDCs upon 24 h stimulation with live or PFA-fixed *H. pylori* G27or PFA-fixed *E. coli* at MOI 5was determined by ELISA. Data are presented as mean ± S.D. of 6 independent experiments. (t-Test: * $p \leq 0.05$; ** $p \leq 0.005$; ns: not significant)

Moreover, even stimulation with PFA-fixed *H. pylori* did not induce a comparable cytokine release by MoDCs as caused by the challenge with *E. coli,* which was explained by the weak immune stimulatory capacity of *H. pylori* LPS and Flagellin.

Interestingly, *E. coli* also induced high levels of IL-10, however, when the ratio between IL-12p70 and IL-10 secretion was assessed, *E. coli* was found to favour a pro-inflammatory cytokine response, while *H. pylori* induced a strong anti-inflammatory cytokine secretion in MoDCs (Figure 15).

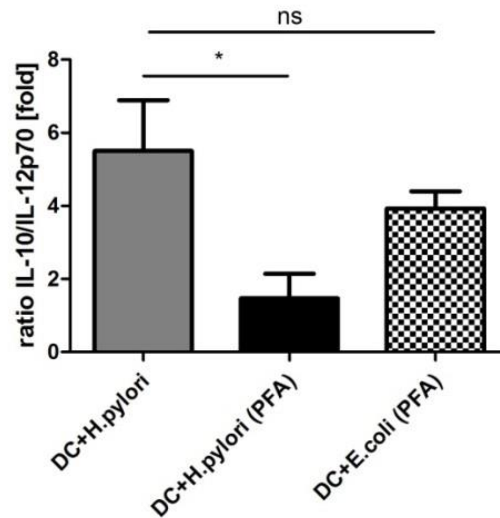

Figure 15: IL-10 and IL-12p70 ratio of MoDCs induced by *H. pylori* and *E. coli*

Ratio of released IL-10 and IL-12p70 upon stimulation with live or PFA-fixed *H. pylori* G27 or *E. coli* K12 was calculated after 24 h of infection. Data are presented as mean ± S.D. of 4 independent experiments.
(t-Test: * p ≤ 0.05; ns: not significant)

4.5. *H. pylori*-primed MoDCs instruct a tolerogenic T cell response

To examine the functional relevance of DC semi-maturation and the induced anti-inflammatory cytokine response upon infection with *H. pylori*, stimulated MoDCs were co-cultured with $CD4^+$ T cells. Since DC generation required 6 days, T cells could not be obtained from the same donor without losing cell viability. Thus, MoDCs were co-incubated with allogenic T cells (Figure 16).

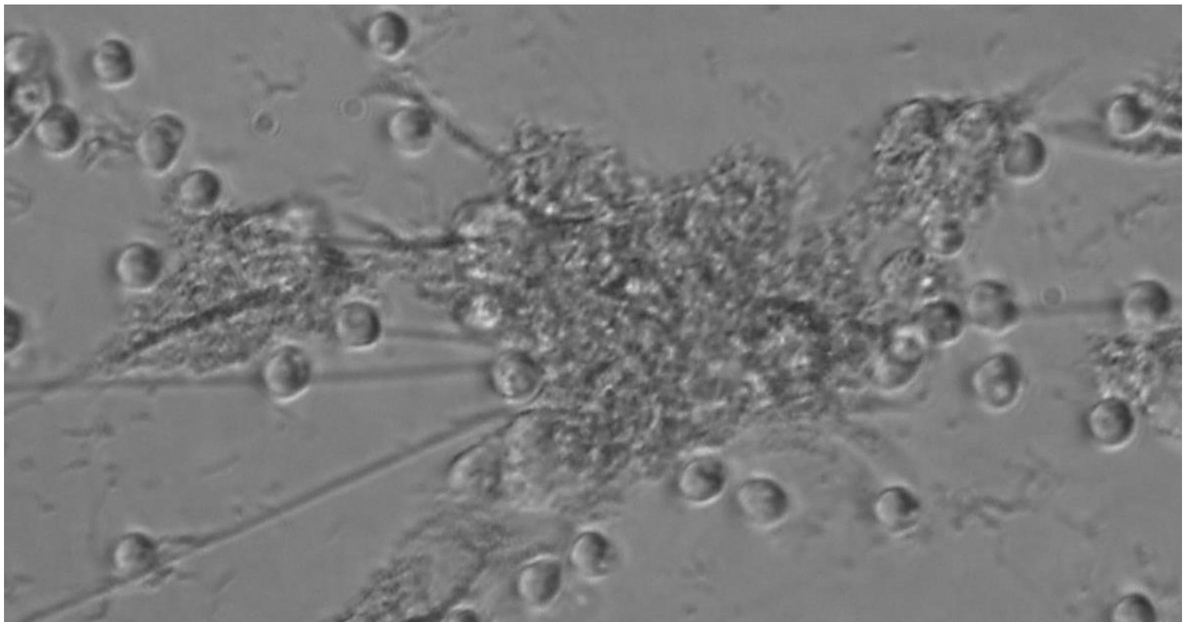

Figure 16: Co-cultivation of *H. pylori*-infected MoDCs with allogenic $CD4^+$ T cells

Immature MoDCs were infected with *H. pylori* G27 at MOI 5 for 24 h and subsequently co-incubated with the two fold amount of allogenic $CD4^+$ T cells. Microscopic image shows co-cultivation after 24 h.

Initially, the effect of *H. pylori*-challenged MoDCs on T cell proliferation and cytokine release was determined. Proliferation of T cells was analysed by the quantification of ATP present in the supernatant of co-cultured cells upon cell lysis. The levels of ATP correlated with the presence of metabolic active cells.

Infection of MoDCs with live *H. pylori* strongly inhibited cell proliferation of co-cultivated T cells (Figure 17). This was accompanied by significantly lower levels of IL-2 (Figure 18), a cytokine secreted by activated T cells, which is known to induce T cell proliferation and differentiation. However, this effect was only observed upon co-culture with MoDCs infected

with live bacteria. MoDCs stimulated with PFA-fixed *H. pylori* induced similar T cell proliferation and IL-2 secretion as *E. coli*-challenged MoDCs. Moreover, T cell proliferation and IL-2 secretion were drastically reduced in response to co-cultivation with *H. pylori*- and *E. coli*-primed MoDCs, indicating that *H. pylori*-infected MoDCs were able to actively suppress T cell proliferation. Importantly, T cell proliferation was also blocked upon *H. pylori* infection in the absence of MoDCs, while increased cell viability was observed upon stimulation with PFA-fixed *Helicobacter* and *E. coli*. These data indicated that live *H. pylori* alone was able to inhibit T cell proliferation. However, T cell stimulation with *H. pylori* or *E. coli* in the absence of MoDCs did not result in the secretion of IL-2, suggesting that DCs are needed for optimal T cell activation.

Notably, co-incubation of *H. pylori*-infected MoDCs or T cell stimulation with *H. pylori* in the absence of gGT restored T cell proliferation, but also IL-2 secretion (Figure 55), suggesting that inhibited T cell proliferation was related to reduced IL-2 production. Furthermore, these data confirmed the findings of Schmees et al. (63), who described the inhibitory effect of *H. pylori* gGT on T cells for the first time.

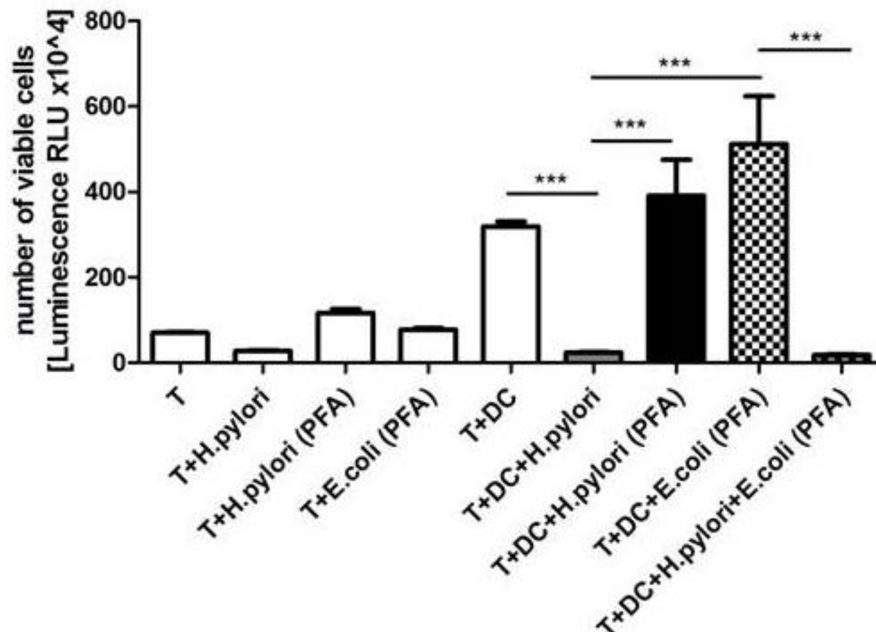

Figure 17: Proliferation of CD4$^+$T cells upon co-culture with *H. pylori*-infected MoDCs

MoDCs were incubated with live or PFA-fixed *H. pylori* G27, *E. coli* K12 or both simultaneously for 24 h and afterwards co-cultured with allogenicCD4$^+$ T cells for further 72 h. Cell viability was determined by measuring ATP with CellTiter-Glo. Data are presented as mean ± S.D. of 9 independent experiments.
(t-Test: *** $p \leq 0.001$)

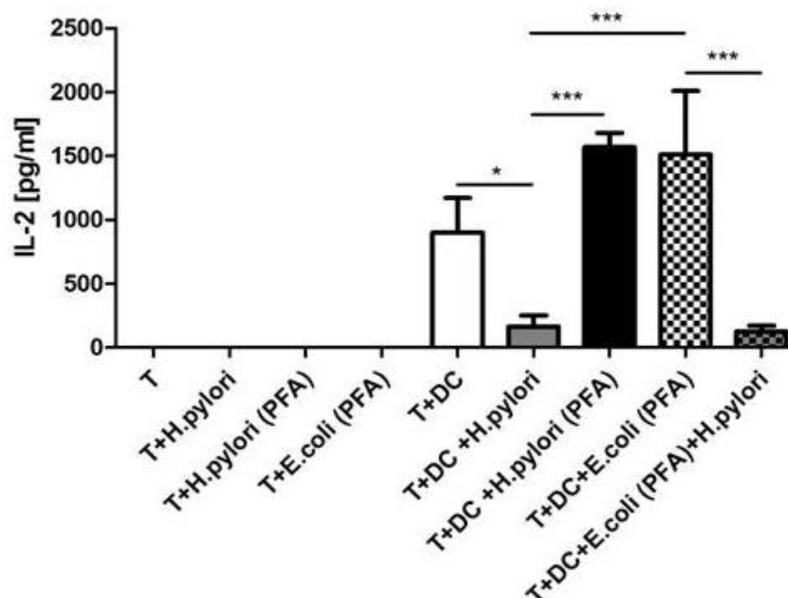

Figure 18: IL-2 release by T cells upon co-culture with *H. pylori*-infected MoDCs

MoDCs were incubated with live or PFA-fixed *H. pylori*G27, *E. coli* K12 or both simultaneously for 24 h and afterwards co-cultured with allogenic $CD4^+$ T cells for further 72 h. The secretion of IL-2 was quantified by ELISA. Data are presented as mean ± S.D. of 9 independent experiments. (t-Test: * $p \leq 0.05$; *** $p \leq 0.001$)

Additionally, also the release of IFN-γ and IL-17A by T cells was determined, since a mixed Th1/Th17 response during *H. pylori* infection has been described.

In response to co-cultivation with *H. pylori*-infected MoDCs, $CD4^+$T cells secreted only low amounts of IFN-γ (Figure 19A), while MoDCs primed with PFA-fixed *Helicobacter* caused increased IFN-γ secretion by T cells. Moreover, also simultaneous stimulation of MoDCs with PFA-fixed *E. coli* and live *H. pylori* induced lower IFN-γ response than *E. coli*-primed DCs, suggesting that lower numbers of T cells that have been observed upon stimulation with live *H. pylori* were responsible for the weak cytokine production of T cells. However, co-cultivation of T cells with MoDCs in the absence of gGT that restored T cell proliferation did not increase the release of IFN-γ as it was observed upon stimulation with PFA-fixed *H. pylori* (Figure 57), indicating that another mechanism was responsible for the weak IFN-γ secretion.

Co-incubation of uninfected allogenic MoDCs induced IL-17A production in T cells, while the levels were significantly lower when *H. pylori*-primed MoDCs were present (Figure 19B). Furthermore, stimulation of MoDCs with PFA-fixed *H. pylori* resulted in a similar IL-17A production than uninfected control, whereas stimulation of MoDCs with *H. pylori* and *E. coli* at the same time caused lower levels of IL-17A than *E. coli*-primed MoDCs alone. These data

indicate that differences in the cytokine response of T cell can be explained by different numbers of viable cells. Hence, killing of bacteria prior T cell co-culture will circumvent this limitation of analysis and offers a better insight in the effect of *Helicobacter* infection of DC for the subsequent T cell response. Moreover, the use of autologous T cells could reduce the basal levels of IL-17A induced by allogeneic stimulation, thereby facilitating the analysis of the effect of *H. pylori*-infected MoDCs on Th17 secretion.

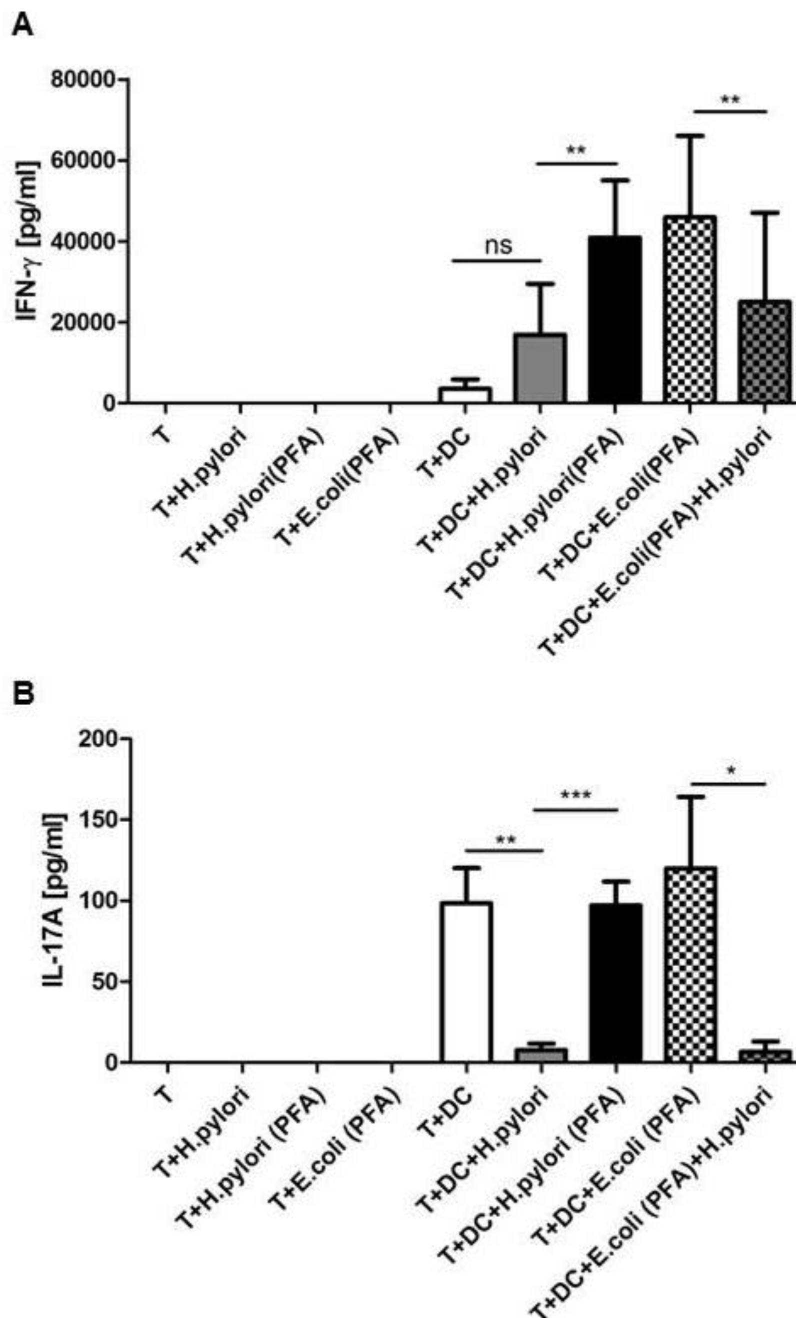

Figure 19: Secretion of IFN-γ and IL-17A by CD4$^+$ T cells in response to *H. pylori*-stimulated MoDCs

MoDCs were primed for 24 h with live or PFA-fixed *H. pylori* G27 or *E. coli* K12 and co-cultivated with allogenic CD4$^+$ T cells for 72 h. Secretion of IFN-γ (A) and IL-17A (B) was measured by ELISA. Data are presented as mean ± S.D. of 5 independent experiments.
(t-Test: * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$; ns: not significant)

The absence of MoDCs during stimulation of $CD4^+$ T cells with *H. pylori* or *E. coli* did not lead to the release of IFN-γ and IL-17A at all, suggesting that DCs are required for the induction of T cell-mediated cytokine production. Regarding IFN-γ the data further indicated that DC maturation status and DC cytokine response could strongly influence the subsequent Th1 response, since significant differences were observed upon DC infection with live and PFA-fixed *H. pylori*.

Notably, T helper cell responses can be suppressed by T_{regs} (202), mainly through IL-10 and TGF-β signalling (203). For that reason it was studied whether the weak Th1 /Th17 responses upon co-cultivation with *H. pylori*-infected MoDCs could not only be explained by lower cell numbers, but also by the presence of T_{regs}, which was monitored by the expression of the specific transcription factor Foxp3and the secretion of TGF-β and IL-10. Since IL-10 was also released by MoDCs upon *H. pylori* infection, this amount was compared with the levels found in the supernatant of co-cultivated cells.

Stimulation of MoDCs with *H. pylori* resulted in an increased Foxp3 expression by co-cultivated $CD4^+$ T cells (Figure 20). In contrast, co-cultivation with MoDCs primed with PFA-fixed *Helicobacter* or *E. coli* caused significantly lower numbers of Foxp3-expressing T cells.

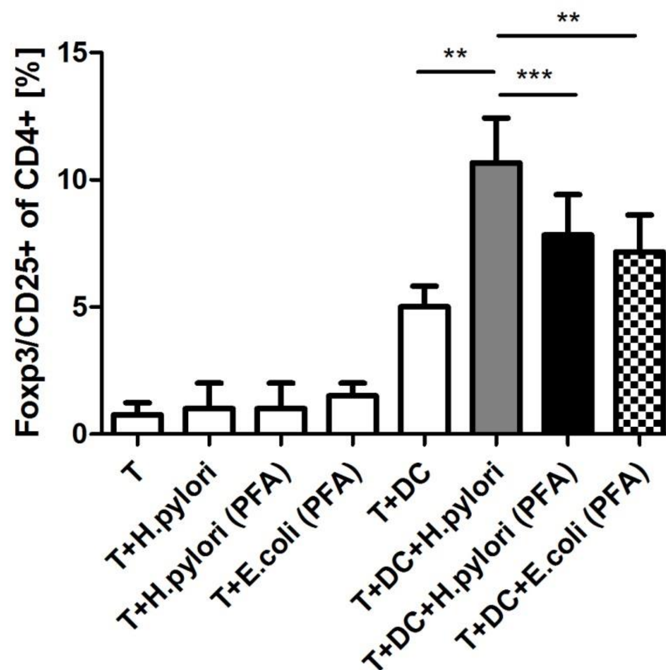

Figure 20: Expression of Foxp3 on T cells in response to *H. pylori*-primed MoDCs

Expression of Foxp3 on $CD4^+$ T cells upon 72 h of co-cultivation with MoDCs pre-stimulated with live or PFA-fixed *H. pylori* G27 or PFA-fixed *E. coli* K12 for 24 d*a*ta are presented as mean ± S.D. of 6 independent experiments. (t-Test: ** $p \leq 0.005$; *** $p \leq 0.001$)

In addition, increased levels of IL-10 were detected in the supernatant upon co-cultivation of T cells with *H. pylori*-primed MoDCs, while PFA-fixed *H. pylori* or *E. coli* did not induce additional IL-10 production (Figure 21). Importantly, in the supernatant of co-cultivated cells no TGF-β could be detected.

These data indicated that *H. pylori*-infected MoDCs are tolerogenic, since they induced a regulatory T cell response that was accompanied with increased IL-10 secretion. Nevertheless, further experiments are required to determine if *H. pylori*-induced T_{regs} were responsible for the weak T helper cell responses.

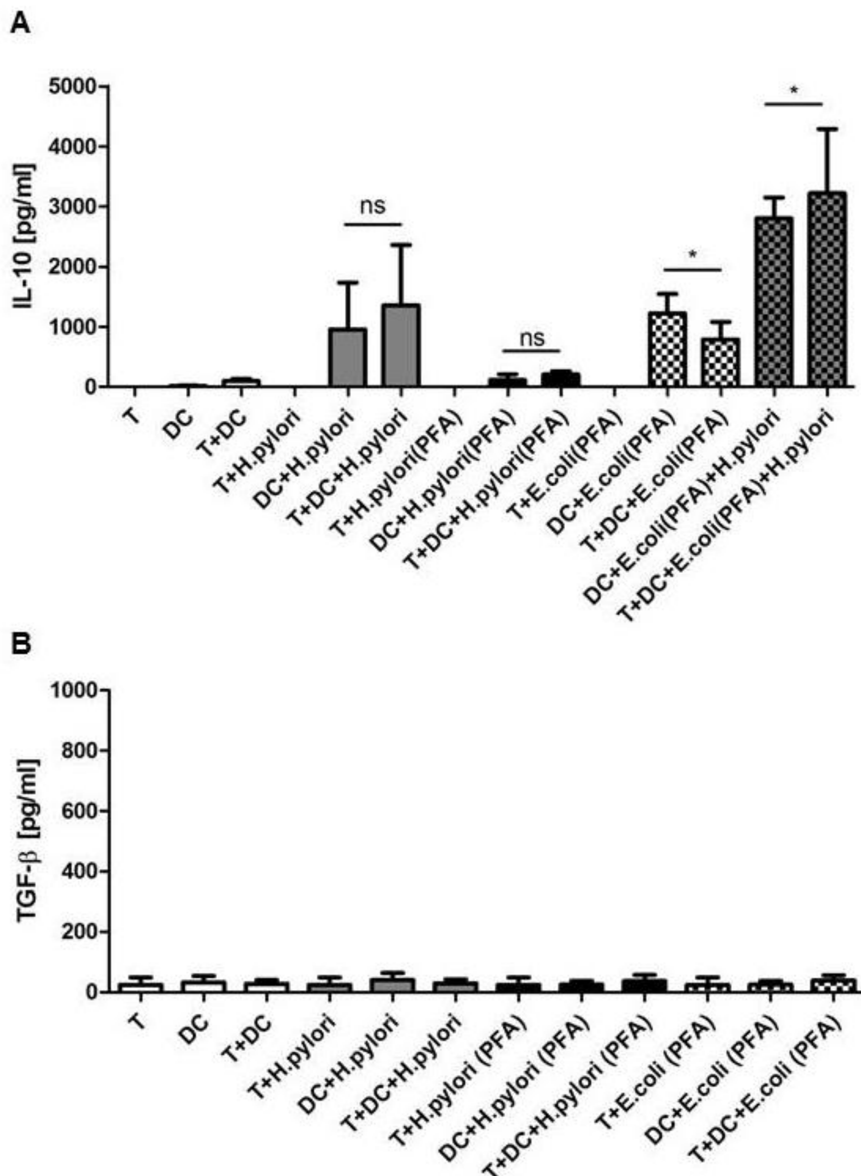

Figure 21: Release of IL-10during co-culture of T cells with *H. pylori*-primed MoDCs

Upon infection of immature MoDCs with live or PFA-fixed *H. pylori* G27 or PFA-fixed *E. coli* K12 for 24 h, allogenic $CD4^+$ T cells were co-cultivated for further 72 h. Secretion of (A) IL-10 and (B) TGF-β was quantified by ELISA. Data are presented as mean ± S.D. of 6 independent experiments.
(t-Test: * $p \leq 0.05$; ns.: not significant)

4.6. *H. pylori*-induced DC semi-maturation is CagA dependent

Live *H. pylori* caused only partial maturation of MoDCs, whereas PFA-fixed bacteria did not have an inhibitory effect. This led to the hypothesis that *H. pylori* virulence factors influenced DC maturation. In order to identify which factors could be involved, immature MoDCs were infected with *H. pylori* strains deficient for either of the virulence factors CagA, BabA, VacA, gGT or UreA/B that have been associated with bacterial colonisation and/or increased pathology.

Significantly increased levels of CD86/CD83 double-positive cells were detected when DCs were infected with CagA-deficient *H. pylori* G27 compared to the wild type strain, indicating that CagA plays an important role in the inhibition of DC maturation (Figure 22). Moreover, augmented DC maturation was also observed upon infection with BabA-deficient *H. pylori*, suggesting that impaired adhesion to the host cells could be responsible for reduced CagA translocation.

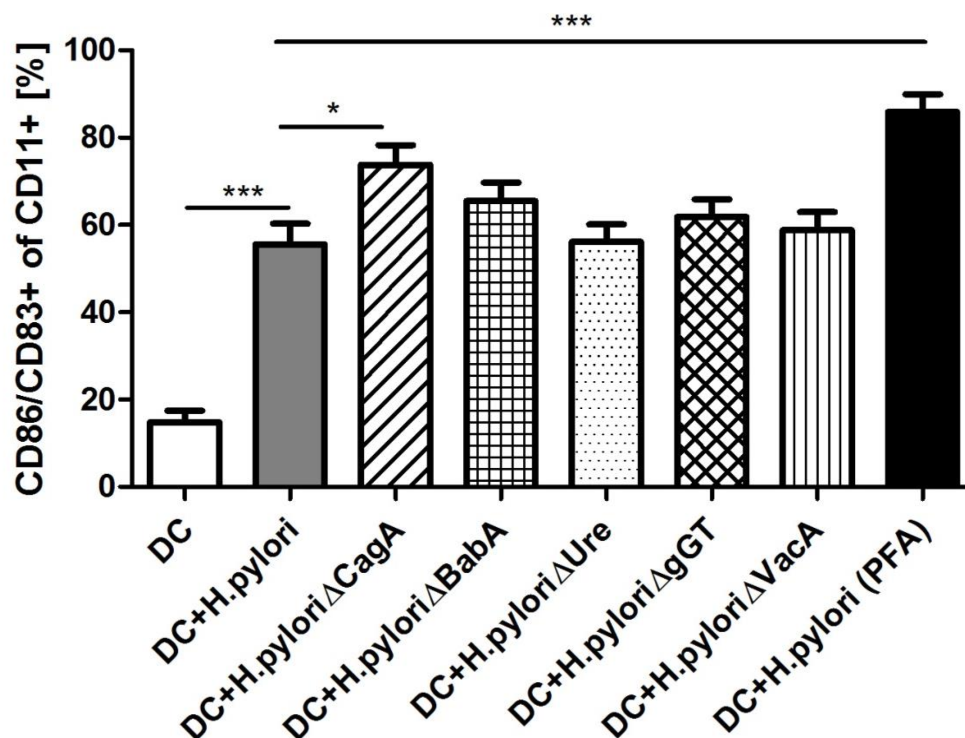

Figure 22: Maturation of MoDC in response to *H. pylori* deficient strains

The amount of CD86/CD83 double-positive MoDCs was determined by flow cytometry upon 24 h of infection with live or PFA-fixed *H. pylori* wild type strain G27 or isogenic mutants (CagA, BabA, UreA/B, gGT and VacA) at MOI 5. Data are presented as mean ± S.D. of 6 independent experiments.
(ANOVA: * $p \leq 0.05$; *** $p \leq 0.001$)

It is well documented that upon direct contact with epithelial cells an active type IV secretion system mediates the translocation of CagA, followed by its tyrosine-phosphorylation (204). Upon stimulation with *H. pylori,* phosphorylated CagA was also detected in MoDCs (Figure 23), whereas no tyrosine-phosphorylation was observed upon incubation with the isogeneic CagA mutant. In addition, also the BabA adhesion mutant showed lower CagA translocation, which could be explained by its impaired ability to bind to Lewis[b] antigens that are expressed on human MoDCs (Figure 25), probably causing a diminished adhesion to MoDCs and subsequently reduced CagA translocation.

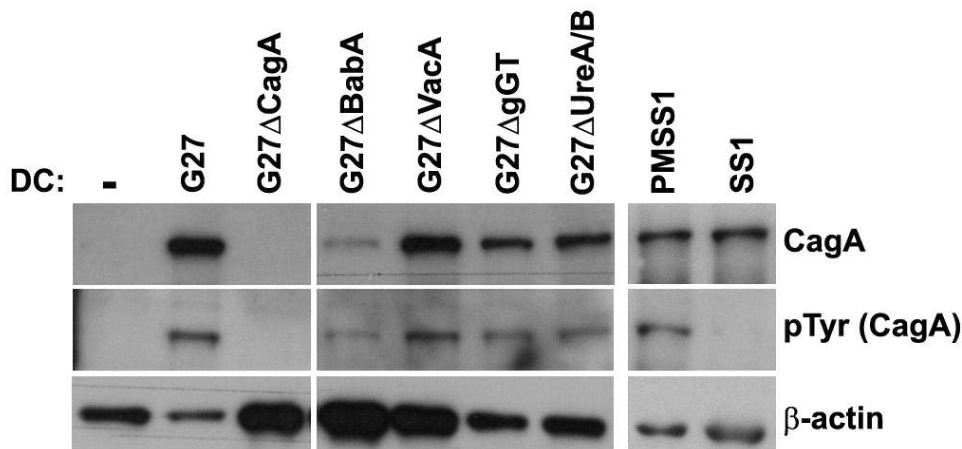

Figure 23: CagA translocation in MoDCs upon *H. pylori* infection

Immature MoDCs were infected with the *H. pylori* wild type strain G27 or isogenic mutants lacking CagA, VacA, BabA or gGT for 24 h at MOI 5. Presence of CagA and its tyrosine-phosphorylation upon translocation (p-Tyr) was analysed by Western Blot. β-actin was used as protein loading control. Validation of the success of the infection was carried out by determining the expression of maturation markers by flow cytometry. Western Blot image shows one representative experiment.

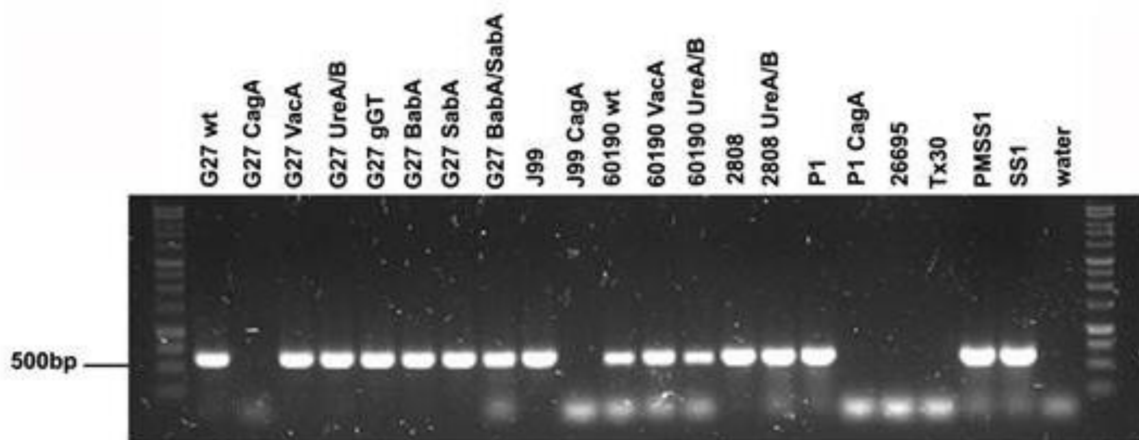

Figure 24: Detection of *cagA* in *H. pylori*

Genomic *H. pylori* DNA of different wild type and mutant strains was analysed for *cagA* by PCR. The predicted size of the amplified c*agA* fragment was 600 base pairs (bp).

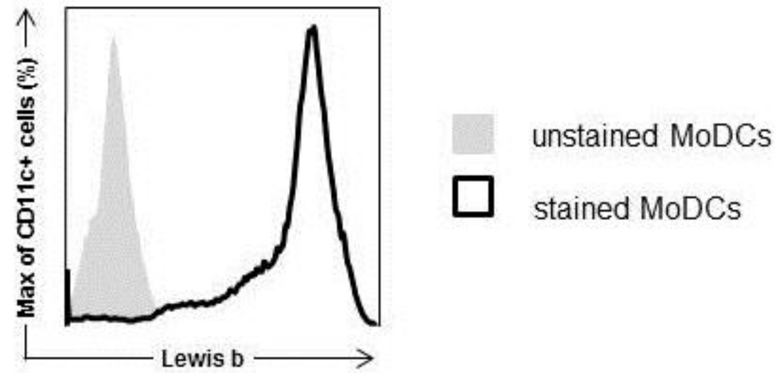

Figure 25: Expression of Lewis b antigens on human MoDCs

Surface-expression of Lewis b antigens on unstimulated MoDCs was determined by flow cytometry. Graph shows one representative experiment of n=2.

The involvement of CagA in *H. pylori*-induced DC semi-maturation was confirmed by infection experiments with the SS1 strain, which, although being CagA positive (Figure 24) lacks a functional type IV secretion system and cannot inject CagA into DCs (Figure 23). Incubation of MoDCs with the *H. pylori* SS1 strain induced higher amounts of CD86/CD83 double-positive cells compared to *H. pylori* PMSS1 strain (Figure 26).These data further suggested that DC maturation was influenced by the translocation of CagA into the host cell.

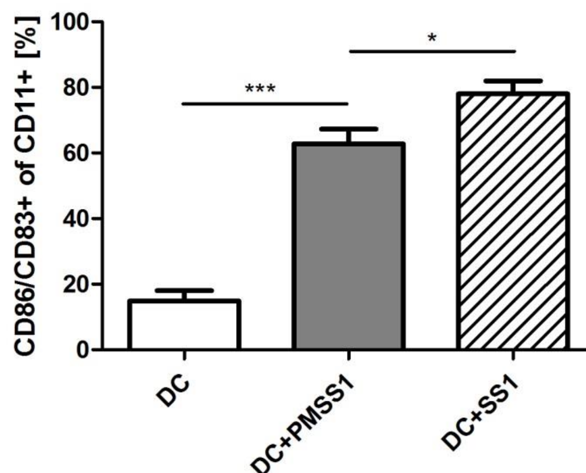

Figure 26: Maturation of MoDCs in response to *H. pylori* PMSS1 and SS1

Immature MoDCs were infected with *H. pylori* wild type strain PMSS1 or SS1. Maturation of MoDCs was analysed by the amount of CD86/CD83 double-positive cells determined by flow cytometry. Data are presented as mean ± S.D. of 4 independent experiments.(ANOVA: * $p \leq 0.05$; *** $p \leq 0.001$)

4.7. CagA translocation in MoDCs causes an anti-inflammatory cytokine response

Since CagA was shown to play a major role in DC semi-maturation, the influence of CagA translocation on the associated cytokine response upon *H. pylori* infection was also investigated. Following stimulation of MoDCs, supernatants were harvested and analysed for pro- and anti-inflammatory cytokines.

MoDCs secreted significantly higher amounts of the pro-inflammatory cytokine IL-12p70 upon infection with CagA-deficient *H. pylori* G27 compared to the wild type, while the levels of IL-10 were markedly lower, indicating that the anti-inflammatory cytokine response of MoDCs upon *H. pylori* infection was influenced by CagA (Figure 27).In contrast, IL-6secretion of MoDCs was hardly altered by the infection with CagA-deficient *Helicobacter*. However, high levels of IL-6 were observed upon stimulation with PFA-fixed *H. pylori*, suggesting that other factors apart from CagA were involved in the release of IL-6.

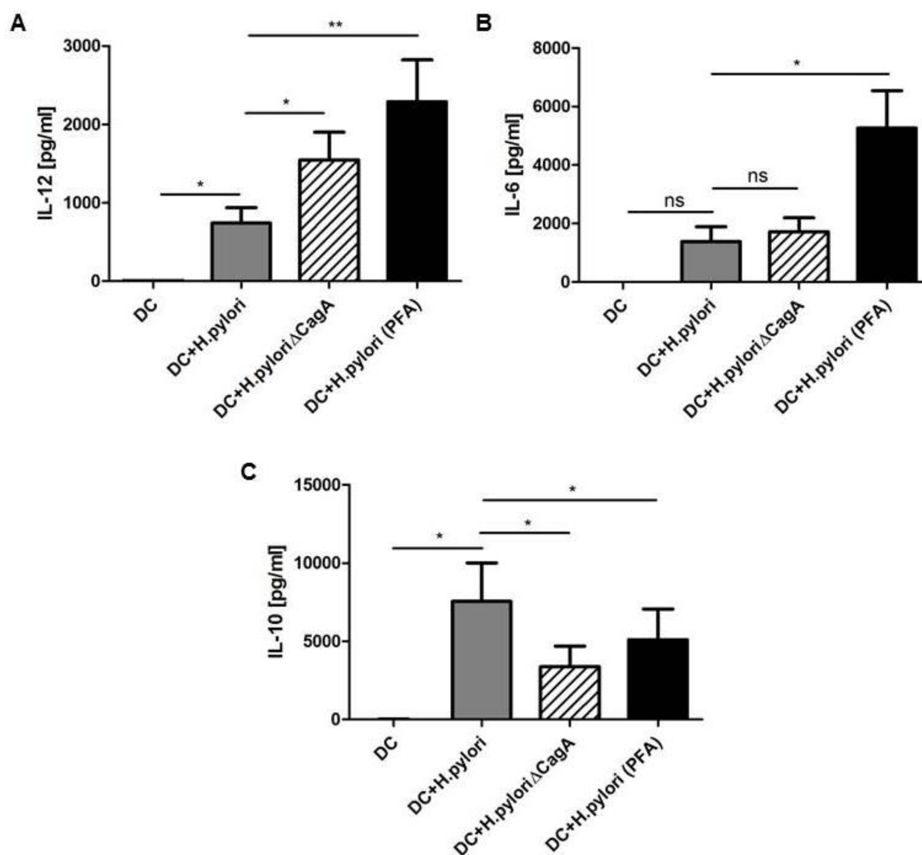

Figure 27: Cytokine response of MoDCs upon infection with CagA-deficient *H. pylori*

Upon 24 h stimulation of immature MoDCs with live or PFA-fixed *H. pylori* G27 or its isogenic CagA mutant at MOI 5, the secretion of (A) IL-12p70, (B) IL-6 and (C) IL-10 was quantified by ELISA. Data are presented as mean ± S.D. of 8 independent experiments. (t-Test: * $p \leq 0.05$; ** $p \leq 0.005$; ns: not significant)

Similar results were obtained when MoDCs were co-incubated with *H. pylori* SS1 that is deficient in CagA translocation. Also here, increased IL-12p70 release and decreased IL-10 production was observed, whereas IL-6 secretion did not change significantly (Figure 28).

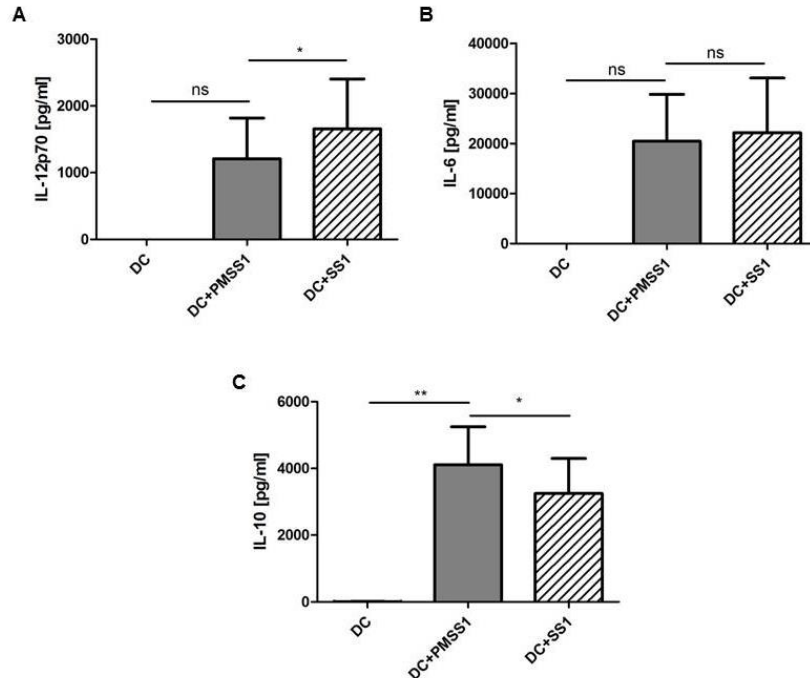

Figure 28: Cytokine secretion of MoDCs in response to *H. pylori* PMSS1 and SS1

Immature MoDCs were incubated with *H. pylori*PM-SS1 or SS1 for 24 h at MOI 5. The secretion of (A) IL-12p70, (B) IL-6 and (C) IL-10 was quantified by ELISA. Data are presented as mean ± S.D. of 4 independent experiments. (t-Test: * $p \leq 0.05$; ** $p \leq 0.005$; ns: not significant)

4.8. CagA translocation in MoDCs causesaregulatory T cell response

Previous experiments showed the involvement of *H. pylori* CagA in DC semi-maturation that was accompanied by an anti-inflammatory cytokine response. In order to investigate the impact of CagA translocation in *H. pylori*-primed MoDCs on the subsequent T cell response, immature MoDCs were stimulated with CagA-deficient *H. pylori* and thereafter-cultured with allogenic $CD4^+$ T cells. Subsequently, T cell proliferation and the release of IL-2 were determined after 72 h of co-incubation.

Live *H. pylori* was shown to actively inhibit T cell proliferation through the secretion of gGT (Figure 55). The absence of CagA during *H. pylori* stimulation of MoDCs did not restore T cell proliferation or production of IL-2 (Figure 29), indicating that translocation of CagA did not affect T cell proliferation. Since similar cell numbers were obtained during DC/T co-culture in the presence of wild type and CagA-deficient *H. pylori*, cytokine responses of T cells were comparable. For completeness, the following graphs contain also PFA-fixed *H. pylori*, although the amount of cells was not the same.

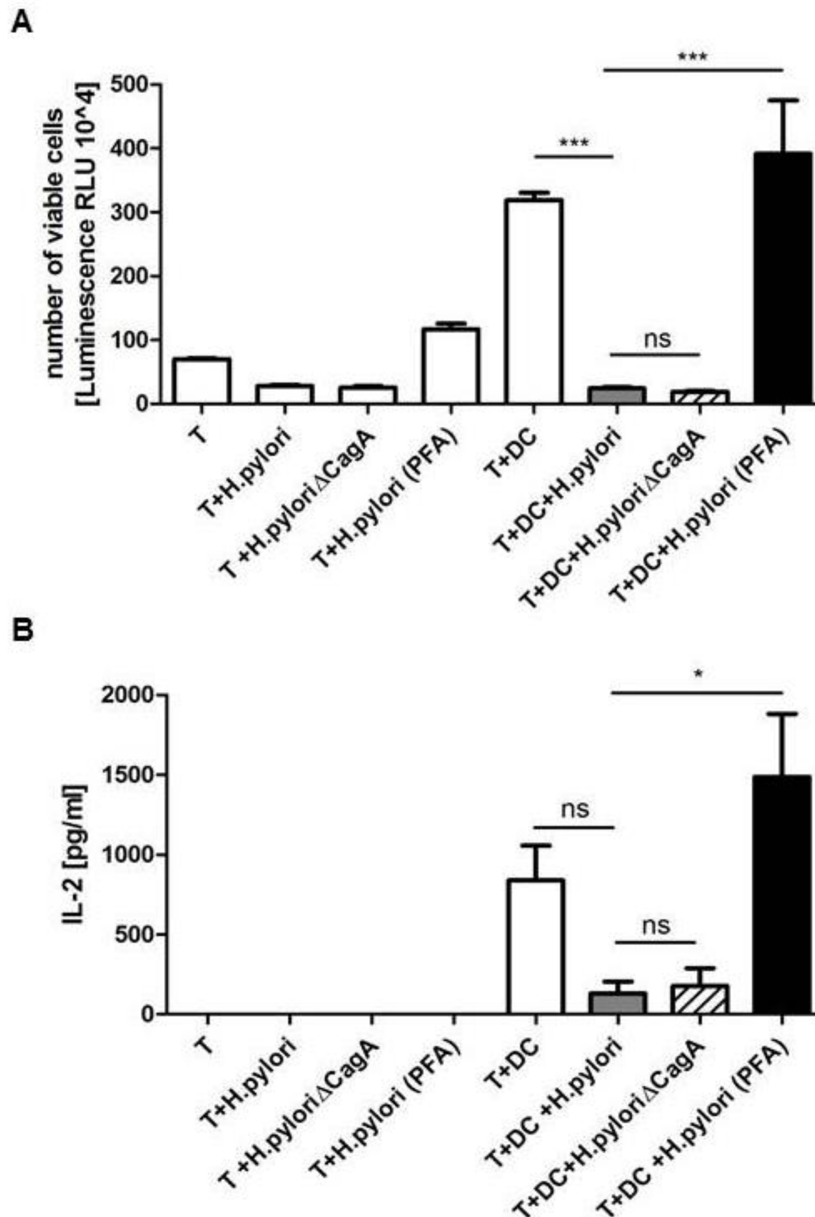

Figure 29: Involvement of CagA on T cell proliferation and IL-2 secretion upon co-culture with *H. pylori*-primed MoDCs

After incubation with live or PFA-fixed *H. pylori* G27 or its isogenic CagA deletion mutant at MOI 5 for 24 h, MoDCs were co-cultivated with allogenic $CD4^+$ T cells for 72 h. (A) Cell viability was determined by measuring ATP with CellTiter-Glo. (B) The release of IL-2 was determined by ELISA. Data are presented as mean ± S.D. of 5 independent experiments. (t-Test: *** $p \leq 0.001$; ns: not significant)

Since *H. pylori* was shown to induce tolerogenic DCs, the involvement of CagA on the expansion of T_{regs} was evaluated. As demonstrated in Figure 30, MoDCs primed with CagA-deficient *H. pylori* induced less Foxp3 expression in $CD4^+$ T cells compared to the wild type strain, reflecting a decrease in the amount of T_{regs}. Moreover, absence of CagA during co-culture of *H. pylori*-infected MoDCs and T cells resulted in lower levels of anti-inflammatory IL-10, suggesting the CagA translocation in MoDCs was responsible for the induction of tolerogenic DCs.

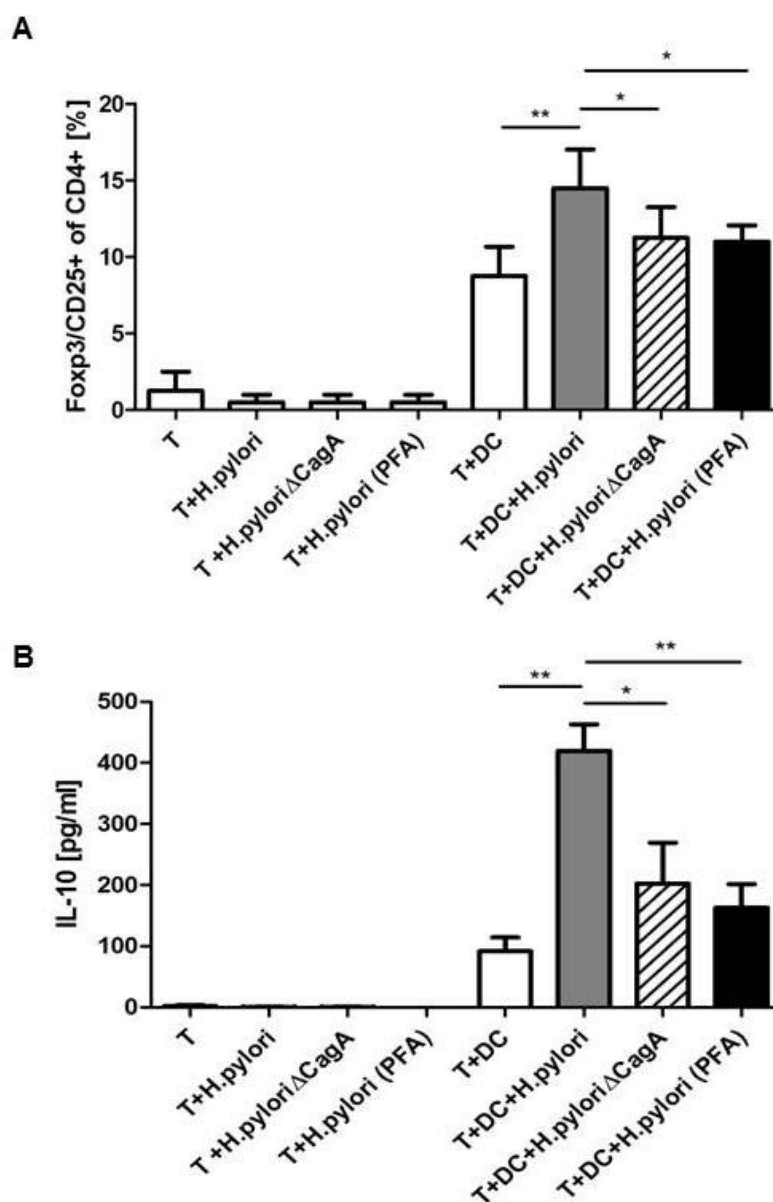

Figure 30: Role of CagA on Foxp3 expression and IL-10 release by T cells upon co-culture with *H. pylori*-stimulated MoDCs

Analysis of Foxp3 expression (A) and IL-10 production (B) by $CD4^+$T cells in response to 72 h co-cultivation with MoDCs that were pre-stimulated for 24 h with *H. pylori* G27 or its isogenic CagA deletion mutant at MOI 5. Foxp3 expression was determined by flow cytometry. IL-10 production was measured by ELISA. Data are presented as mean ± S.D. of 6 independent experiments (t-Test: * $p \leq 0.05$; ** $p \leq 0.005$).

As a decrease in the amount of T_{regs} and IL-10 was assumed to enhance T helper cell responses, the release of IFN-γ and IL-17A was consequently analysed.

The absence of CagA during *H. pylori* infection of MoDCs resulted in a significantly increased IFN-γ production by co-cultivated $CD4^+$ T cells (Figure 31). However, the amount of IL-17A did not change. These results suggested that CagA-translocation conferred MoDCs a tolerogenic phenotype that led to the expansion of T_{regs} on the one hand, and to the suppression of T cell-mediated IFN-γ secretion on the other. The reduced IL-17A production by T cells did not depend on CagA-translocation in MoDCs, but on other factors, since the release of IL-17A could be restored by infecting MoDCs with PFA-fixed *Helicobacter*.

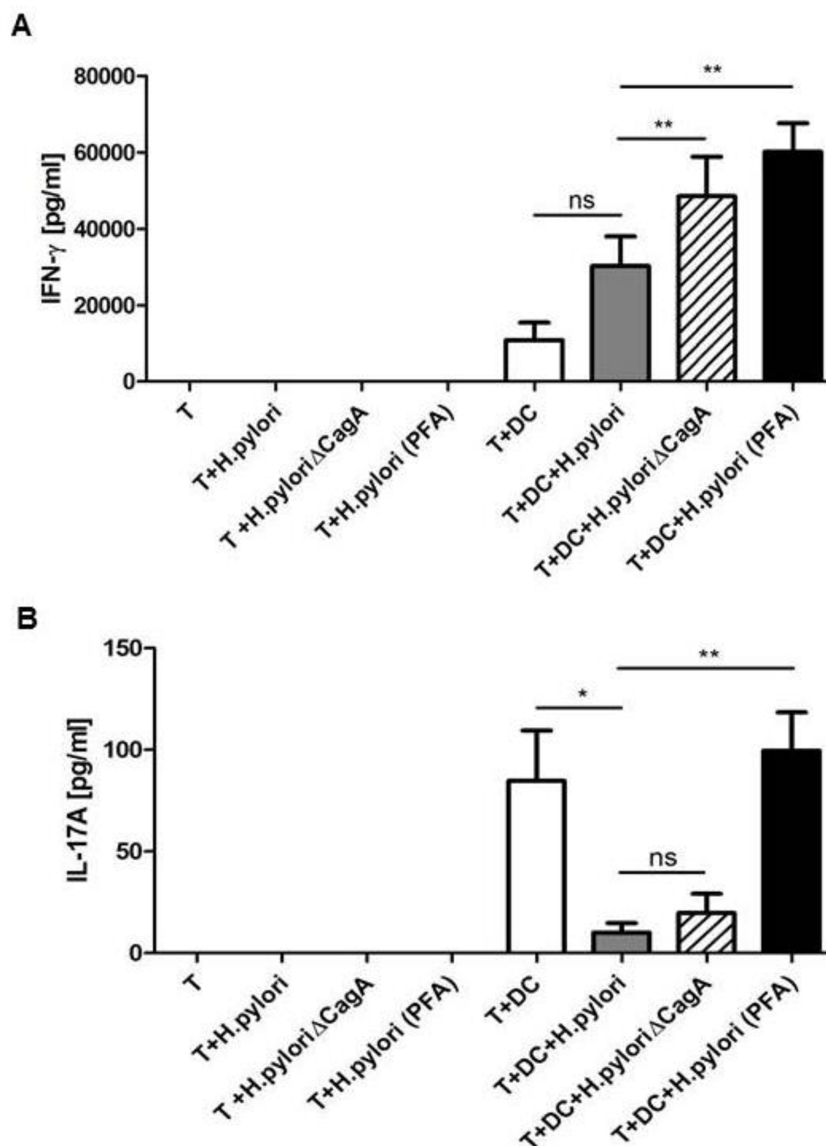

Figure 31: Effect of CagA translocation on the release of IFN-γ and IL-17A by T cells in response to co-culture with *H. pylori*-infected MoDCs

Production of IFN-γ (A) and IL-17A (B) by $CD4^+$ T cells in response to co-cultivated MoDCs pre-stimulated with CagA-deficient *H. pylori* at MOI 5. Cytokines were measured by ELISA. Data are presented as mean ± S.D. of 3 independent experiments. (t-Test: * $p \leq 0.05$, ** $p \leq 0.005$; ns: not significant)

4.9. *H. pylori* activates STAT3 in MoDCs

The maturation status as well as the cytokine response of MoDCs upon *H. pylori* infection significantly affected the subsequent T cell response. The molecular mechanisms that could be responsible of the tolerogenic phenotype of *H. pylori*-infected MoDCs remained unknown. In 2010, Melillo et al. described STAT3 depletion in DCs to be involved in impaired mucosal tolerance in mice (205). In the same year, Manicassamy et al. published that ß-catenin in intestinal DCs is required for T_{reg} induction (206). Recently, the inactivation of NFκB has been described by Zhu et al. to induce DC tolerance (207). In order to verify whether one of these mechanism played a role in the induction of tolerance in *H. pylori*-primed MoDCs, lysates of stimulated DCs were analysed by Western Blot for activated STAT3 (p-STAT3), ß-catenin or the inhibitor of NFκB (p-IKBα).

After challenging MoDCs with the *H. pylori* wild type strains G27 and PMSS1 no changes in the levels of ß-catenin and p-IKBα were observed, indicating that neither ß-catenin nor the inhibition of NFκB were implicated in the induction of DC semi-maturation during *H. pylori* stimulation (Figure 32). On the other hand, infection with G27 and PMSS1 resulted in the phosphorylation of STAT3, suggesting an involvement of STAT3 in *H. pylori*-induced DC semi-maturation.

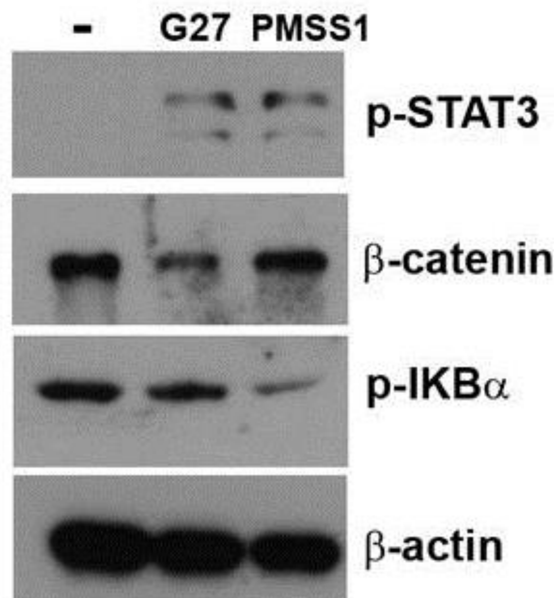

Figure 32: *H. pylori*-induced levels of p-STAT3, β-Catenin and p-IκBα in MoDCs

Immature MoDCs were infected with *H. pylori* G27 or PMSS1 at MOI 5 for 24h. Cells were lysed and analysed for p-STAT3, β-Catenin and p-IKBα expression by Western Blot. β-actin was used as protein loading control. Western Blot image shows one representative experiment of n=3.

Since STAT3 was demonstrated to be activated by *H. pylori* G27 and PMSS1, also other wild type strains were tested to see whether they could induce STAT3 phosphorylation in MoDCs. Therefore, the *H. pylori* wild type strains P1, 2808, 60190 and J99 were used for infection of immature MoDCs.

Upon stimulation of immature MoDCs with different *H. pylori* wild type strains, p-STAT3 was always detected, indicating that activation of STAT3 was induced by all wild type strains (Figure 33).

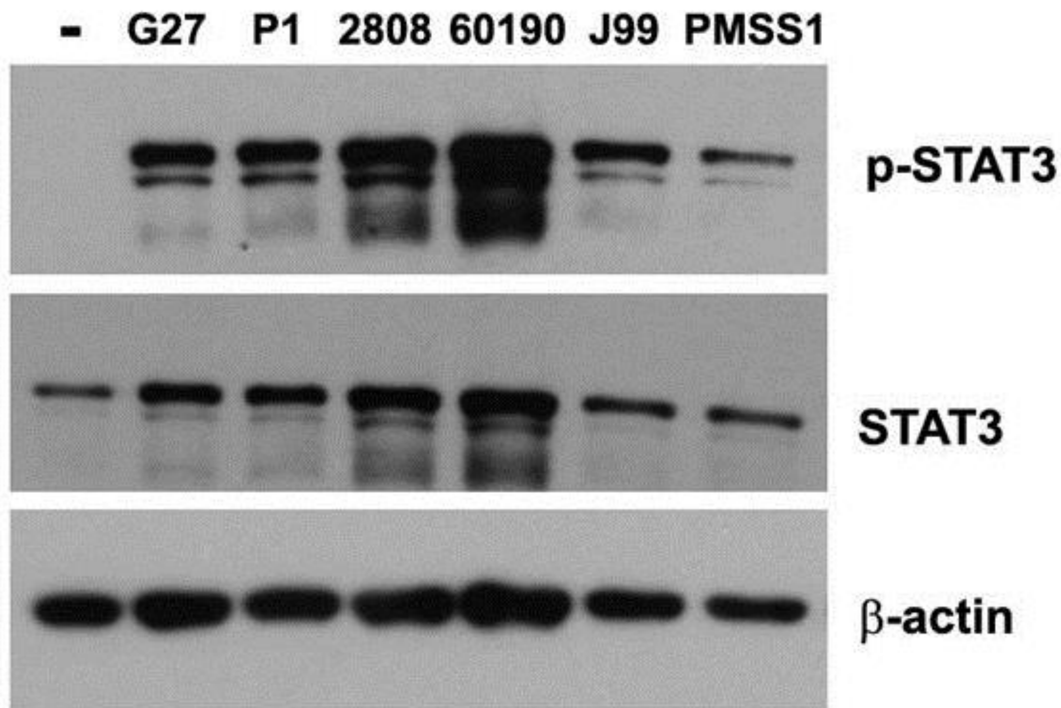

Figure 33: Activation of STAT3 in MoDCs in response to different *H. pylori* strains

Upon challenge with the *H. pylori* wild type strains G27, P1, 2808, 60190, J99 and PMSS1 at MOI 5 for 24h, MoDCs were lysed and the expression of STAT3 and p-STAT3 was analysed by Western Blot. β-actin was used as protein loading control. One representative experiment is shown of n=2.

Time course experiments revealed that STAT3 became activated after a few hours upon *H. pylori* infection. Already after 2 hours of DC stimulation with *H. pylori* G27 phosphorylated STAT3 was observed (Figure 34) and maintained, since after 96 hour infection high levels of p-STAT3 were detected (Figure 35).

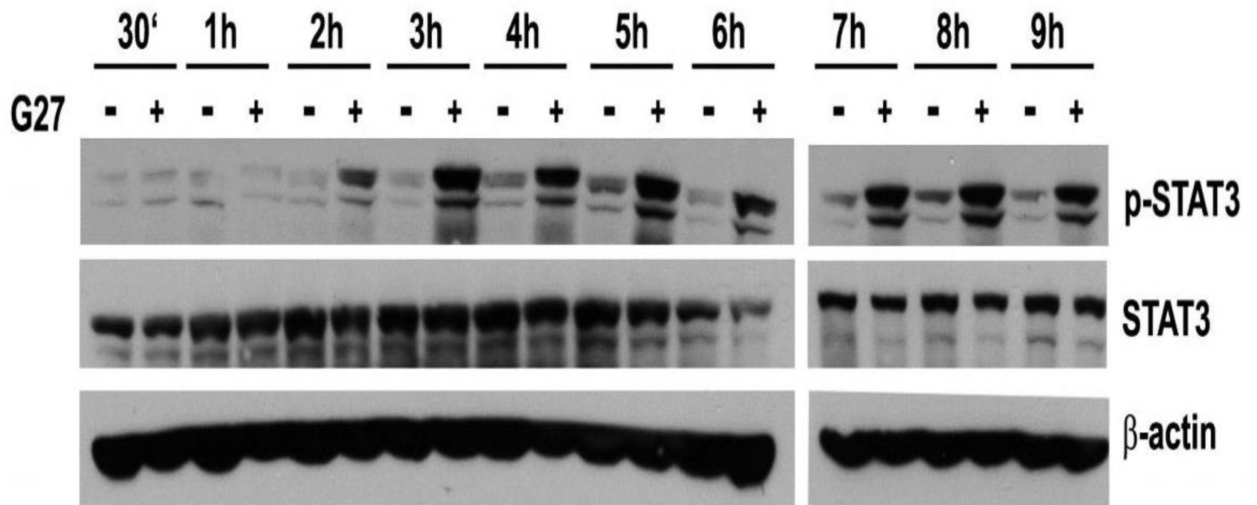

Figure 34:STAT3 phosphorylation in MoDCs upon 30 min to 9 h infection with *H. pylori*

Immature MoDCs were infected with *H. pylori* G27 at MOI 5 at different time points (from 30min to 9h). Cells were lysed and the expression of STAT3 and p-STAT3 was analysed by Western Blot. β-actin was used as protein loading control. Western Blot image shows one representative experiment of n=3.

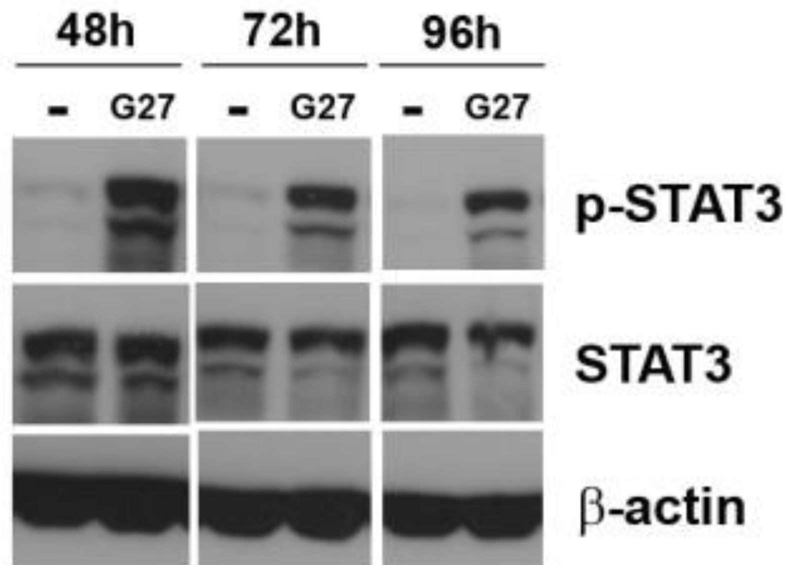

Figure 35: *H. pylori*-induced phosphorylation of STAT3 in MoDCs after 48 h to 96 h

Immature MoDCs were stimulated with *H. pylori* G27 at MOI 5 for 48 h, 72 h and 96h. Upon cell lysis, protein levels of STAT3 and p-STAT3 were determined by Western Blot. β-actin was used as protein loading control. Western Blot image shows one representative experiment of n=2.

Upon *H. pylori* infection MoDCs developed a semi-mature phenotype and STAT3 became activated. Since CagA translocation was shown to be responsible for DC semi-maturation, the influence of CagA on STAT3 activation was investigated.

H. pylori CagA and BabA mutants that were characterised by no or reduced CagA translocation, respectively, induced markedly lower levels of activated STAT3 than wild type bacteria or mutants deficient in gGT, VacA and UreA/B, indicating an involvement of CagA in the induction of STAT3 phosphorylation (Figure 36). The involvement of CagA in STAT3 activation was further confirmed by the stimulation of MoDCs with the *H. pylori* strain SS1, which is unable to translocate CagA. Upon infection of MoDCs with *H. pylori* SS1 lower levels of p-STAT3 were detected (Figure 37), compared to the challenge with PMSS1.

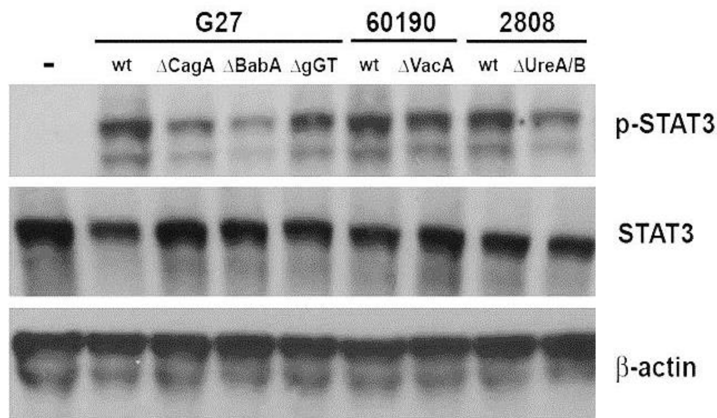

Figure 36: STAT3 phosphorylation in MoDCs in response to isogenic *H. pylori* mutants

Immature MoDCs were infected with the *H. pylori* wild type strains G27 or its isogenic CagA, BabA or gGT deletion mutants, 60190 or its isogenic VacA deletion mutant, 2808 or its isogenic UreA/B deletion mutant for 24 h at MOI 5. Cells were lysed and expression of STAT3 and p-STAT3 was analysed by Western Blot. β-actin was used as protein loading control. Western Blot image shows one representative experiment of n=4.

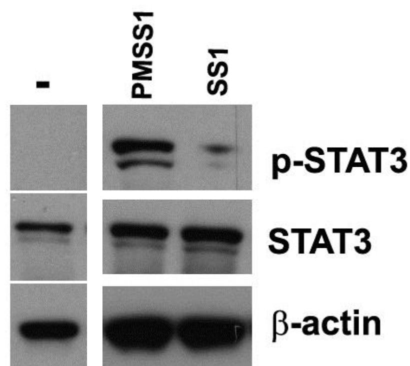

Figure 37: CagA-dependent STAT3 phosphorylation of MoDCs in response *H. pylori*

Following 24 h incubation with *H. pylori*PMSS1 or SS1, MoDCs were lysed and analysed for expression of STAT3 and p-STAT3 by Western Blot. β-actin was used as protein loading control. Western Blot image shows one representative experiment of n=4.

Moreover, it was analysed if the lower p-STAT3 levels that were observed upon *H. pylori* infection of MoDCs in the absence of CagA could be a consequence of delayed activation. Hence, phosphorylation of STAT3 induced by wild type bacteria and Cag-deficient *H. pylori* was compared at different time points (Figure 38).

STAT3 was activated after two to three hours by wild type and mutant bacteria, but in the absence of CagA always lower levels of p-STAT3 were observed, suggesting that CagA might be directly inducing STAT3 phosphorylation, as it has been previously described in gastric cancer epithelial cells (208). On the other hand, the cytokines IL-6 and IL-10 were also shown to activate STAT3 in DCs, leading to the induction of tolerance (209) (210).

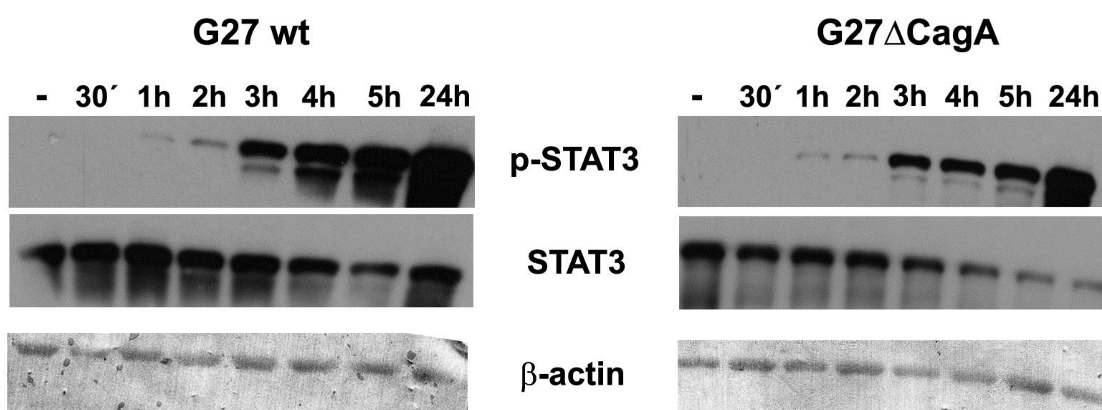

Figure 38: Time course of STAT3 phosphorylation in MoDCs upon infection with wild type and CagA-deficient *H. pylori*

Immature MoDCs were infected with the *H. pylori* wild type strain G27 or the isogenic CagA deletion mutant for different time points (30min to 24h). Cells were lysed and STAT3 and p-STAT3 levels were analysed by Western Blot. Detection of β-actin was used as protein loading control. Western Blot image shows one representative experiment of n=3.

To determine if STAT3 activation was due to secreted cytokines, immature MoDCs were incubated with supernatants obtained from control and *H. pylori*-infected MoDCs. STAT3 phosphorylation was only detected in cells incubated with supernatants from infected MoDCs (Figure 39), indicating that cytokines released upon infection might be responsible of STAT3 activation.

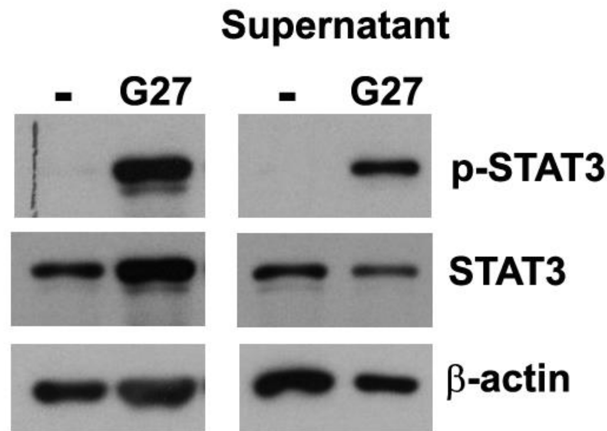

Figure 39: Activation of STAT3 upon stimulation with supernatants from *H. pylori*-infected MoDCs

Supernatants of *H. pylori*-infected or uninfected MoDCs were harvested and used for stimulation of immature MoDCs. Cell lysates were analysed for STAT3 and p-STAT3 expression. β-actin was used as protein loading control. Western Blot image shows one representative experiment of n=4.

To elucidate the specific effect of IL-6 and IL-10 on STAT3 activation upon *H. pylori* infection, immature MoDCs were incubated with anti-IL-6 and/or anti-IL-10 neutralising antibodies prior to infection. First, the efficiency of neutralisation was checked. Therefore, MoDCs were incubated with neutralising antibodies followed by stimulation with recombinant IL-6 or IL-10. As shown in Figure, pSTAT3 induced by recombinant proteins was mainly blocked in the presence of anti-IL-6 or anti-IL-10, indicating that cytokines were mostly neutralised.

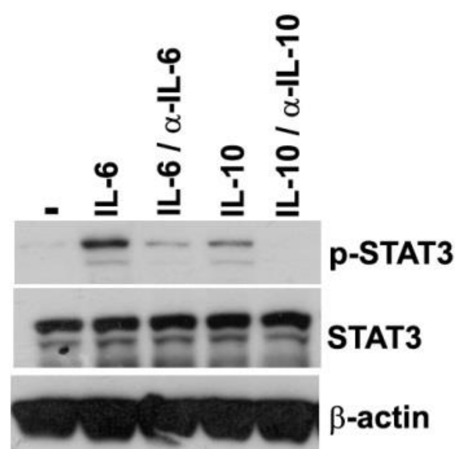

Figure 40: STAT3 activation in MoDCs after α-IL-6/α-IL-10 neutralisation

Neutralisation efficiency was controlled by stimulating MoDCs with recombinant IL-6 and IL-10 upon 1 h of pre-incubation with α-IL-6 or α-IL-10 antibodies. Cell lysates were analysed after 24 h for STAT3 phosphorylation. β-actin was used as protein loading control. Western Blot image shows one representative experiment of n=3.

Next, IL-6 and IL-10 were neutralised prior *H. pylori* infection. Reduced levels of p-STAT3 were observed when IL-10 and IL-6 were blocked (Figure 41). However, IL-10 neutralisation had a stronger effect, indicating that mainly IL-10 was responsible of activating STAT3 in DCs after *H. pylori* infection. This was also supported by the observation that CagA-deficient strains induced lower levels of IL-10, which correlated with lower levels of p-STAT3.

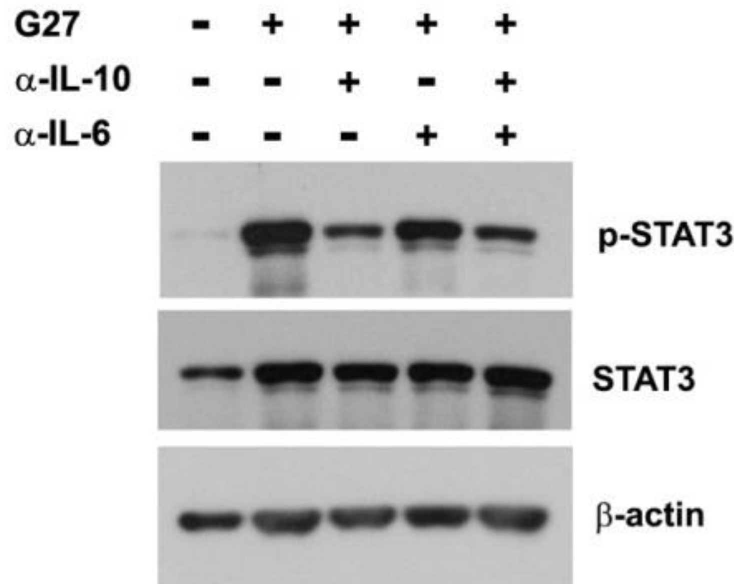

Figure 41: *H. pylori*-induced STAT3 activation in MoDCs in the presence of α-IL-6/α-IL-10 neutralising antibodies

Immature MoDCs were incubated with α-IL-6 and/or α-IL-10 neutralising antibodies prior to infection with *H. pylori* G27 wild type strain. Cells were lysed after 24h and analysed for STAT3 phosphorylation by Western Blot. β-actin was used as protein loading control. Western Blot image shows one representative experiment of n=4.

4.10. *H. pylori* induces tolerogenic MoDCs through STAT3 activation

To investigate whether activation of STAT3 in MoDCs upon *H. pylori* infection was responsible for the induction of a tolerogenic T cell response, STAT3 phosphorylation was inhibited, either by pre-stimulation with anti-IL-10 or anti-IL-6/ IL-10 neutralising antibodies or by the use of the specific STAT3 inhibitor Stattic that selectively inhibits activation, dimerisation and nuclear translocation of STAT3 (211).

The blockage of STAT3 activation by neutralising antibodies resulted in significantly higher levels of the co-stimulatory molecule CD86 as well as the activation marker CD83, indicating that STAT3 activation strongly inhibited the maturation of MoDCs in response to *H. pylori* (Figure 42). However, Stattic did not have any effect on DC maturation.

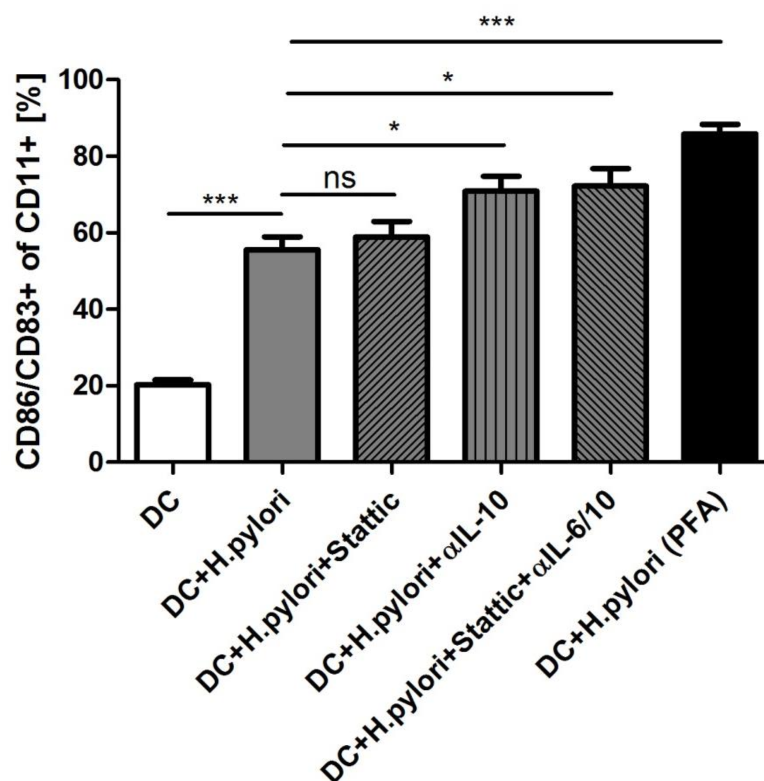

Figure 42: Maturation of MoDCs upon *H. pylori* infection in the presence of α-IL-6/α-IL-10 neutralising antibodies and Stattic

Immature MoDC were pre-incubation of with α-IL-10 or α-IL-6/α-IL-10 neutralising antibodies and/or 0.5 µM Stattic for 1 h. After 24h of infection with *H. pylori* G27, maturation of MoDCs was determined by the expression of CD86 and CD83 by flow cytometry. Data are presented as mean ± S.D. of 7 independent experiments. (ANOVA: * $p \leq 0.05$;*** $p \leq 0.001$; ns: not significant)

Since Stattic alone did not show a prominent effect on restoring DC maturation upon *H. pylori* infection, it was investigated whether the amount of Stattic was sufficient to completely block STAT3 phosphorylation. Although lower levels of STAT3 were detected after incubating MoDCs with Stattic, complete inhibition of STAT3 phosphorylation was only achieved when cells were simultaneously incubated with anti-IL-6/-IL-10 antibodies and Stattic (Figure 43). Interestingly, MoDCs of some donors secreted only very low amounts of IL-6 and IL-10 in response to *H. pylori*. Pre-incubation of their MoDCs with Stattic prior infection was sufficient to restore DC maturation. These findings indicated that the amount of Stattic was not always sufficient to overcome the effect of the high cytokine levels released by MoDCs of the majority of the donors. Importantly, increasing amounts of Stattic were shown to induce cell death (Figure 44), limiting the amount of inhibitor that can be used.

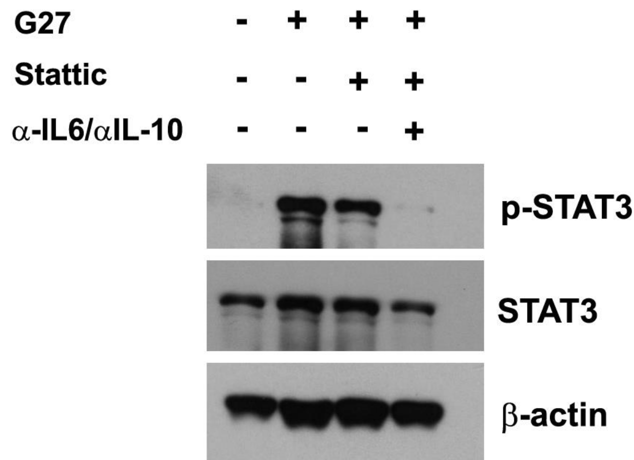

Figure 43: Activation of STAT3 in MoDCs upon *H. pylori* infection in the presence of α-IL-6/ α-IL-10 neutralising antibodies and Stattic

Prior to infection with *H. pylori* G27 at MOI 5, immature MoDCs were pre-incubated with α-IL-6/α-IL-10 neutralising antibodies and/or 0.5 µM Stattic for 1 h. Cells were lysed after 24 h and analysed for STAT3 phosphorylation by Western Blot. Western Blot image shows one representative experiment of n=3.

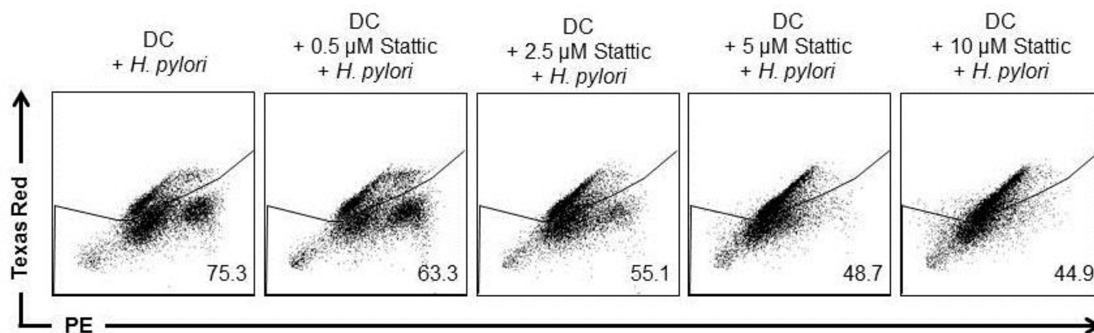

Figure 44: Effect of Stattic on vitality of MoDCs during *H. pylori* challenge

Prior to infection with *H. pylori* G27 at MOI 5, immature MoDCs were pre-incubated with different amounts of Stattic (0.5 µM – 10 µM) for 1 h. Cell vitality after 24 h was analysed by EMA staining (PE/ Texas Red) using flow cytometry. Picture shows one representative experiment.

Moreover, inhibition of STAT3 activation was accompanied by a different cytokine secretion of MoDCs in response to *H. pylori* (Figure 45). While the release of IL-6 was hardly affected, the levels of IL-12p70 were significantly increased and the amount of IL-10 was markedly lower, indicating that IL-10 suppressed IL-12p70 secretion, as described by Koch and colleagues (212). Furthermore, these findings suggested that *H. pylori*-induced DC semi-maturation was predominantly induced by IL-10-mediated STAT3 activation, which blocked the expression of co-stimulatory molecules and activation marker on the one hand, and repressed the secretion of IL-12p70 on the other hand.

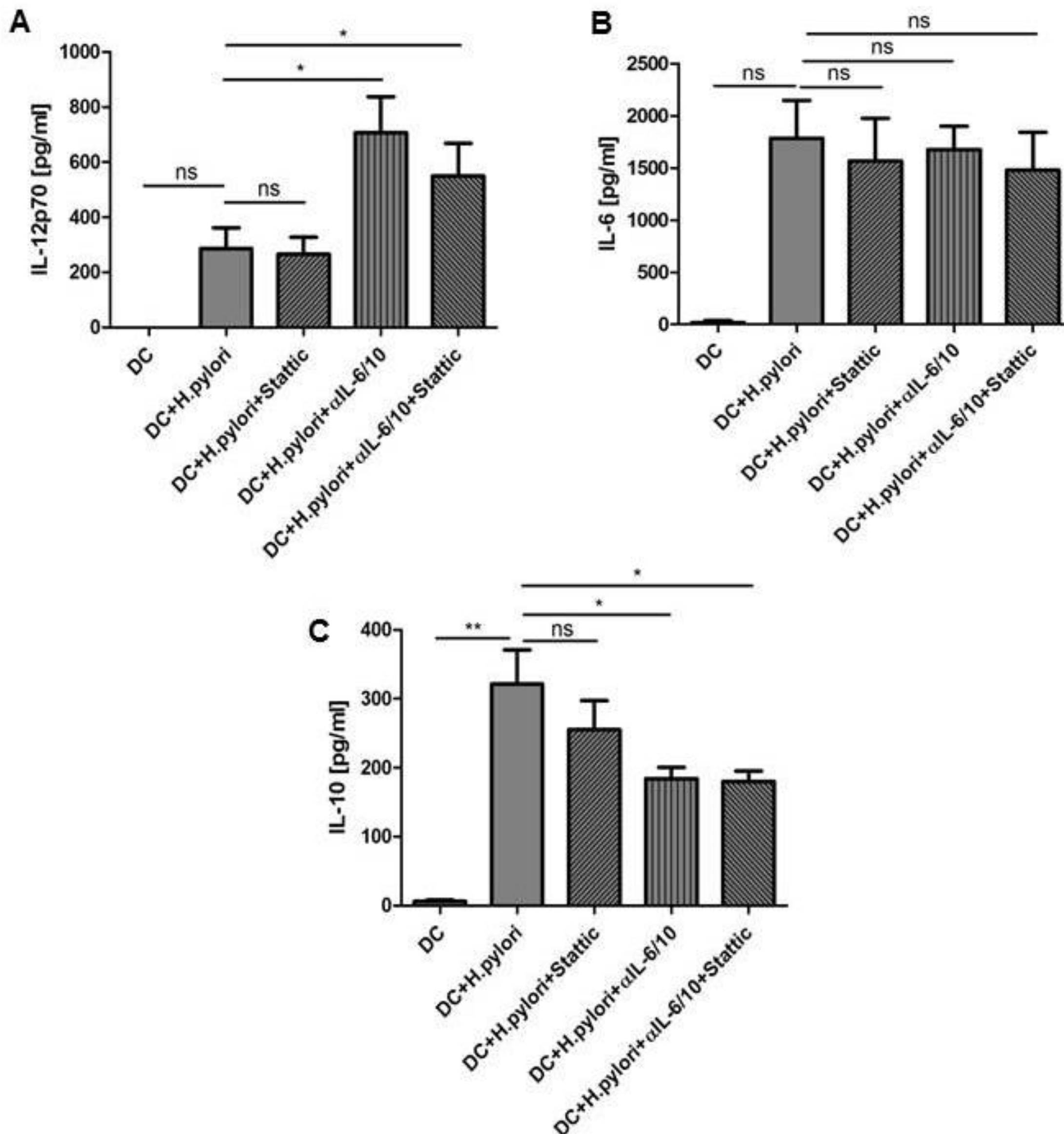

Figure 45: *H. pylori*-induced cytokine secretion of MoDCs in the presence of α-IL-6/ α-IL-10 neutralising antibodies and Stattic

Immature MoDC were pre-incubated with α-IL-6/α-IL-10 neutralising antibodies and/or 0.5 µM Stattic for 1 h. After 24 h of infection with *H. pylori* G27, secretion of IL-6, IL-10 and IL-12p70 was measured ELISA. Data are presented as mean ± S.D. of 3 independent experiments. (t-Test: * $p \leq 0.05$; ** $p \leq 0.005$; ns: not significant)

Next, the effect of inhibiting STAT3 phosphorylation in *H. pylori*-primed MoDCs on the subsequent T cell response was analysed. Blocking of STAT3 activation in *H. pylori*-infected MoDCs led to an increased secretion of IFN-γ (Figure 46A) and reduced Foxp3expression (Figure 47) in co-cultured T cells, while the release of IL-17A was not affected (Figure 46B). Consequently, it was postulated that *H. pylori*-induced STAT3 activation caused the suppression of IL-12p70 and DC maturation, which in turn was responsible for the expansion of T_{regs} over IFN-γ-producing T cells.

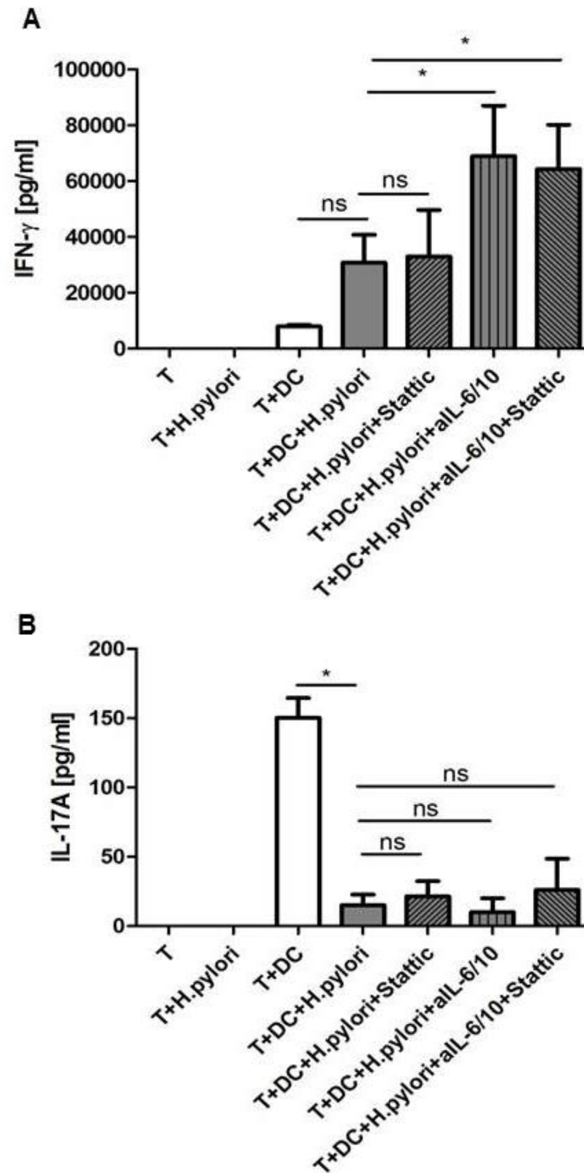

Figure 46: *H. pylori*-induced T cell response upon co-culture with MoDCs in the presence of α-IL-6/ α-IL-10 neutralising antibodies and Stattic

Immature MoDCs were pre-incubated with α-IL-6/α-IL-10 neutralising antibodies with or without Stattic before challenge with *H. pylori* G27. After 24h, allogenic $CD4^+$ T cells were co-cultured for further 72h. Release of (A) IFN-γ and (B) IL-17A was quantified by ELISA. Expression of (C) Foxp3 was determined by flow cytometry. Data are presented as mean ± S.D. of 4 independent experiments. (t-Test: * $p \leq 0.05$; ns: not significant)

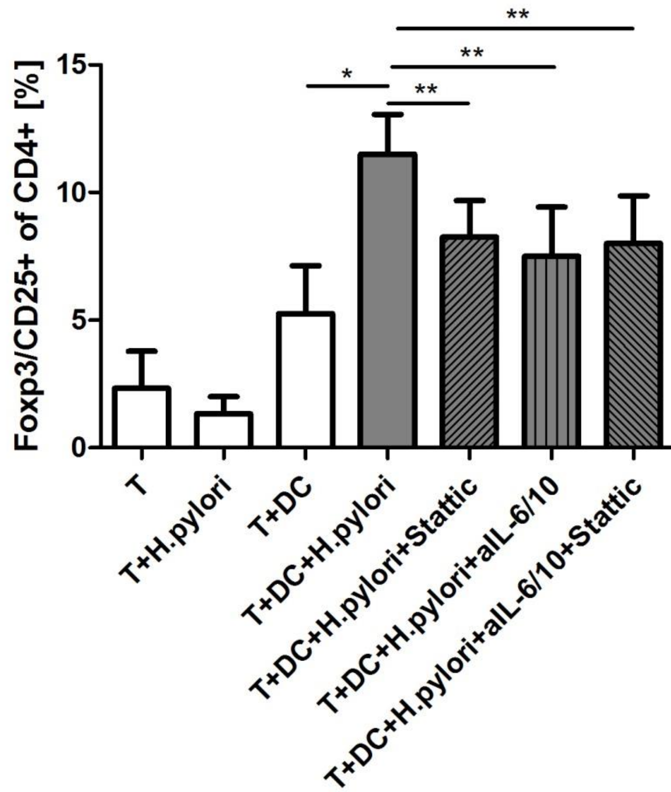

Figure 47: *H. pylori*-induced Treg expansion upon co-culture with MoDCs in the presence of α-IL-6/ α-IL-10 neutralising antibodies and Stattic

Immature MoDCs were pre-incubated with α-IL-6/α-IL-10 neutralising antibodies with or without Stattic before challenge with *H. pylori* G27. After 24 h, allogenic $CD4^+$ T cells were co-cultured for further 72 h. Expression of Foxp3 was determined by flow cytometry. Data are presented as mean ± S.D. of 4 independent experiments. (t-Test: * $p \leq 0.05$; ns: not significant)

4.11. TLR-4 on MoDCs is involved in *H. pylori*-mediated T cell response

The presence of CagA during *H. pylori* infection has been shown to cause DC semi-maturation, leading to the expansion of T_{regs} and a decreased IFN-γ release by co-cultured T cells. This phenotype was probably caused by secreted IL-10 and could be restored by cytokine neutralisation. However, it remained unclear how IL-10 production was induced in MoDCs upon *H. pylori* infection.

The expression of cytokines can be induced by TLR or non-TLR signalling in myeloid DCs. Activation of TLRs was shown to result in the activation of the extracellular signal-regulated kinase 1 (ERK1) and ERK2, p38 and NFκB pathways, leading to the induction of cytokine expression (213) (214) (215).

An involvement of TLRs in the recognition of *H. pylori* by murine DCs was already shown (76); however, there are no data about activation of extracellular TLRs in human MoDCs. Therefore, TLR neutralising antibodies were used to block TLR signalling. Initially, neutralisation efficiency was controlled by the use of TLR agonists. Following TLR-neutralisation and stimulation with anti-TLR-4 and LPS, anti-TLR-2 and Pam3Cys or anti-TLR-5 and Flagellin, MoDCs were analysed for IL-6 secretion, which is downstream of all respective TLRs. Lower levels of IL-6 upon neutralisation and stimulation indicated that TLR-signalling was mainly inhibited (Figure 48).

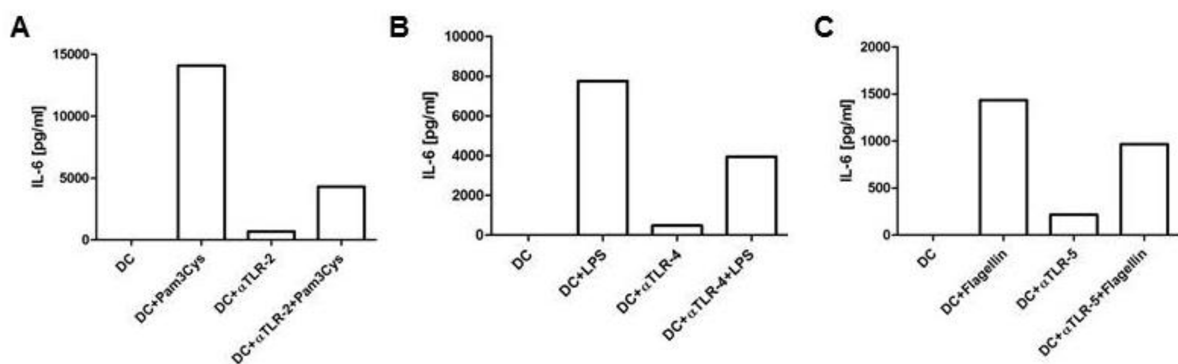

Figure 48: Release of IL-6 upon neutralisation of TLRs in MoDCs

Prior to stimulation with the TLR agonists (A) Pam3Cys, (B) LPS and (C) Flagellin, extracellular TLRs expressed on MoDCs were neutralised with antibodies for 1 h. Secretion of IL-6 was determined by ELISA after 24 h. Data show one representative experiment of n=2.

Subsequently, TLR-signalling was blocked prior *H. pylori* infection of MoDCs and cytokine secretion was analysed.

No notable effect onIL-12p70, IL-6 and IL-10 release by MoDCs was observed upon TLR-5 neutralisation, while a slight decrease was detected after blocking TLR-2 (Figure 49). However, the strongest reduction in cytokine secretion was observed when anti-TLR-4 antibodies were used; especially the release of IL-12p70 and IL-10 were predominantly dependent on TLR-4. These results indicated that activation of TLR-4 signalling by *H. pylori* was mainly responsible for the induction of pro- and anti-inflammatory cytokine secretion by MoDCs and identified TLR-4 as major inducer of IL-10 secretion in response to *H. pylori*.

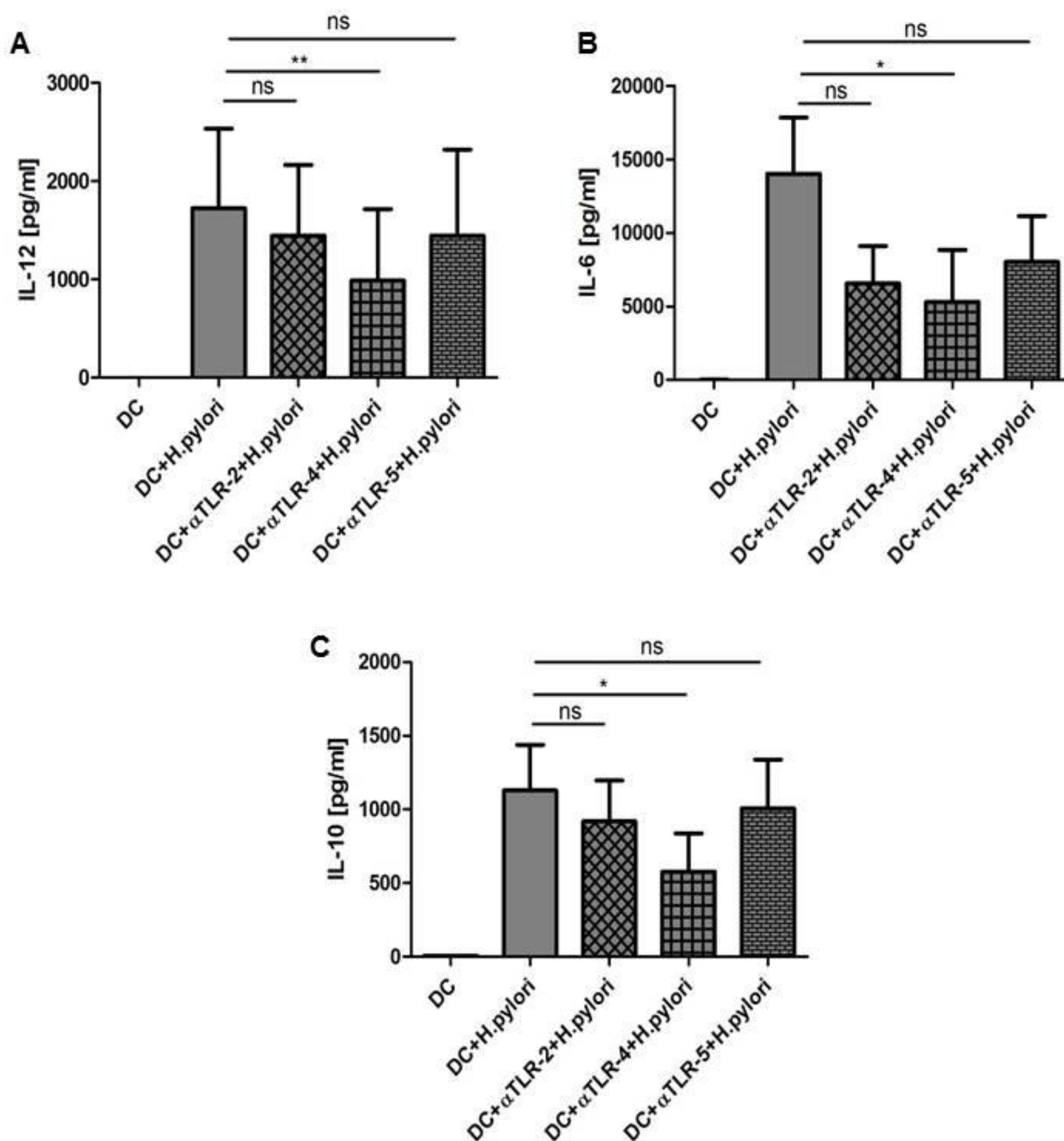

Figure 49: Cytokine release by *H. pylori*-primed MoDCs in the presence of TLR-neutralising antibodies

Extracellular TLRs (TLR-2/-4/-5) of immature MoDCs were neutralised for 1 h, followed by stimulation with *H. pylori* G27 for 24 h. Cytokine release was determined by ELISA. Data are presented as mean ± S.D. of 4 independent experiments. (t-Test: * $p \leq 0.05$; ** $p \leq 0.005$; ns: not significant)

It was shown before that *H. pylori*-induced DC semi-maturation was dependent of IL-10-mediated STAT3 activation. Moreover, IL-10 secretion was predominantly dependent on activation of TLR-4 and in part also upon TLR-2 signalling.

Inhibition of TLR signalling during *H. pylori* infection also affected DC maturation. The expression of CD86 and CD83 was significantly increased when TLR-2 and TLR-4 were blocked (Figure 50), suggesting that the secretion of IL-10 in response to TLR-2 and -4 activation was responsible for the induced semi-maturation of DCs.

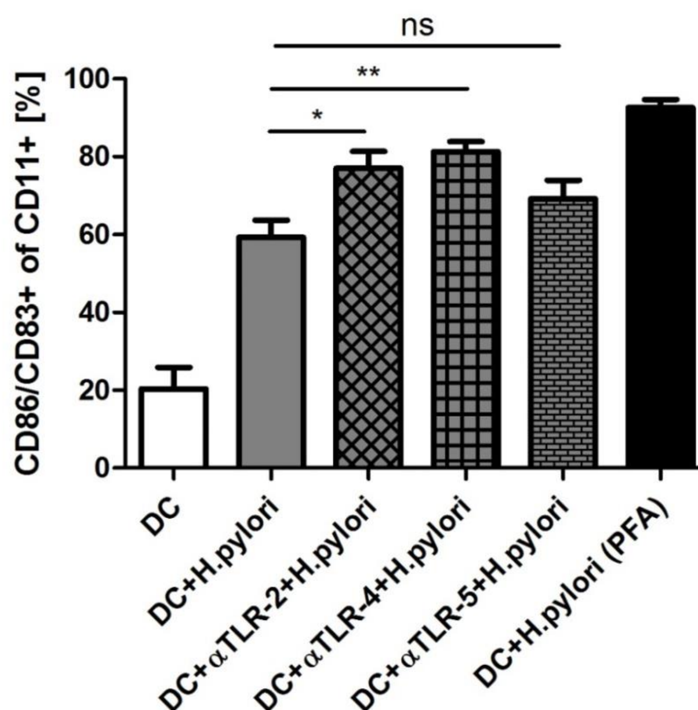

Figure 50: Maturation of *H. pylori*-infected MoDCs in the presence of TLR-neutralising antibodies

TLR-2 /-4 or -5 on MoDCs were blocked with neutralising antibodies for 1 h. Subsequently, MoDCs were infected with *H. pylori* G27 for 24 h. Maturation was determined by measuring the amount of CD86/CD83 double-positive cells by flow cytometry. Data are presented as mean ± S.D. of 4 independent experiments. (ANOVA: * $p \leq 0.05$; ** $p \leq 0.005$; ns: not significant)

TLR signalling was shown to influence DC maturation but also cytokine secretion of MoDCs in response to *H. pylori*. Especially reduced levels of IL-10 were observed, suggesting an effect on the subsequent T cell response.

Neutralisation of TLR-2 and -5 on MoDCs prior *H. pylori* challenge did not change the amount of T_{regs} significantly. However, when TLR-4 was blocked, lower amounts of Foxp3^{+}cells were observed (Figure 51). An impaired induction of T_{regs} was accompanied by reduced levels of IL-10 in the supernatant of co-cultivated cells.

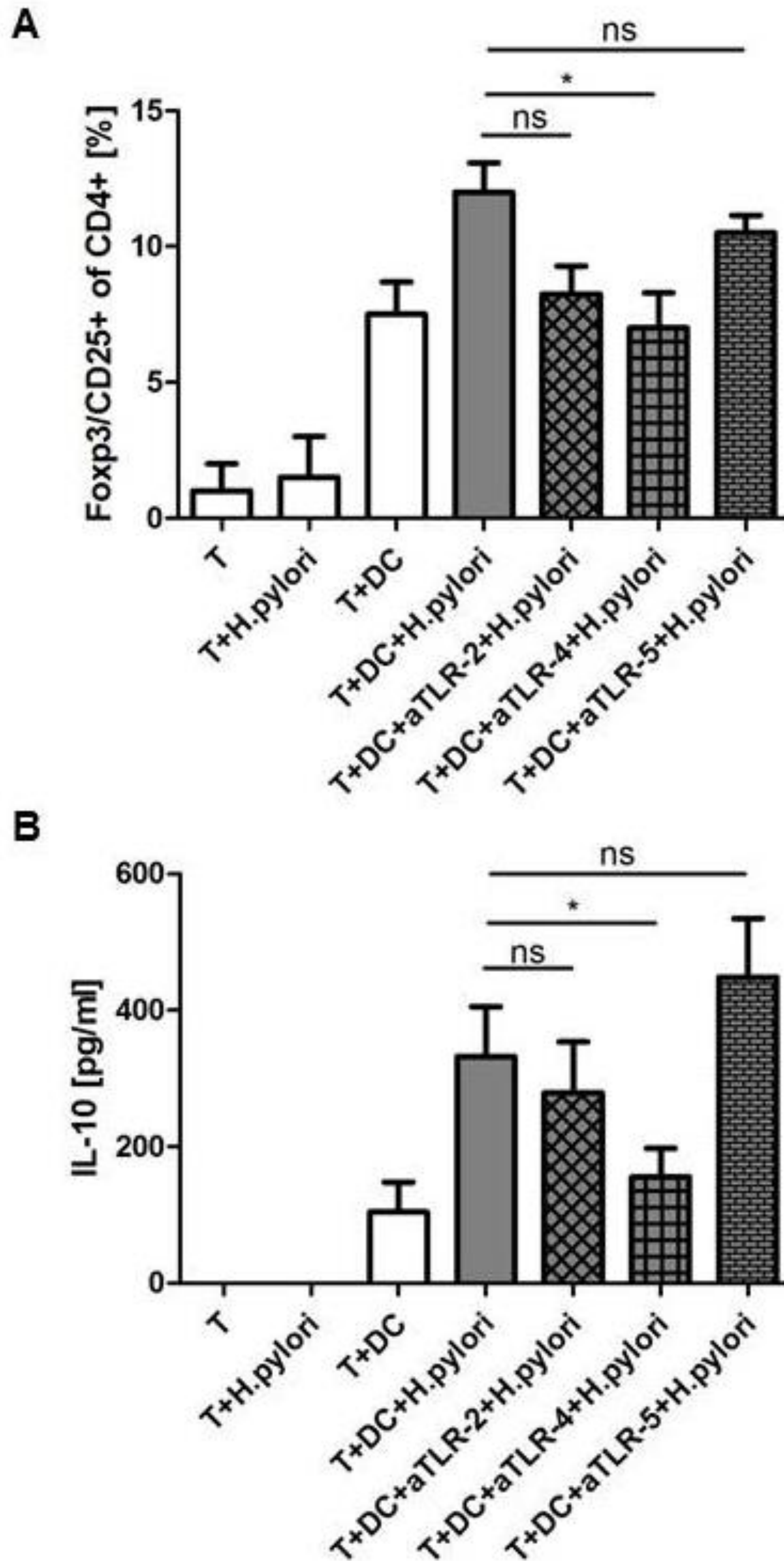

Figure 51: Foxp3 expression and IL-10 secretion by T cells upon co-culture with TLR-neutralised *H. pylori*-primed MoDCs

Upon neutralisation of TLR-2 /-4 /-5, MoDCs were stimulated with *H. pylori* G27 and afterwards co-cultured with allogenic $CD4^+$ T cells. (A) The Expression of Foxp3 was determined by flow cytometry, (B) the release of IL-10 was measured by ELISA. Data are presented as mean ± S.D. of 4 independent experiments.
(t-Test: * $p \leq 0.05$; ns: not significant)

Moreover, co-culture with TLR-neutralised *H. pylori*-infected MoDCs also led to an impaired IFN-γ and IL-17A production by T cells (Figure 52), indicating that the weak cytokine response of TLR-neutralised MoDCs in response to *H. pylori* impaired the subsequent T cell response. On the other hand, these findings suggested that blocking of TLR-4 might be considered as a tool for inhibiting T_{reg} expansion.

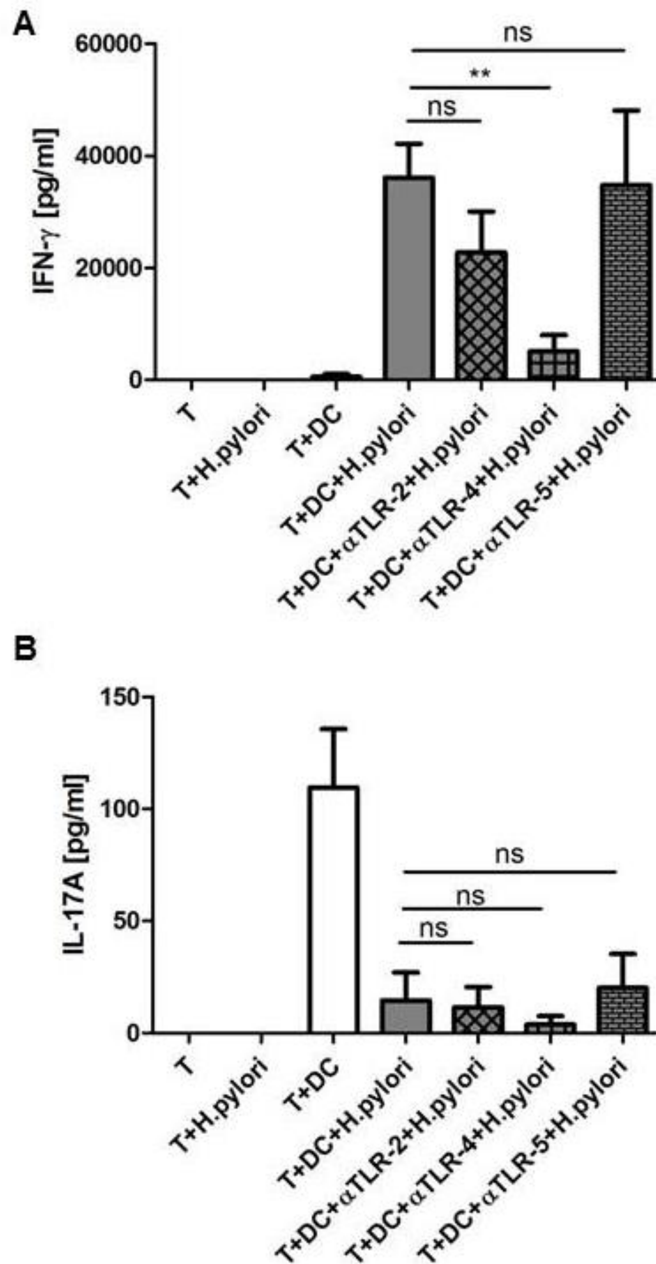

Figure 52: IFN-γ and IL-17A secretion of T cells in response to co-cultivation with TLR-neutralised *H. pylori*-primed MoDCs

Upon neutralisation of TLR-2 /-4 /-5with neutralising antibodies, MoDCs were stimulated with *H. pylori* G27 for 24 h and subsequently co-cultured with allogenic $CD4^+$ T cells for further 72h. Secretion of (A) IFN-γ and (B) IL-17A by co-cultured T cells was quantified by ELISA. Data are presented as mean ± S.D. of 4 independent experiments. (t-Test: ** $p \leq 0.005$; ns: not significant)

4.12. *H. pylori* gGT inhibitsT cell proliferation and induces tolerance

Co-cultivation of *H. pylori*-primed MoDCs resulted in an inhibited T cell proliferation, decreased IFN-γ and IL-17A production as well as an induction of T_{regs}. So far, it could be shown that the CagA-induced IL-10 production caused the phosphorylation of STAT3 that led to DC semi-maturation and T_{reg} expansion. In mice however, also γ-Glutamyltranspeptidase has been described to be involved in the induction of tolerance by changing the maturation of DCs. (192). However, when human immature MoDCs were challenged with either wild type or mutant *H. pylori*, it could be shown that the absence of gGT hardly influenced the *H. pylori*-induced DC semi-maturation (Figure 53).

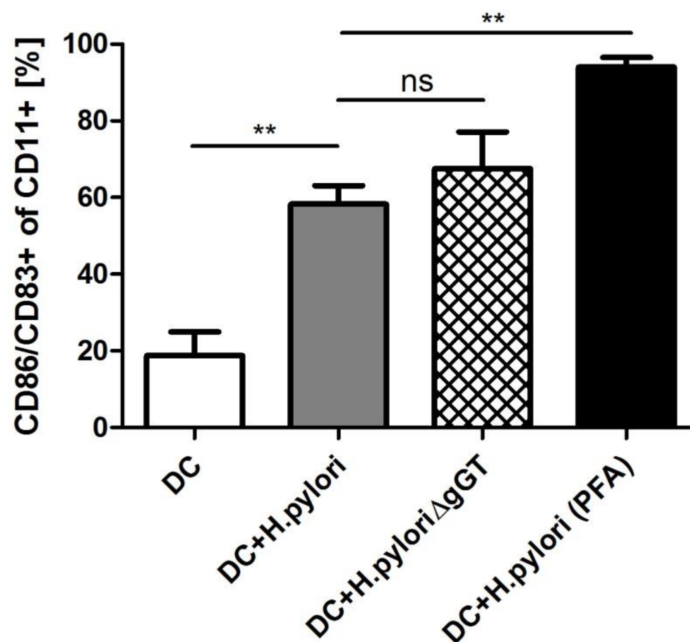

Figure 53: Maturation of MoDCs in response to gGT-deficient *H. pylori*

Immature MoDCs were infected with *H. pylori* G27 wild type strain or its isogenic gGT mutant. Maturation of MoDCs was illustrated by the amount of CD86/CD83 double-positive cells. Data are presented as mean ± S.D. of 5 independent experiments. (ANOVA: ** $p \leq 0.005$; ns: not significant)

In contrast, infection of human MoDCs with gGT-deficient *H. pylori* led to a significant increase in the secretion of the pro-inflammatory cytokines IL-12p40, IL-23 and IL-6 when comparing to wild type bacteria, while the production of IL-12p70and IL-10was hardly affected (Figure 54). Although no differences in DC maturation were observed upon challenge with gGT-deficient *H. pylori,* changes in the cytokine response suggested possible effects on the subsequent T cell response.

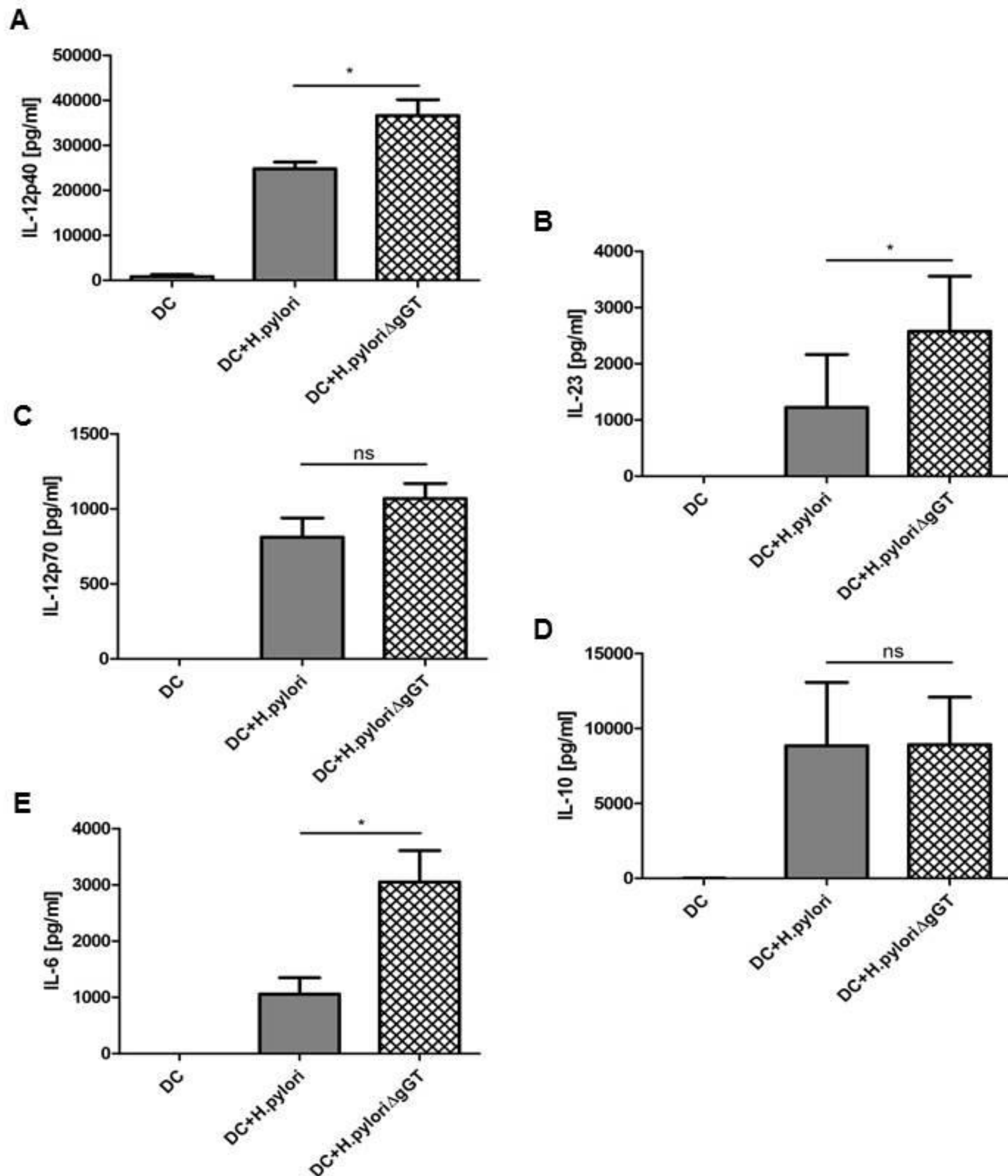

Figure 54: Cytokine release of MoDCs upon stimulation with gGT-deficient *H. pylori*

Immature MoDCs were infected with *H. pylori* wild type strain G27 or its isogenic gGT mutant. Secretion of (A) IL-12p40, (B) IL-23, (C) IL-12p70, (D) IL-10 and (E) IL-6 was quantified by ELISA. Data are presented as mean ± S.D. of 4 independent experiments. (t-Test: * $p \leq 0.05$; ns: not significant)

For that reason MoDCs were infected with gGT-proficient and gGT-deficient *H. pylori* and subsequently co-cultivated with CD4$^+$ T cells. Cell viability and cytokine responses of T cells were evaluated.

Co-culture experiments revealed that *H. pylori*-primed MoDCs induced an inhibition of T cell proliferation that was completely restored in the absence of gGT (Figure 55). Additionally, significantly increased levels of IL-2 were observed, indicating that gGT was the major virulence factor responsible for the *H. pylori*-induced inhibition of T cell proliferation, and confirming the published data of Schmees et al. (63).

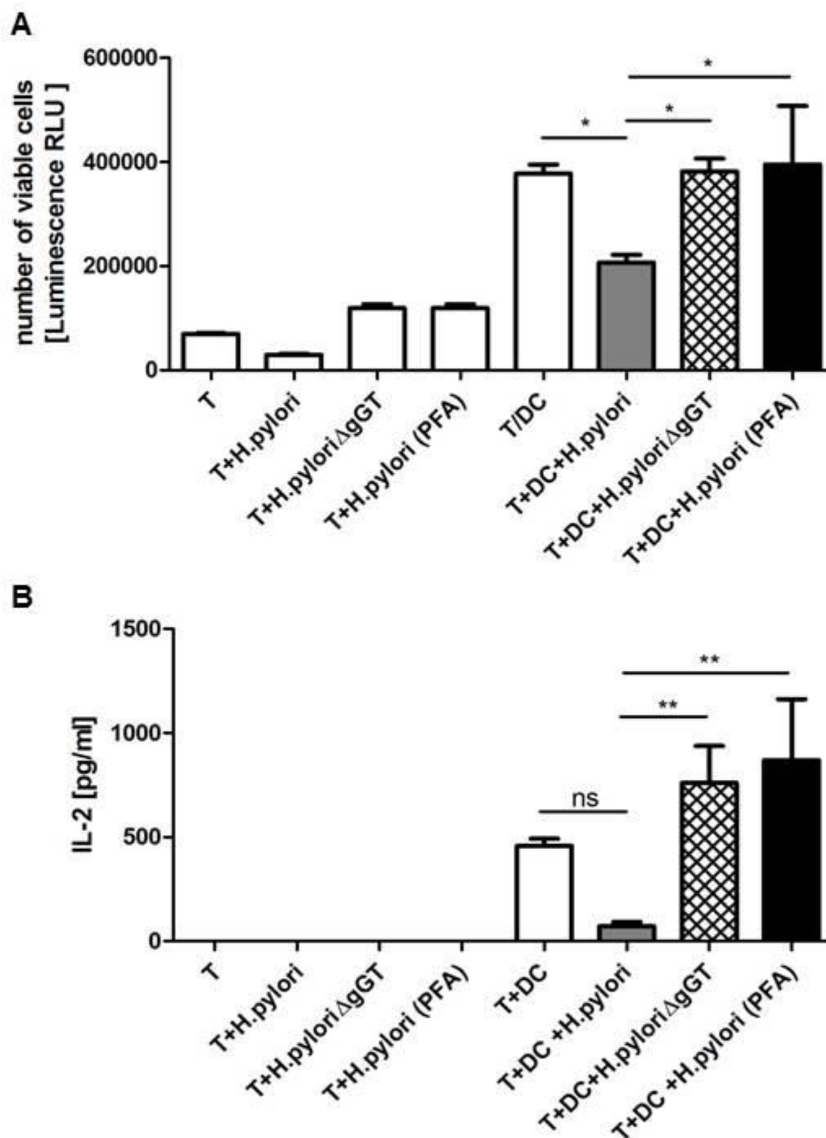

Figure 55: Proliferation and IL-2 release of T cells upon co-culture with *H. pylori*-primed MoDCs in the absence of gGT

Immature MoDCs were challenged with the *H. pylori* wild type strain G27 or its isogenic gGT mutant and subsequently co-cultivated with allogenic CD4$^+$ T cells for 72 h. (A) Viability of co-cultivated cells was measured using CellTiter-Glo. (B) Secretion of IL-2 was analysed by ELISA. Data are presented as mean ± S.D. of 3 independent experiments. (t-Test: * $p \leq 0.05$; ** $p \leq 0.005$; ns: not significant)

Besides its inhibitory effect on T cell proliferation, gGT has been described to be involved in the induction of T cell tolerance and in the inhibition of Th1/Th17 immunity (192). For that reason, the expression of Foxp3 in $CD4^+$ T cells was analysed.

Indeed, the absence of gGT during *H. pylori* infection of MoDCs resulted in significantly decreased numbers of Foxp3-expressing T cells (Figure 56), indicating that gGT was able to induce tolerogenic DCs.

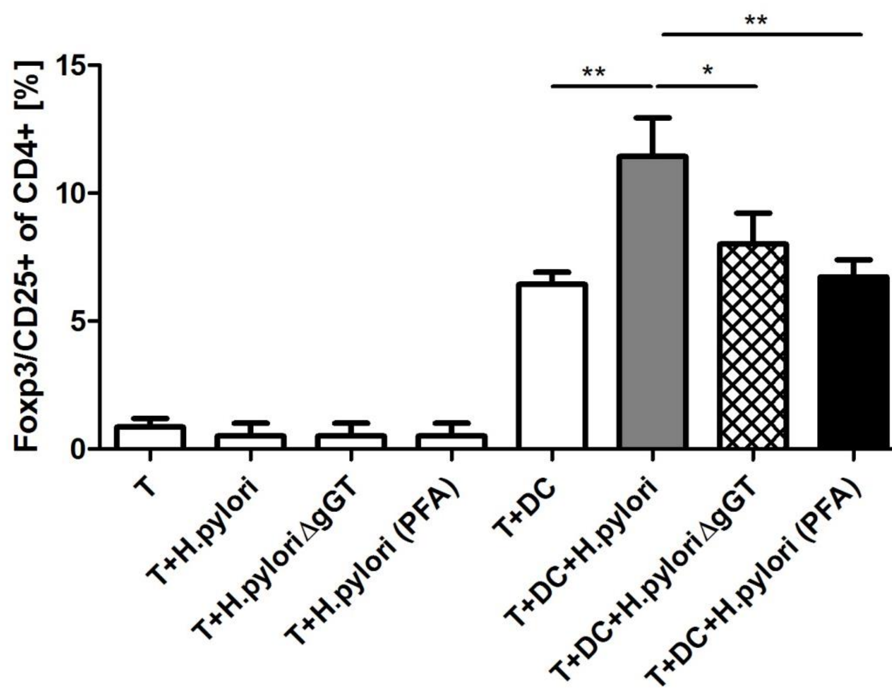

Figure 56: Expression of Foxp3 in T cells upon co-culture with *H. pylori*-primed MoDCs in the absence of gGT

Immature MoDCs were challenged with the *H. pylori* wild type strain G27 or its isogenic gGT mutant and subsequently co-incubated with allogenic $CD4^+$ T cells. Expression of Foxp3 was determined by flow cytometry. Data are presented as mean ± S.D. of 6 independent experiments. (ANOVA: * $p \leq 0.05$; ** $p \leq 0.005$)

Furthermore, also the effector T cells response was analysed. Unexpectedly, co-incubation with *H. pylori*-primed allogeneic MoDCs in the absence of gGT induced similar levels of IFN-γ and IL-17A (Figure 57). In contrast, DC stimulation with PFA-fixed bacteria that was shown to induce comparable proliferation of co-cultivated T cells than DCs primed with gGT-deficient bacteria, resulted in markedly higher amounts of IFN-γ and IL-17A, suggesting that the secretion of gGT during DC/T co-culture did not affect Th1 and Th17 responses.

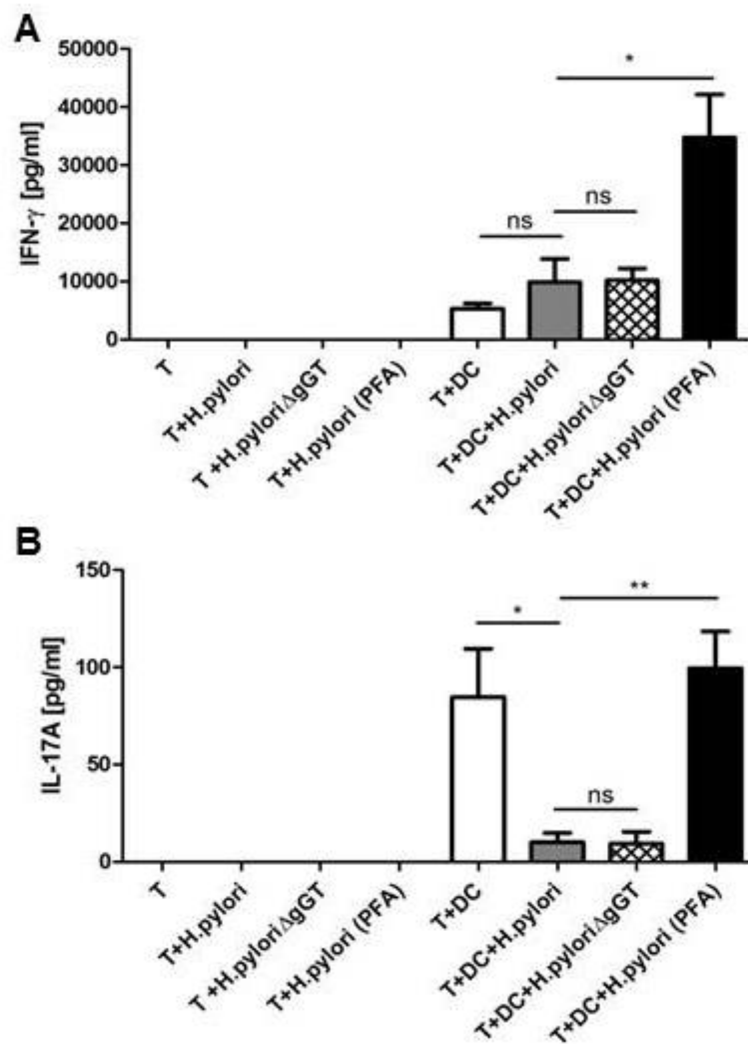

Figure 57: Production of IFN-γ and IL-17A of T cells in response to *H. pylori*-primed MoDCs in the absence of gGT

Prior co-culture with allogenic CD4$^+$ T cells, immature MoDCs were challenged with *H. pylori* wild type strain G27 or its isogenic gGT mutant. The secretion of (A) IFN-γ and (B) IL-17A was analysed by ELISA. Data are presented as mean ± S.D. of 5 independent experiments. (t-Test: * $p \leq 0.05$; ** $p \leq 0.005$; ns: not significant)

H. pylori was shown to induce DC semi-maturation and anti-inflammatory cytokine secretion, leading to the expansion of T_{regs}. CagA-deficient bacteria restored maturation and induced a pro-inflammatory cytokine response, causing decreased Foxp3 expression in T cells and favoured the release of IFN-γ. The *H. pylori* gGT-mutant also reduced the amount of T_{regs} however, it did not induce changes on DC maturation, suggesting another mechanism for the gGT-mediated induction of tolerance.

4.13. gGT-induced T cell tolerance depends on glutamine deprivation

It has been described that glutamate activation of metabotropic glutamate receptor 4 (mGluR4) signalling in DCs prevents the production of IL-6 and IL-23, favouring the development of T_{regs} over Th17 cells in Experimental Autoimmune Encephalomyelitis (EAE) (216).

As previously shown, *H. pylori* also induced lower IL-6 and IL-23 secretion in MoDCs compared to gGT-deficient bacteria, leading to an expansion of T_{regs}. In parallel, gGT has been described to enable *H. pylori* to use extracellular glutamine and glutathione as a source of glutamate (59). Hence, the effect of glutamine deprivation during *H. pylori* infection was analysed. Generally, decreasing levels of glutamine were shown to induce apoptosis in host cells (217) and to inhibit T cell proliferation by limiting IL-2 (218). Here, it was examined whether gGT-dependent glutamine deprivation could induce T_{regs} expansion. Therefore, MoDCs were stimulated with gGT-deficient *H. pylori*. The addition of recombinant gGT (rgGT) in the presence or absence of glutamine (Gln) was used to control if the presence of gGT indeed was responsible for the induction of T_{regs} and whether this effect was dependent on glutamine deprivation.

Incubation of *H. pylori*-infected MoDCs induced higher levels of Foxp3-expressing T cells than DCs primed with gGT-deficient bacteria. Interestingly, Foxp3 expression was increased when DCs were further stimulated with recombinant gGT, however, only in the presence of

glutamine in the medium (

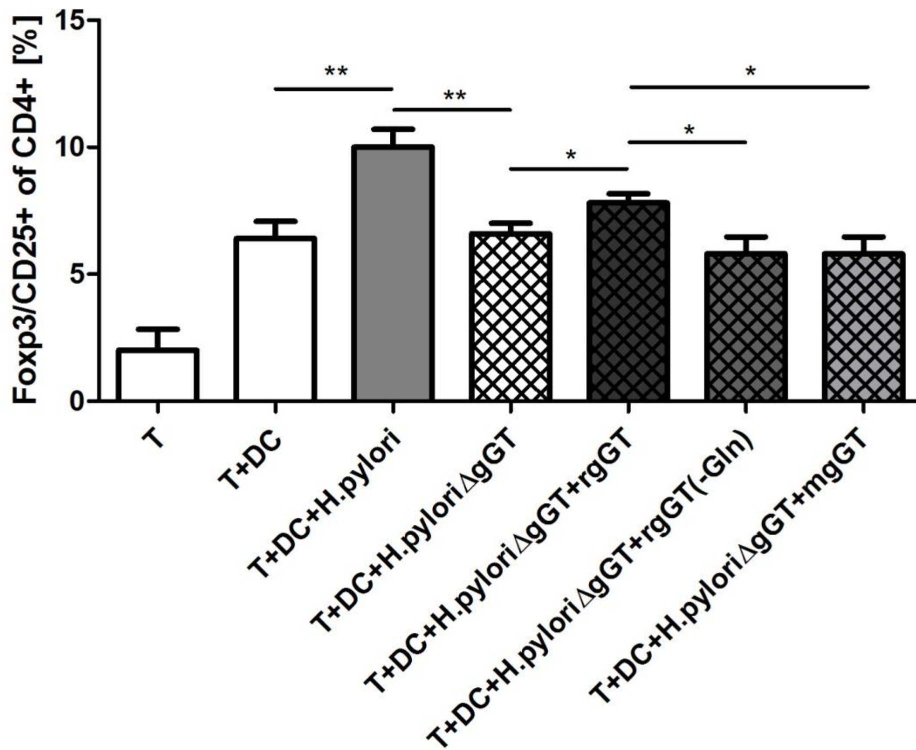

Figure 58). Absence of glutamine or the use of an enzymatic inactive enzyme (mgGT) did not induce higher amounts of Foxp3, indicating that gGT-dependent glutamine deprivation was responsible for the induction of T_{regs} during co-culture of T cells with *H. pylori*-primed MoDCs, since either no substrate or no active enzyme was present. Nevertheless, glutamine and glutamate levels in the supernatant of co-cultivated cells have to be determined in order to verify this hypothesis.

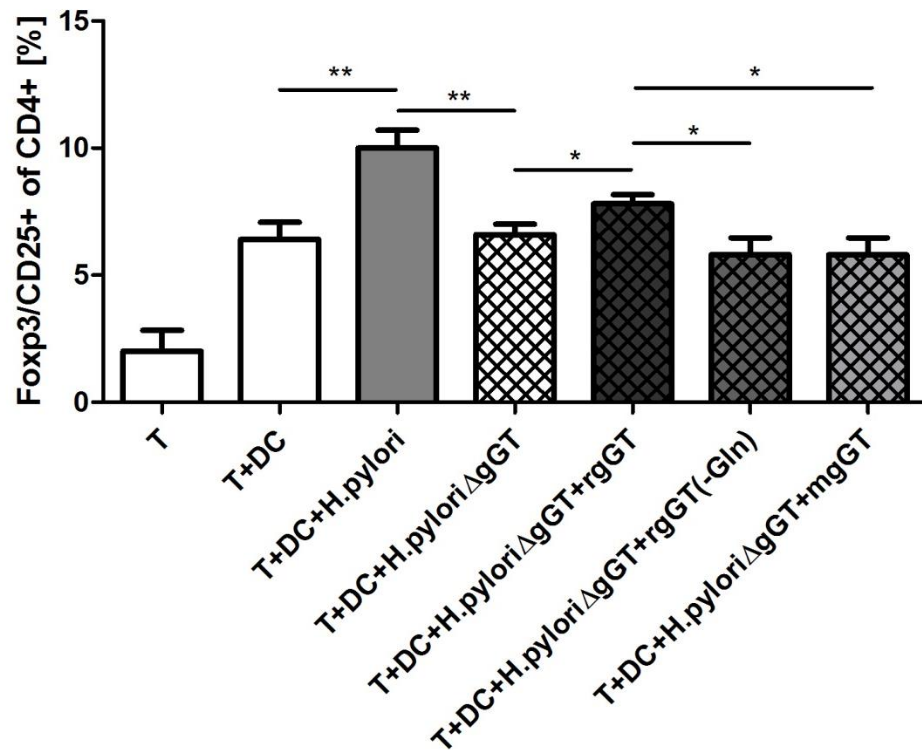

Figure 58: Expression of Foxp3 in T cells in dependence of glutamine deprivation

Immature MoDCs were stimulated with gGT-deficient *H. pylori* G27 and recombinant gGT (0.1 µg/ml) in glutamine free (-Gln) or glutamine containing medium (2 mM). The expression of Foxp3 was evaluated by flow cytometry. Data are presented as mean ± S.D. of 4 independent experiments. (t-Test: * $p \leq 0.05$; ** $p \leq 0.005$)

Wild type *H. pylori* was shown to induce lower levels of IL-6 and IL-23 in MoDCs compared to gGT-deficient bacteria. Whether this impaired cytokine release was also caused by glutamine deprivation was analysed next.

Upon stimulation of MoDCs with recombinant gGT it could be shown that the weak IL-6 and IL-23 secretion depended on glutamine deprivation, since lower cytokine levels were only detected when glutamine was used as substrate for the enzyme (Figure 59). This result indicated that enzymatic active gGT used up glutamine, leading to lower IL-6 and IL-23 secretion. In contrast, when glutamine was absent, there was no substrate available, hence higher amounts of IL-6 and IL-23 were observed.

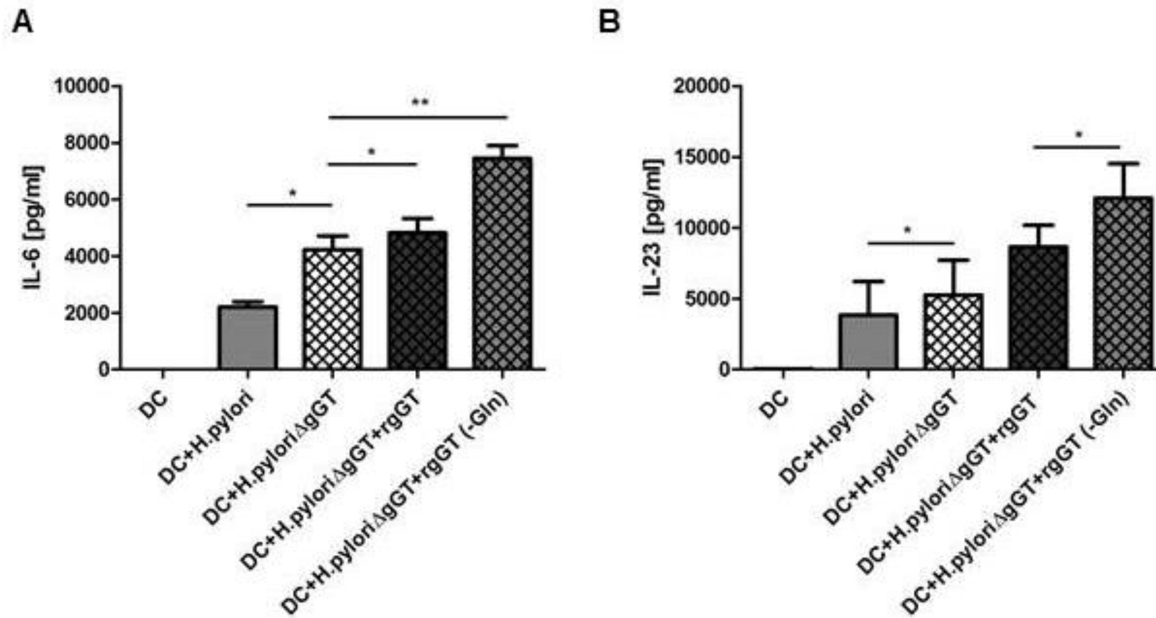

Figure 59: Influence of glutamine deprivation on the release of IL-6 and IL-23 by MoDCs

Immature MoDCs were stimulated with wild type or gGT-deficient *H. pylori* G27. Influence of glutamine deprivation was analysed by stimulation of MoDCs with gGT-deficient *H. pylori* and recombinant gGT (0.1 µg/ml) in glutamine free (-Gln) or glutamine containing medium (2 mM). Secretion of (A) IL-6 and (B) IL-23 was quantified by ELISA. Data are presented as mean ± S.D. of 3 independent experiments.(t-Test: * $p \leq 0.05$)

It can be concluded that gGT-dependent glutamine deprivation during *H. pylori* infection of MoDCs induced lower levels of IL-6 and IL-23 that were accompanied by an expansion of T_{regs}. Nevertheless, there are further experiments needed to clarify if the regulatory T cell response was caused by the altered cytokine secretion of DCs or if glutamine deprivation alone was sufficient to induce the expression of Foxp3.

Discussion

5. Discussion

H. pylori is a globally spread bacterium that is associated with peptic ulcer disease and gastric cancer (15). Although *H. pylori* infection causes host immune responses (8), the pathogen cannot be eliminated from the stomach in many cases (9). However, it remains still unclear how *H. pylori* manages to persist in the stomach for decades.
Recent reports proposed that chronic infection with *H. pylori* may also be of benefit to the host by conferring protection against allergies and asthma (163). It has been shown in mice that the protective effects of *H. pylori* depend on highly suppressive T_{regs} (189) that are predominantly induced by *H. pylori*-stimulated DCs (84) (190). Naïve T cells differentiate into $Foxp3^+$ T_{regs} upon contact with DCs having tolerogenic rather than immunogenic properties (200). T cell conversion into T_{regs} occurs through antigen presentation by DCs in the absence of co-stimulatory molecules and mainly in the presence of IL-10, TGF-β, retinoic acid and/or programmed cell death ligands (200) (201).
So far, there are a few reports showing that *H. pylori*-infected murine DCs are tolerogenic and instruct T_{reg} expansion. However, there are several discrepancies when analysing human DCs and, furthermore, there are not much data on the molecular mechanisms involved. Consequently, one of the main objectives of this thesis was the characterisation of the response of human DCs to *H. pylori*. A better understanding of the influence of *H. pylori* on DCs might help to comprehend how *H. pylori* is influencing the adaptive immune response of the host.

Initially, human DCs were generated from monocytes of *H. pylori*-negative donors. Cultivation of monocytes in a GM-CSF and IL-4 containing medium induced the differentiation of monocyte-derived DCs (MoDCs), which differ from monocytes by losing the ability to express CD14 (195).
Immature MoDCs are characterised by low surface expression levels of the co-stimulatory molecules CD80/ CD86 and the maturation marker CD83. The comparison of different DC generation protocols revealed that the initial maturation status of MoDCs deeply influenced the response to the bacterium; hence MoDCs needed to be immature in order to analyse DC maturation upon bacterial stimulation. While the generation of MoDCs in FCS-free medium caused pre-activation of DCs that was translated to an up-regulation of CD86 and pro-inflammatory cytokine response upon bacterial exposure, the use of FCS-containing medium

resulted in the generation of immature MoDCs. Consequently, the establishment of a robust DC generation protocol allowed the reliable analysis of DC maturation upon *H. pylori* infection.

The exposure to *H. pylori* induced semi-maturation of human MoDCs that was characterised by an induction of the co-stimulatory molecules CD80/CD86 and the activation marker CD83, while low levels of MHC class II molecules were detected. Semi-maturation of human DCs was independent of the duration of infection, bacterial dose or the used *H. pylori* strain. Thereby it could be shown for the first time that *H. pylori* confers a semi-mature phenotype to human DCs, as it was described for murine DCs (189) (219).

Notably, DC semi-maturation was only observed upon infection with live *H. pylori*, while stimulation of human immature MoDCs with PFA-fixed *H. pylori* induced a stronger increase in DC maturation, suggesting that virulence factors actively influenced the expression of activation markers. Indeed, co-incubation of MoDCs with live and PFA-fixed *H. pylori* at the same time, caused lower DC maturation when compared to dead bacteria alone, suggesting that bacterial virulence factors actively induce semi-maturation of DCs thereby preventing full DC activation triggered by PFA-fixed *H. pylori*. Comparable results were obtained when live *H. pylori* was added in combination to PFA-fixed *E. coli*.

DCs not only play a crucial role as primary responders to microbial infection due to their ability of capturing and transferring antigens, but they also determine the type of T cell-mediated response to be mounted (220). Interestingly, *in vitro* stimulation of human MoDCs with *H. pylori* caused reduced activation of co-cultivated allogenic $CD4^+$ T cells, which was illustrated by low numbers of metabolic active cells and reduced secretion of IL-2.The latter was shown to depend on the presence of DCs. However, impaired antigen-presentation was not responsible for the inhibited T cell proliferation and activation, but the secretion of gGT by *H. pylori*. Stimulation of T cells with live *H. pylori* in the absence of MoDCs also led to impaired numbers of metabolic active T cells that could be restored by using gGT-deficient bacteria, as it was described by Schmees et al. (63). Since the pathogen was shown to be able to survive aerobic conditions for days, gGT was still produced during the DC/T co-culture and could also affect the expansion and cytokine response of T cells.

Maturation and antigen presentation of MoDCs is usually accompanied by cytokine secretion that can further influence T cell polarisation, thereby shaping the adaptive immune response towards *H. pylori*. In infected individuals increased levels of IL-12p70 were observed (149) that could be shown to be the major stimulator of Th1 cells (120) (221), which represent the

largest group of infiltrating $CD4^+$ T cells during *H. pylori* infection (222). Furthermore, IL-23 is highly produced in *H. pylori*-infected patients. Together with IL-6 and TGF-β, IL-23 is needed for human T cell polarisation towards Th17 (126) (127) (128) (223). In mice Th17 cells have been shown to act synergistically with Th1 cells to induce gastritis (149).

In response to live *H. pylori* human MoDCs secreted lower levels of the pro-inflammatory cytokines IL-12p70 and IL-6 compared to MoDCs primed with PFA-fixed *Helicobacter*. Conversely, increased levels of anti-inflammatory IL-10 were observed in response to live *H. pylori*, suggesting that this cytokine repertoire might favour a regulatory T cell response. Indeed, emerging evidence suggests that *H. pylori* may also induce a regulatory T cell response against T helper cell immunity, since increased expression of Foxp3 was observed in the gastric tissue of *H. pylori*-infected individuals compared with uninfected controls (224). *H. pylori*-specific T_{regs} have been shown to suppress IFN-γ production (225) and memory T cell responses to *H. pylori* (226) in infected individuals. In mice, *Heliocobacter*-primed DCs induced T_{regs} which were capable of inhibiting Th17 differentiation (84). Interestingly, T_{reg} depletion was associated with a higher *H. pylori*-specific Th17 response and lower bacterial density than controls. These findings established an association between *H. pylori* infection and the induction of T_{regs}, which, by counteracting the host's immune response, may lead to *H. pylori* persistence. However, the exact mechanism of the effector cell suppression by *H. pylori*-induced T_{regs} is not completely understood. Several cell-surface molecules, such as CD25 (227) or CTLA-4 (228), were proposed to play a role as mediators of T_{reg}-mediated suppression. In addition, several secreted proteins have been implicated in the suppression by T_{regs} too, including IL-10, IL-35, granzyme B, IL-9, and TGF-β (229) (230).

In this study it was shown that co-cultivation of *H. pylori*-infected MoDCs with allogenic $CD4^+$ T cells resulted in the release of higher levels of IL-10 compared to uninfected DCs. On the other hand, moderate levels of IFN-γ, but almost no IL-17A were detected. In contrast, DC/T co-culture in the presence of PFA-fixed *H. pylori* caused lower expression of Foxp3 and less IL-10 secretion, but was characterised by large amounts of IFN-importantly, no gGT inhibition was observed when using PFA-fixed *H. pylori*, suggesting that higher cytokine levels could be caused by increased T cell numbers. However, decreased IL-10 levels were observed in DC/T co-culture when DCs were primed with PFA-fixed *H. pylori* compared to live bacteria. Interestingly, no enhanced secretion of IL-17A from $CD4^+$ T cells co-cultured with *H. pylori*-primed DCs was detected, in contrast to the observations by Khamri et al. (67). They showed that autologous $CD4^+$ T cells were indeed able to produce IL-17A in response to *H. pylori*-infected MoDCs. Thus, one possible explanation to our observation could be that

the release of IL-17A, which is already effective at very low levels, was already saturated due to the co-incubation with allogenic MoDCs.

Although differences in T cell proliferation might have an effect on the amount of cytokines released, no influence was expected concerning T_{reg} expansion, since total cell numbers were compared. Co-culture of *H. pylori*-infected MoDCs with allogenic naïve $CD4^+$ T cells led to the expansion of Foxp3-expressing T cells. In contrast, stimulation of MoDCs with PFA-fixed bacteria led to lower numbers of T_{regs}. Thereby it was shown that *H. pylori* induced semi-maturation of human DCs conferring a tolerogenic phenotype that elicited expansion of regulatory T cells. These results are in line with former studies in murine models showing impaired DC maturation and tolerogenic reprogramming of DCs by *H. pylori* (219) (192) (231).

Since *H. pylori* was shown to actively inhibit human DC maturation, it needed to be clarified how the pathogen was influencing DC cytokine secretion and the expression of activation markers. In mice, there is disagreement about the molecular mechanisms by which DCs become tolerogenic. John Kao and colleagues, who did not focus on DC maturation but cytokine secretion, claimed that T_{reg} skewing in mice depends on TGF-β and IL-10 secretion and that the virulence factors VacA and CagA had no influence (84). In contrast, the group of Anne Müller argues that the presence of *H. pylori* gGT and VacA promotes the induction of murine T_{regs} (192). During *H. pylori* infection of murine bone marrow-derived DCs they observed reduced expression of CD80 and low levels of IL-12p40. However, they did not study other maturation markers or cytokines involved in DC maturation. Interestingly, another study by Tanaka et al. indicated an involvement of CagA on the induction of IL-10 producing T cells and impaired Th1 polarisation, using LPS-stimulated CagA-overexpressing murine DCs (191). CagA-transgenic DCs were characterised by impaired DC maturation, weak secretion of pro-inflammatory cytokines but strong production of IL-10.

In the present work, human immature MoDCs were stimulated with several *H. pylori* mutant strains and analysed for DC maturation. Interestingly, only the mutants lacking CagA or BabA induced an increased DC maturation compared to wild type *H. pylori*. Both mutants are characterised by an impairment of CagA translocation. Using CagA-deficient bacteria it could be shown that increased DC maturation was accompanied by an enhanced IL-12p70 and lower IL-10 secretion.

Co-cultivation of T cells with *H. pylori*-infected MoDCs revealed CagA translocation in human DCs important for T_{reg} expansion and reduction of IFN-γ secretion. These findings indicate that CagA negatively influences human DC maturation and pro-inflammatory

cytokine release, leading to the expansion of a regulatory T cell response. This in turn may contribute to the *H. pylori*-induced persistence and pathogenesis. These finding are in line with the observation of Tanaka (191) who showed the effect of CagA on DC maturation and cytokine secretion in mice, leading to impaired IFN-γ production but increased IL-10 secretion by T cells.

In contrast to the data of Oertli and colleagues (192), stimulation of human MoDCs with gGT-deficient *H. pylori* did not lead to increased DC maturation, but resulted in a different cytokine profile that also induced T_{reg} expansion. This will be explained in a following section.

Although differentiation of DCs into immunogenic or tolerogenic DCs has not been fully characterised, it is generally accepted that DC maturation status rather than DC lineage alone determines the functionality of DCs (232). Different signalling cascades are involved in DC maturation, predominantly the NFκB pathway. In this context, RelB has been reported to be a critical factor involved in DC maturation, and tolerogenic DCs have been generated by RelB silencing using shRNA (233). Other signalling networks involved in programming DCs into a tolerogenic state include Wnt-β-catenin and Jak/STAT3. In intestinal DCs, β-catenin was reported to be required for regulatory T cell induction while suppressing inflammatory effector T cells (206). On the other hand, different studies have demonstrated an important role of STAT3 in DC differentiation and function under physiological conditions and cancer. For instance, hyperactivation of STAT3 was shown to lead to an abnormal differentiation of DCs in cancer (234), while inhibition of Jak2/STAT3 or STAT3 silencing induced a dramatic improvement of DC differentiation (235) (236). Also, in different mouse models, including the IL-6 knockout and gp130 knock-in mice, the IL-6/STAT3 signalling pathway was shown to regulate DC differentiation *in vivo* (237). Furthermore, DC-specific STAT3 deletion in mice was associated with impaired mucosal tolerance, and revealed STAT3 as a negative regulator of DC function (205).

In an attempt to identify the signalling pathways involved in the *H. pylori*-induced DC tolerance, protein lysates from infected MoDCs were assessed by Western Blot. Interestingly, this analysis revealed a correlation between *H. pylori*-induced DC semi-maturation and STAT3 activation. It was shown that *H. pylori* activated STAT3 in MoDCs after a few hours upon infection, independent of the *H. pylori* strain used. Importantly, lower levels of phosphorylated STAT3 were observed in the absence of CagA translocation, indicating that CagA-induced inhibition of DC maturation and STAT3 activation are related.

Cytokines and growth factors are known to induce phosphorylation of STAT3, causing its dimerisation and translocation into the nucleus, where it modulates the expression of target genes. While *H. pylori* has been described to induce phosphorylation of STAT3 in gastric cancer cells (208), no data on STAT3 activation upon *H. pylori* uptake in DCs have been reported to date. In fact, it has been previously described in gastric cancer epithelial cells that CagA is able to induce STAT3 phosphorylation (208). In addition, also IL-6 and IL-10 that were secreted by *H. pylori*-primed DCs could mediate activation of STAT3 in DCs (209) (210).

Since high IL-6/ IL-10 levels were observed in DCs upon *H. pylori* infection, supernatants of *H. pylori*-infected MoDCs were used for stimulation of immature MoDCs. It was primarily demonstrated that secreted cytokines of human MoDCs were sufficient to induce STAT3 phosphorylation. However, it remained unclear which cytokines were responsible for STAT3 phosphorylation in human MoDCs in response to *H. pylori.* Neutralisation of IL-10 by using neutralising antibodies decreased STAT3 activation, indicating that IL-10 is the main cytokine responsible for STAT3 phosphorylation in DCs after *H. pylori* challenge. This observation was further supported by the fact that impairment of STAT3 activation by the specific STAT3 inhibitor Stattic was only achieved in few samples obtained from donors secreting low levels of IL-10 upon *H. pylori* challenge. In the majority of donors however, no differences in STAT3 activation were observed when using Stattic, suggesting that the amount of Stattic was not sufficient to block STAT3 activation because of the high levels of IL-10 secreted. Several reports have shown STAT3 to negatively regulate DC differentiation and function *in vivo* (238) (209). Thus, it is hypothesized that STAT3 activation upon *H. pylori* infection is responsible for DC tolerisation. This assumption is supported by data showing that blocking of STAT3 phosphorylation confers a less tolerogenic DC phenotype favoring a Th1 immune response.

Increased levels of IL-10 after incubating DCs with *H. pylori* were concomitant to low levels of IL-12 and depended on the presence of CagA. This observation is in agreement with previous reports describing reduced IL-12 secretion by *H. pylori*-primed DCs (219) (239) (67) and the involvement of CagA in the suppression of IL-12p40 expression (191); however the possible signaling pathways implicated remain unclear. Yet it was speculated that activation of STAT3 might be also involved in preventing IL-12p70 release, since STAT3 was found to negatively regulate NFκB binding to the IL-12p40 promoter (240), while in tumour-associated DCs STAT3 inhibits NFκBdependent IL-12p35 expression (241). Indeed, increased levels of IL-12p70 were observed after blocking STAT3. Nevertheless, additional

experiments are required to further characterise the *H. pylori* virulence determinants as well as the downstream signalling cascades engaged in IL-12p70 suppression during *H. pylori* infection.

In addition to increased IL-12p70 secretion and DC maturation, blockage of STAT3 by IL-10 neutralising antibodies also favoured the polarisation of Th1 cells over $T_{regs.}$ Hence, the shift of secreted pro- and anti-inflammatory cytokines of MoDCs in response to wild type or CagA-deficient *H. pylori* may strongly influence the maturation of the DC themselves, but also the polarisation of co-cultivated T cells. Thus, it was shown for the first time that *H. pylori* infection leads to semi-maturation of human DCs through an IL-10-mediated activation of STAT3, resulting in the expansion of regulatory T cells. Therefore, STAT3 is involved in programming DCs into a tolerogenic state in response to *H. pylori* stimulation.

In contrast, β-catenin that has been described to cause tolerogenic DCs in the gut (206) was not involved during *H. pylori* infectionof human MoDCs that exclusively takes place in the stomach, suggesting that the location of DCs and the resulting detection of different antigens are important. Intestinal DCs recognise exogenous pathogens, but also commensal bacteria, which are necessary for the development and maintenance of a healthy immune system (242). Hence, a β-catenin-dependent induction of tolerance might be necessary to protect commensal bacteria from an attack by effector T cells. In the human stomach however, where only a minority of bacteria is able to persist the gastric acid, *H. pylori* survival is ensured by inducing tolerogenic DCs through IL-10-mediated STAT3 activation, leading to impaired secretion of IL-12p70 and decreased Th1 induction.

As described before, activation of STAT3 might be involved in preventing IL-12p70 release; either through negative regulation ofNFκB binding to the IL-12p40 promoter (240) or by the inhibition of NFκB dependent IL-12p35 expression, as it has been observed in tumour-associated DCs (241). However, NFκB activation in DCs was not inhibited by *H. pylori* infection, suggesting that other mechanisms were involved in the strong anti-inflammatory cytokine response.

The cytokine profile upon *H. pylori* infection excited a crucial role in defining the polarisation of T cells. However, it was unclear how cytokines were induced in human MoDCs upon *H. pylori* infection. Generally, cytokines are produced in response to bacterial recognition by several receptors. During *H. pylori* infection, Rad and colleagues described MyD88-dependent DC maturation and cytokine secretion in mice (76), suggesting that TLRs play a crucial role. Hence, the involvement of TLR signalling in human MoDCs upon *H. pylori* infection was analysed.

Blocking of TLR signalling of human MoDCs by neutralising antibodies was shown to inhibit pro- and anti-inflammatory cytokine secretion; but distinct TLRs were shown to induce different cytokine secretion. While the release of IL-6 by MoDCs upon *H. pylori* stimulation was affected by the blockage of TLR-2 /-4 and -5, since all are able to activate IL-6 expression (243), the release of IL-12p70 was mainly influenced by TLR-4 neutralisation, suggesting that predominantly TLR-4 was responsible for the secretion of IL-12p70 upon *H. pylori* infection.

The release of IL-12p70 is synergistically enhanced by the activation of MyD88 and TRIF (244). Importantly, TLR-4 is the only receptor that can activate both. Initially, TLR-4 recruits TIRAP at the plasma membrane and facilitates the recruitment of MyD88 to trigger the initial activation of NFκB and MAPK (245). Subsequently, TLR-4 undergoes endocytosis and is transported to the endosome, where it forms a signalling complex with TRAM and TRIF, to initiate the TRIF-dependent pathway that leads to IRF3 activation as well as the late-phase activation of NFκB and MAPK (246) (247) (248).Thus, TLR-4 activation generates both, type I interferons and inflammatory cytokines. While NFκB signaling pathway is essential for the induction of IL-12p35 transcription, IRF plays a major role in the transcriptional activation of the IL-12p35 gene (249). Hence, type I interferons were shown to stimulate IL-12p70 production through an autocrine-paracrine loop (250). In contrast, TLR-2 activation can only induce the expression of messenger RNA (mRNA) encoding the p40 and p19 subunits of IL-23, but not the mRNA expression of the p35 subunit of IL-12 (215).

TLR signalling did not only influence IL-12p70 secretion, but also affected the production of IL-10. Neutralisation of TLRs on MoDCs upon *H. pylori* infection identified TLR-4 and in part TLR-2 as inducers of IL-10 secretion, suggesting that their activation might be responsible for the acquisition of a tolerogenic phenotype. Indeed, neutralisation of TLR-2 and -4 led to the restoration of DC maturation upon *H. pylori* infection, indicating that the secretion of IL-10 was responsible for the weak expression of co-stimulatory molecules and activation markers. Indeed, in line with our data, Chang and colleagues showed that *H. pylori* was able to induce IL-10 production via p38 MAPK and NFκB activation downstream of DC-SIGN, TLR-2 and TLR-4 signalling in human DCs (251). Furthermore, it has been shown by Buelens et al. that IL-10 is involved in the down-regulation of MHC class II and CD86 expression on the surface of DCs that finally has an inhibitory effect on alloreactive T cell responses (252).

Reduced IL-10 secretion in response to *H. pylori* caused by TLR neutralisation was concomitant to increased DC maturation that might influence the subsequent T cell response.

Indeed, neutralisation of TLR-4 and in part TLR-2 on MoDCs prior *H. pylori* stimulation resulted in lower number of Foxp3-expressing $CD4^+$ T cells, suggesting that blockage of TLR signalling in DCs, leading to low IL-10 production, is responsible of impaired expansion of T_{regs}. Nevertheless, lower levels of other cytokines, e.g. IFN-γ and IL-17A were measured upon T cell co-culture with TLR-4 neutralised *H. pylori*-infected MoDCs, indicating that *H. pylori*-induced DC cytokine secretion rather than the maturation status strongly influenced the subsequent T cell polarisation. Moreover, these data show that *H. pylori*-mediated DC cytokine release was TLR dependent.
The importance of TLR activation during *H. pylori* infection is furthermore reflected by infected patients carrying TLR-4 single nucleotide polymorphisms (SNPs). Those individuals possess high amounts of infiltrating plasma cells that correlate with increased gastritis and a higher risk for atrophy and intestinal metaplasia (253). Interestingly, Figueroa and colleagues discovered TLR-4 SNP (Asp299Gly) to interfere with the recruitment of MyD88 and TRIF in murine macrophages, leading to an impaired induction of TNF-α and IFN-β mRNA (254) that could explain the reduced responsiveness to *H. pylori* LPS (253). The results presented here show for the first time that impaired TLR-4 stimulation during *H. pylori* infection, as it was induced upon TLR neutralisation, led to lower amounts of regulatory T cells. Hence, it can be speculated that increased gastritis in patients carrying TLR-4 SNPs might be caused by decreased numbers of $T_{regs,}$ since depletion of T_{regs} in infected mice was reported to cause an increased gastritis that in turn led to impaired bacterial colonisation (151) (84). Nevertheless, further analysis for the presence of regulatory T cells in samples from TLR-4 SNP carriers should be conducted in order to confirm this hypothesis.

Remarkably, TLR-4 neutralisation and deficient CagA translocation during *H. pylori* infection induced a similar DC phenotype: bacterial stimulation was associated with increased DC maturation and impaired T_{reg} expansion that can be explained by decreased IL-10 secretion, suggesting a crosstalk between CagA and TLR-4 signalling. This hypothesis is reinforced by the findings of Tanaka et al. who demonstrated that *H. pylori* CagA is able to suppress DC function in mice through limiting IRF3 (191), which is downstream of the TRIF pathway. Notably, the activation of both, the MyD88- and TRIF-dependent pathways is necessary for the induction of inflammatory cytokines (244) and DC maturation (255) via TLR-4 signalling in mice. Since no differences in the activation of NFκB in DCs upon *H. pylori* infection were observed, it can be speculated that the impairment of TRIF signalling in MoDCs by CagA might be responsible for the acquisition of a tolerogenic phenotype. However, further experiments are needed to clarify this hypothesis.

In a murine model of *H. pylori* infection, gGT was reported to induce tolerogenic DCs by changing the maturation status (192). However, infection of human MoDCs with wild type or gGT-deficient *H. pylori* led to a similar expression of maturation markers, indicating that DC maturation was not affected by gGT. In contrast, the presence of gGT caused a decreased production of IL-6 and IL-23 by human MoDCs, while the levels of IL-12p70 and IL-10 were hardly affected. Generally, IL-6 and IL-23 are known to induce Th17 cells. Nevertheless, co-cultivation of *H. pylori*-infected DCs with $CD4^+$ T cells in the absence of gGT did not lead to the production of IL-17A. Instead, the expression of Foxp3 was significantly impaired, indicating that *H. pylori* gGT was able to induce T_{reg} expansion upon co-incubation with human MoDCs. Also in the mouse model a gGT-dependent T_{reg} expansion has been described (192), however, the mechanism remained unclear. As CagA- and gGT-deficient *H. pylori* differently affected human DC maturation and cytokine release, especially IL-10, which is known to induce T_{reg} expansion, different mechanisms for the induction of tolerance were suspected.

H. pylori gGT is an enzyme which is able to hydrolyse glutamine and glutathione to glutamate (59). Glutamine deprivation has been reported to induce tolerogenic DCs during EAE (216). Activation of the glutamate receptor led to impaired IL-6 and IL-23 production, favouring the expansion of T_{regs}. During *H. pylori* infection wild type bacteria induced lower levels of IL-6 and IL-23 compared to gGT-deficient *Helicobacter*, leading to the assumption that glutamine deprivation and T_{reg} polarisation might also be related during *H. pylori* infection. Indeed, it could be shown that glutamine deprivation was responsible for reduced IL-6 and IL-23 secretion of human MoDCs upon *H. pylori* infection. Furthermore, the expansion of Foxp3-expressing T cells was shown to be dependent on tolerisation of DCs upon glutamine deprivation by *H. pylori* gGT. In mice, Fallarino *et al.* showed that activation of mGluR4 by glutamate inhibits adenylyl cyclase and the production of cAMP, preventing the expression of IL-6 and IL-23 (256). Decrease of IL-6 and IL-23, in turn, enables the upregulation of Foxp3 (257). Nevertheless, further experiments are needed to clarify the repression of IL-6 and IL-23 as well as the induction of T_{reg} expansion in human MoDCs as a result of glutamine conversion into glutamate by gGT.

Together these data show that *H. pylori* induces tolerogenicity in human MoDCs, thereby favouring a regulatory T cell response. The main virulence factors involved in the induction of tolerance are CagA and gGT. This is sustained with *in vivo* data from human patients, showing only a weak prevalence of CagA/gGT-double-deficient *H. pylori* strains in infected

patients (Formicella et al.; Manuscript submitted) and thereby confirming that these two virulence determinants are needed for bacterial persistence.

Summary

6. Summary

H. pylori infection is one of the most prevalent infections worldwide and is characterised by its persistence, which is presumably facilitated through the induction of tolerance. So far, there are insufficient data about the influence of *H. pylori* on the human immune response. However, DCs are attributed a special role for the initiation of tolerance in mice. The investigation of the implication of *H. pylori* on human DCs might help to understand how *H. pylori* is influencing the immune response of its host. Therefore, MoDCs represent a useful mean for the *in vitro* analysis.

In the present study it has been shown for the first time that *H. pylori* infection induces a tolerogenic phenotype in human MoDCs, thereby causing a regulatory T cell response which in turn can favour *H. pylori* persistence. These findings emphasize the importance of human DCs for the stimulation of the adaptive immune response towards *H. pylori*.

Moreover, CagA and gGT were identified as the main virulence factors responsible for DC tolerisation. In addition, two different molecular mechanisms were described. Primarily, it was shown that activation of TLR signalling on human MoDCs by *H. pylori* induces cytokine secretion, influencing DC maturation themselves and T cell polarisation in parallel. Furthermore, the production of IL-10 upon *H. pylori* infection in the presence of CagA was demonstrated to cause STAT3 activation and the inhibition of DC maturation, favouring the expansion of T_{regs}. Additionally, an increase of T_{regs} was also observed upon *H. pylori* infection in the presence of gGT. The secretion of gGT influenced DC cytokine release through glutamine deprivation and thereby influenced the subsequent T cell response.

In conclusion these data show for the first time two independent molecular mechanisms involved in the induction of tolerance during *H. pylori* infection in human.

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Lebenslauf

Lebenslauf

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