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DISSERTATION

Inhibition of antigen presentation by primary antigen presenting cells through *Helicobacter pylori*

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ABBREVIATIONS

Ag	antigen
AP-1	activator protein 1
APC	antigen presenting cell
APZ	antigenpräsentierende Zelle
BabA	blood group antigen-binding adhesin
BHI	brain heart infusion
BSA	bovine serum albumin
c-RAF	cellular Rapidly Accelerated Fibrosarcoma
cag-PAI	cytotoxicity associated pathogenicity island
CagA	cytotoxin-associated gene A
Cam	chloramphenicol
CCL21	C-C motif ligand 21
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
Cdk	cyclin-dependent kinase
CLIP	class-II-associated invariant-chain peptide
CTLA4	cytotoxic T lymphocyte antigen 4
DC	dentritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-i
DMEM	Dulbecco’s modified Eagle's medium
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal receptor kinase
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
Foxp3	forkhead box P3
GGT	γ-glutamyl transpeptidase

GILT	IFN-γ-induced lysosomal thiol reductase
GM-CSF	granulocyte-macrophage colony-stimulating factor
H. pylori	*Helicobacter pylori*
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hi	heat inactivated
HLA	human leukocyte antigen
ICAM	Intercellular adhesion molecule
IFN	interferon
Ii	invariant chain
IL	interleukin
iNOS	inducible nitric oxide synthetase
JAK-STAT	Janus kinase-Signal Transducer and Activator of Transcription
Kan	kanamycin
LB medium	Lurie-Bertani medium
LFA-1	leukocyte functional antigen-1
LPS	lipopolysaccharide
M. tuberculosis	*Mycobacterium tuberculosis*
MALT	mucosa associated lymphoma tissue
MAPK	mitogen-activated protein kinase
mDC	myeloid dendritic cell
MFI	median of the fluorescence intensity
MHC	major histocompatibility complex
MOI	multiplicity of infection
Myc	myelocytomatosis oncogene
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
NFAT	nuclear factor of activated T-cells
NI	not infected
NKT	natural killer T-cell
NO	nitric oxide
NOD I	nucleotide-binding oligomerisation domain protein I
PAMP	pathogen-associated molecular patterns

PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PMA	phorbol 12-myristate 13-acetate
PRR	pattern (or pathogen) recognition receptor
RNA	ribonucleic acid
Rpm	rounds per minute
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
SabA	sialic acid-binding adhesin
SD	standard deviation
SEM	standard error of the mean
T4SS	type IV secretion system
TCR	T-cell receptor
TGF-β	transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
Tregs	regulatory T-cells
UreB	Urease-β subunit
VacA	vacuolating cytotoxin A
WHO	World Health Organization
WT	wildtype

ABSTRACT

Helicobacter pylori (*H. pylori*) colonizes the stomach of approximately half of the world's population. Even though the infection remains asymptomatic in most cases, it is strongly associated with the development of chronic gastritis, peptic ulcer disease and gastric cancer or lymphoma. Gastric colonization by *H. pylori* leads to local inflammation and the induction of a specific immune response in which antigen-presenting cells (APCs) are most likely involved. This immune response, however, is not able to prevent the persistence of the bacteria. *H. pylori* may interfere with antigen presentation, and bacterial virulence factors, e.g., vacuolating cytotoxin A (VacA), gamma-glutamyl transpeptidase (GGT) or urease, might be involved in this process. The aim of this study was to investigate effects of *H. pylori* on antigen presentation by human monocyte-derived macrophages and dendritic cells in comparison with APCs of the THP-1 cell line.

To overcome known direct alterations of phagocytosis and the T-cell response by *H. pylori,* we modified a previously described assay, which enables assessing human APC functions by using murine T-cell hybridoma cells restricted by human leucocyte antigen (HLA) alleles. THP-1 cells or monocyte-derived macrophages and dendritic cells generated from HLA-DR1-positive donors were exposed to the wildtype *H. pylori* P12 strain (or mutant strains lacking virulence factors) and pulsed with the antigen 85B (AG85B) of *Mycobacterium tuberculosis*. The interleukin-2 secretion by Ag85B-specific hybridoma cells was then evaluated as a correlate of antigen presentation.

H. pylori-incubated macrophages and dendritic cells but not cells left without incubation with *H. pylori* showed an impaired capacity to present AG85B, which was not due to down-regulation of cellular HLA-DR surface expression by the bacteria. In contrast, antigen presentation by THP-1 cells was not inhibited by *H. pylori*. Further experiments demonstrated that presentation of AG85B was inhibited when it was added to dendritic cells up to 24 hours before, or 48 hours after the incubation with *H. pylori*. The inhibitory effect of *H. pylori* was not related to the expression of VacA-, GGT-, or urease, since bacterial mutants with depletions of these proteins yielded similar results. The observed inhibition of antigen presentation, however, was not *H. pylori*-specific; when we used LPS or *Escherichia coli* instead of *H. pylori* antigen presentation

also was inhibited.

In conclusion, the newly established assay allows studies on antigen presentation in the context of *H. pylori* infection and should be helpful in gaining further information on the molecular mechanisms involved in the inhibition of antigen presentation by APCs incubated with *H. pylori*. The observed inhibitory effect of *H. pylori* (and *E. coli* or LPS) might be related to the ability of professional APCs to discriminate between potentially harmful inflammatory antigens and non-inflammatory antigens.

ZUSAMMENFASSUNG

Das Bakterium *Helicobacter pylori* (*H. pylori*) besiedelt den Magen von circa der Hälfte der Weltbevölkerung. Erkrankungen wie chronische Gastritis, Magenulzera und Magenkarzinome oder -lymphome werden sehr stark mit der Besiedlung mit *H. pylori* assoziiert, auch wenn die Infektion in den meisten Fällen asymptomatisch verläuft. Die Besiedlung des Magens führt zu einer lokalen Entzündung sowie einer systemischen Immunantwort, in die antigenpräsentierende Zellen (APZ) sehr wahrscheinlich involviert sind. Dennoch verhindert die Immunantwort nicht die Persistenz des Bakteriums im Organismus. *H. pylori* kann die Antigenpräsentation beeinflussen, und Virulenzfaktoren wie das vakuolisierende Zytotoxin (VacA), die Gamma-Glutamyl-Transpeptidase (GGT) oder Urease sind wahrscheinlich in diesen Prozess involviert. Ziel dieser Arbeit war es, den Effekt von *H. pylori* auf die Antigenpräsentation von humanen, aus Monozyten gewonnenen Makrophagen und dendritischen Zellen mit APZ der THP-1-Zelllinie zu vergleichen.

Um bekannte hemmende Effekte von *H. pylori* gegenüber der Phagozytose und der T-Zell-Antwort auszuschließen, modifizierten wir einen zuvor beschriebenen Antigenpräsentations-Assay, welcher ermöglicht, die Zellfunktion humaner APZ mit Hilfe muriner T-Zell-Hybridomzellen zu beurteilen. THP-1 Zellen oder aus humanen Monozyten differenzierte Makrophagen und dendritische Zellen (gewonnen von HLA-DR-1-positiven Blutspendern) wurden mit dem *H. pylori* Wildtyp des P12 Stammes (oder Mutanten welchen Virulenzfaktoren fehlen) infiziert und mit dem Antigen 85B von *Mycobakterium tuberculosis* inkubiert. Die Interleukin 2-Sekretion der Antigen 85B-HLA-DR-1-spezifischen T-Zell-Hybridomzellen wurde als Korrelat für die Antigenpräsentation gemessen.

Mit *H. pylori* inkubierte Makrophagen und dendritische Zellen zeigten, im Vergleich zu Zellen, welche nicht mit *H. pylori* inkubiert wurden, eine verminderte Kapazität, Ag85B zu präsentieren, wobei die Expression der HLA-DR-Moleküle an der Zelloberfläche unverändert blieb. Im Vergleich hierzu war die Antigenpräsentation von mit *H. pylori* inkubierten THP-1 Zellen nicht inhibiert. Weiterführende Experimente zeigten, dass die Präsentation von Ag85B durch dendritische Zellen bis zu 24 Stunden vor und 48 Stunden nach der Inkubation mit *H. pylori* reduziert blieb. Dieser hemmende Effekt von *H. pylori* konnte nicht auf die Expression von

VacA, GGT oder Urease zurückgeführt werden, da Bakterienmutanten, denen diese Proteine fehlten, vergleichbare Ergebnisse erzielten. Allerdings war die beobachtete Hemmung der Antigenpräsentation nicht *H. pylori*-spezifisch; LPS oder *Escherichia coli* an Stelle von *H. pylori* verringerten ebenfalls die Antigenpräsentation.

Zusammenfassend kann man festhalten, dass der neu etablierte Assay Studien zur Antigenpräsentation im Kontext einer *H. pylori*-Infektion ermöglicht. Außerdem sollte der Assay helfen, weitere Informationen über die molekularen Mechanismen, welche in der Hemmung der Antigenpräsentation durch mit *H. pylori* inkubierte APZ eine Rolle spielen, zu gewinnen. Der beobachtete, hemmende Effekt durch *H. pylori* (und *E. coli* oder LPS) mag mit der Fähigkeit von professionellen antigenpräsentierenden Zellen, zwischen möglicherweise schädlichen entzündlichen Antigenen und nicht entzündlichen Antigenen zu unterscheiden, in Zusammenhang stehen.

1 INTRODUCTION

1.1 *Helicobacter pylori* - the human stomach pathogen

Helicobacter pylori (*H. pylori*) has colonized the human stomach since the appearance of modern humans in Africa 200.000 years ago [1]. It was first discovered by G. Bizzozero in 1893, who saw spiral organisms in the stomach of dogs, a few years later, these organisms could be confirmed in human patients by W. Krienitz [2, 3]. Back then, it was thought impossible for any organism to survive in the acidic environment of the stomach, so it was hypothesized that those organisms were the result of contaminated food. It was not until 100 years later that Barry J. Marshall and J. Robin Warren managed to isolate these bacteria from biopsy samples and confirm that they were not only permanently colonizing the human stomach as commensals but also involved in the development of gastric diseases [4]. In 2005 they were awarded the Nobel Prize in Physiology or Medicine for their work.

1.1.1 *Helicobacter pylori* as a pathogen

Nowadays, it is known that more than 50% of the world's population is infected with this gram-negative bacterium [5, 6]. Its transmission is not fully understood but it is known that infection is usually acquired in early childhood and normally persists throughout life if not eradicated by antibiotic therapy. Longtime infection with *H. pylori* always causes a histologically visible gastritis which can grow into an active chronic gastritis resulting in either peptic ulcer disease or atrophic gastritis [7]. Most patients have no outward symptoms, but 10-20% of patients develop discomforts and gastritis or even different forms of gastric cancer. The risk of distal gastric adenocarcinoma increases with *H. pylori* colonization and there is an up to six fold higher risk of developing gastric mucosa associated lymphoma tissue (MALT) lymphoma [8]. Every year 803.000 people die from gastric cancer which makes it the second most common cause of cancer-related death in the world [6]. These findings led to *H. pylori* as being declared a group 1 carcinogen by the World Health Organization (WHO) in 1994 [9]. Even though this classification was followed by extensive research work, it is still not completely understood how *H. pylori* induces pathogenic mechanisms, and why, although recognized by the human immune system, this does not lead to its eradication. Despite all these unanswered questions, it is possible

to eradicate the bacterium from the human stomach. The first line therapy is a triple medication with a proton pump inhibitor and two antibiotics, which are clarithromycin and either amoxicillin or metronidazole. In general, this led to eradication in 95% of patients, but through bacterial resistance against one of the components, eradication rates are only about 70% in recent studies [10].

Interestingly, some studies show a positive effect of *H. pylori* colonization of the stomach: they report a lower incidence of gastro-esophageal reflux disease in *H. pylori*-positive patients, which could be explained by higher pH levels of gastric juice [11, 12]. It seems probable that two organisms that have co-existed for 200.000 years do not only harm each other but are also useful to in some respects. Still, the damage an *H. pylori* infection can cause leads to the understanding that most patients would profit from an eradication of the bacterium from their stomachs. Antibiotic therapy is helpful here, but it has drawbacks in creating resistances and high costs, and it also does not prevent re-infection. A vaccine would be a cost-effective method to diminish *H. pylori*-associated diseases on a population level, without the disadvantages of antibiotics [13].

1.2 Bacterial virulence factors

H. pylori has adapted to the inhospitable conditions found in the human stomach which include acidity and peristalsis, normally inhibiting bacterial colonization. The Gram-negative, microaerophilic and spiral shaped bacterium has developed several strategies and virulence factors in this adaptation process.

1.2.1 Urease, flagella and adhesins

H. pylori is urease-positive, an enzyme which catalyzes urea cleavage into CO_2 and NH_3. The resulting ammonia buffers the low pH at the gastric mucosal surface, ensuring bacterial survival until it enters the mucosal layer [14]. Due to its helical shape and its possession of two to six unipolar, sheathed flagella, it is highly mobile and can enter the mucosal layer rapidly where the pH level is less acidic [15]. Once below the mucus, *H. pylori* adheres tightly to the underlying epithelial cells via adhesion molecules such as BabA, SabA or AlpAB and via modifications of cell membrane proteins and of cytoskeletal proteins [14]. It can also stay free-living in the gastric mucus layer [6]. Adhesion is crucial in the ability of *H. pylori* to persist and cause disease, therefore, adhesins are seen as virulence factors. In addition to the contribution to colonization, adherence results in signal transduction and activation of nuclear factor kappa-light-chain-

enhancer of activated B-cells (NF-κB) which is important in the inflammatory response during infection [16].

1.2.2 The cytotoxicity-associated pathogenicity island

One of the factors responsible for inflammation is the cytotoxicity-associated pathogenicity island (cag-PAI). This 40 kb genetic fragment contains 31 genes that encode for proteins which form a type IV secretion system (T4SS), capable of transferring bacterial proteins to the host cell's cytoplasm [17]. Until now, two forms of molecules that are brought into the host cell through the T4SS are known: cytotoxin-associated gene A (CagA) and peptidoglycans. Recognition of the gram-negative bacteria peptidoglycans by the intracellular nucleotide-binding oligomerisation domain protein I (Nod I) leads to NF-κB activation and gene expression encoding pro-inflammatory cytokines, including interleukin 8 (IL-8) [17, 18]. CagA tyrosine domains can be phosphorylated and subsequently induce the mitogen-activated protein kinase (MAPK) pathway and the extracellular signal receptor kinase (ERK) signaling pathway [16, 17]. This results in morphological changes and cell proliferation in epithelial cells [15]. CagA also plays a role in the increase of IL-8 secretion via ERK through NF-κB activation [19]. In a phosphorylation-independent pathway, CagA can lead to a disruption of epithelial tight junctions, tissue damage and inhibition of B cell proliferation by suppressing the Janus kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signaling pathway [16, 17].

1.2.3 The vacuolating cytotoxin A

Another important virulence factor is vacuolating cytotoxin A (VacA), so called because of its vacuolating activity in vitro [15]. It is considered responsible for the gastric epithelial erosion observed in infected mice [20]. VacA induces epithelial permeabilization through channel formation in the plasma membrane of the target cell, resulting in release of nutrients such as urea [21]. Enhanced transport of Fe^{3+} and Ni^{2+} ions or larger molecules is possible due to the loosening of tight junctions by VacA [7]. The protein is further found in the inner mitochondrial membrane where it activates the caspase-3-dependant cell death signaling cascade via cytochrome c release, resulting in mitochondria-dependent apoptosis [5]. By activating p38 MAPK, a kinase which participates in inflammation by promoting the expression of pro-inflammatory cytokines and cyclooxigenase-2, VacA also plays a role in the development of inflammation in the gastric mucosa [7]. Another significant way in which VacA contributes to

pathogenesis is by inhibiting the processing of antigens by B cells and their presentation to $CD4^+$ T-cells, as well as T-cell activation and proliferation [22, 23].

H. pylori strains which are CagA- and VacA-positive are called type I strains and are considered to be more pathogenic, which means that patients infected with type I strains more frequently develop ulcers and gastric cancer, whereas infections with type II strains normally remain asymptomatic [24].

1.2.4 The γ-glutamyl transpeptidase

A γ-glutamyl transpeptidase (GGT) was identified as a further virulence factor of *H. pylori*. It impairs T-cell proliferation, probably by disruption of Ras-dependent signaling, leading to induction of a cell cycle arrest [25]. GGT also interferes with DC maturation, contributing to immune tolerance through regulatory T-cell activation [26].

Despite all the strategies *H. pylori* has developed to survive in the human stomach, there is a vigorous innate and acquired immune response, which, however, does not lead to bacterial eradication. This indicates a complex deregulation of the immune system by *H. pylori*. Considering that the bacterium has evolved together with its host, it seems logical that it is able to maintain a balance between activating inflammatory processes and protecting itself from the negative consequences [7].

1.3 Basics of the human immune system and its acting cells

The immune response towards bacterial pathogens can be divided into an innate and an adaptive response. The innate response is generally an initial non-specific process, which reacts quickly to signal infectious danger with the aim of killing bacteria, consistent of different types of phagocytes. By contrast, the adaptive immune response is delayed and antigen-specific, leads to the activation of T-, B- and memory cells and is shaped by the innate immune response [27].

1.3.1 Macrophages – bacterial killers

Macrophages are a mature form of monocytes and resident in almost all tissues. They can be differentiated into two cell types: M1 and M2 macrophages. Whereas M1 macrophages induce inflammation and post-infectious pathogenesis, M2 macrophages are thought to be anti-inflammatory immunomodulators. One of the major functions of M1 macrophages is to engulf and kill invading microorganisms; therefore, they belong to the group of phagocytes together

with granulocytes and dendritic cells (DCs). Recognition of microorganisms or antigenic material in general is possible through pathogen recognition receptors (PRR) which include TLRs (Toll-like receptors), the NOD receptor, the mannose receptor, and several others. These receptors are widely expressed on phagocytic cells and they are able to detect non-specific pathogenic structures such as enzymes or membrane particles which are evolutionaryly conserved and called pathogen-associated molecular patterns (PAMPs) [28]. Besides antigen internalization and antigen disruption, phagocytes in general, but especially macrophages, induce the production of several immune modulatory molecules like cytokines and chemokines. These can increase the permeability of blood vessels, allowing proteins and fluid to pass into the tissue or attract more macrophages and other inflammatory cells like neutrophils to the site of infection. All effects caused by cytokines and chemokines enhance the level of inflammation. Furthermore, engulfment of microorganisms by phagocytosis leads to their degradation, generating peptides that can be presented by major histocompatibility complex (MHC) molecules to T-cells to enhance the immune reaction. There are two major classes of MHC molecules: MHC classes II and I. MHC class I molecules are found on nearly every cell and present proteins to cytotoxic T-cells ($CD8^+$). Only certain immune cells, particularly macrophages and DCs, express MHC class II molecules. These cells present antigens via MHC class II molecules, leading to $CD4^+$ helper T-cell activation and differentiation of B-cells into antibody-producing cells [29].

1.3.2 Dendritic cells – inducers of the adaptive immune system

DCs are professional antigen-presenting cells (APCs) and play an important role in initializing the adaptive immune response due to their ability to stimulate naïve T-cells. They originate from pluripotent hematopoietic stem cells in the bone marrow, where myeloid and lymphoid progenitors exist. DCs differ in shape, phenotype and properties depending on their maturation level. Immature DCs reside mostly in the tissue of the body; initially they are highly phagocytic and express low levels of MHC II or the co-stimulatory ligands B7.1 (CD80) and B7.2 (CD86). Whereas macrophages mainly recognize extracellular antigens, DCs specialized in capturing any antigenic material via different mechanisms like phagocytosis, macropinocytosis and receptor-mediated endocytosis in infected tissue [30]. Once they have engulfed an antigen and received activation stimuli via pathogen PRRs similar to those in macrophages, they mature and express high levels of MHC molecules, B7 co-stimulators, as well as several cytokine receptors, and adhesion molecules [31]. As soon as DCs become activated, they can be called “highly

effective" or "most potent" APCs because their engulfing capacity is drastically reduced and their ability to process antigens and to present them via long lived MHC II and I molecules is augmented. Activated DCs migrate to the secondary lymphoid tissue.

After internalization of the pathogenic material, it is either proteolyzed in the proteasome followed by linkage to the MHC I complex in the endoplasmic reticulum, or transported into the endosome, which matures and fuses with lysosomes and vesicles containing MHC II molecules.

Endosome maturation for MHC class II presentation comprises acidification and fusion with lysosomes. Those endolysosomes contain proteases, e.g. cathepsins that are activated at low pH, which then degrade the antigenic proteins [29, 32, 33]. Disulfide bonds of proteins can be reduced by IFN-γ-induced lysosomal thiol reductase (GILT) before the proteins are degraded in the endolysosome [34]. Newly synthesized MHC class II molecules must be prevented from premature antigen binding in the endoplasmic reticulum. Therefore, a part of the invariant chain (Ii) blocks the peptide-binding groove. The Ii also targets the delivery of the MHC II molecule to an endosomal compartment, where the antigen loading can occur. There, the Ii is cleaved until only a short segment remains, which is called class-II-associated invariant-chain peptide (CLIP) and, which is finally displaced by an antigenic peptide [35].

The traditional view of antigen presentation is that intracellularly synthesized antigens are presented through MHC I molecules and activate $CD8^+$ cytotoxic T-cells, whereas extracellular antigens are presented by APCs via MHC II molecules to $CD4^+$ T helper cells [36]. In addition to these two mechanisms of antigen presentation, DCs possess the ability to process exogenous proteins which are incorporated via endocytosis, and to present them to $CD8^+$ cytotoxic T-cells via MHC I molecules. This process is known as cross-presentation [36]. Under special conditions, specific macrophages and other cells are able to cross-present, but normally this quality is unique to DCs [31, 33].

Likewise for monocytes and macrophages, detection of pathogens occurs through PRRs in DCs, which are widely expressed in and on the surface of immature DCs. An important family of PRRs is the TLR family, an evolutionaryly conserved transmembrane protein family. TLRs commonly activate the MyD88 pathway, resulting in the activation of NF-κB and MAPK signaling. This mediates the immune response toward the induction of inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), IL-12, and interferon. The TLRs

recognize different bacterial patterns e.g. double-stranded RNA, lipopolysaccharide (LPS), single-stranded RNA or unmethylated CpG DNA. Signaling by TLRs results in an alteration of chemokine receptors on DCs, which facilitates their entry into peripheral lymphoid tissue, such as the expression of the receptor CCR7, which binds to the chemokine CCL21 produced by lymphoid tissue [37]. Another group of PRRs is the group of C-type lectins consisting of receptors like the mannose receptor, DEC205, DC-SIGN, and others. These receptors recognize a wide variety of bacteria and viruses and induce the endocytic pathway, where the antigen is processed and presented on MHC I molecules. Recognition of intracellular bacteria may be facilitated by the NOD proteins [38].

The main purpose of antigen processing and presentation by DCs is the initiation of T-cell immunity through activation of naïve T-cells [33]. DCs provide signals that lead to T-cell differentiation into different subgroups of effector T-cells. This process depends on signals of the local environment, especially on cytokines delivered from APCs. For example $CD11c^+$ and $CD11c^-$ DCs can induce Th1 and Th2 responses, respectively, while the Th1 induction is favored by the production of IL-12 of the DCs [38, 39]. Th2 induction can be favored by DCs stimulated by mast cells and prostaglandin E_2 (PGE_2) and by the cytokine IL-4, but there is no evidence that DCs produce IL-4 [39]. Another T-cell subgroup, the regulatory T-cells (Tregs), require TGF-β for differentiation, which can be provided by DCs [40] .

Only about 1% of circulating peripheral blood mononuclear cells (PBMCs) are DCs, hence isolation in sufficient numbers and purity is difficult. Most information about DCs comes from in vitro studies with generated DCs. They can be generated either from bone-marrow-derived $CD14^+$ monocytes or PBMCs by cultivating them in GM-CSF and IL-4 [31, 41]. These cells are regarded as myeloid dendritic cells (mDCs). Immature monocyte-derived DCs can also mature in the presence of LPS, TNF-α, or a cocktail containing IL-6, IL-1β, TNF-α and PGE_2 [42].

1.3.3 T-cell immunity

T-lymphocytes are an essential element of the adaptive and the innate immune system. They accomplish many different functions, which is why there is a variety of different subsets of T-cells. The most important ones are the $CD8^+$ and the $CD4^+$ T-cells as well as Tregs. All subgroups develop from progenitors that are derived from pluripotent hematopoietic stem cells in the bone marrow and they all migrate through the blood to the thymus to start their maturation

[43]. Early in T-cell development two distinct types of T-cells are produced, the major α:β T-cell population and the minor γ:δ T-cells which express different types of T-cell receptors (TCRs). Another group of T-cells, the natural killer T-cells (NKT-cells) which express a NK1.1 receptor, divert in this early state. The α:β T-cells undergo two processes called positive and negative selection in the thymus. Positive selection means that T-cells with TCRs that are able to recognize self-peptide:self-MHC complexes and thus function in a self-MHC restricted response to antigens, were selected not to undergo apoptosis. Negative selection refers to the deletion of those T-cells, which react too strongly with ubiquitous self-antigens presented via a MHC molecule. The outcome are mature, but still naïve T-cells which are either MHC I or MHC II restricted, depending on the co-receptor CD8 or CD4, respectively, and which are self-tolerant [37]. The co-receptors bind to the MHC molecule during TCR:MHC:peptide interaction to stabilize that interaction [44].

After the process of positive and negative selection, T-cells migrate to the secondary lymphoid organs, a process called “homing”, to encounter antigen presented by APCs, preferentially by DCs [45]. The antigenic stimulation is often called “T-cell priming”. Three signals are needed for successful T-cell priming: signal one is the specific binding of the TCR with the MHC:peptide complex at the same time as the ligation of the co-receptor CD4 or CD8 to the MHC class II or I molecule. Signal two is the co-stimulatory non-specific binding of the CD28 receptor of the T-cell to a B7 molecule (CD80 or CD86) on the professional APC. And signal three is realized by the delivery of cytokines such as IL-4, IL-6, IL-12, TGF-β or IFN-γ [46]. Signal one or the interaction between the TCR and the MHC:peptide complex on the APC is specific, meaning that every TCR is restricted to recognize one MHC molecule. In humans, these are also called human leukocytic antigen (HLA) molecules. HLA I molecules are divided into HLA-A, HLA-B, and HLA-C; and HLA II molecules are divided into HLA-DP, HLA-DQ, and HLA-DR. Each group can be yet again divided into more subgroups, and every TCR is restricted to recognize one of these molecules and thus can only be stimulated by this specific one. Several other adhesion interactions exist between a DC and the T-cell, mediated through integrins called leukocyte functional antigen-1 (LFA-1), ICAM-3 and CD2 on the T-cell and ICAM-1, ICAM-2, DC-SIGN and CD58 on the DC [37, 47]. These molecules can be detected on either activated T-cells or mature DCs. The interaction leads to the production of NFAT, AP-1 and NF-κB, following an autocrine IL-2 secretion of the T-cell. This results in the induction of T-cell proliferation and differentiation [48].

Activated cells differentiate into effector T-cells, enabling them to migrate to inflammation sites and to realize an immune attack without the need for co-stimulation. Naïve $CD8^+$ T-cells differentiate into cytotoxic T-cells, which are important in the defense against intracellular pathogens, especially viruses. Virus-infected cells present their antigens through the endogenous pathway via MHC I molecules, and these are recognized by $CD8^+$ T-cells. $CD4^+$ T-cells can differentiate into Th1-, Th2-, and Th17-cells, as well as into Tregs. Th1 differentiation is driven by IL-12 and IFN-γ signaling by the APC, leading to IL-2 and IFN-γ secretion of the Th1-cell itself. Most bacterial infections lead to a Th1 response. Th1-cells work in an antimicrobial way through stimulation of APCs, mainly macrophages, to enhance their phagocytic effect. Signaling through IL-4 induces a functional Th2 response characterized by IL-4 and IL-5 production [37, 49]. Both Th1- and Th2-cells have an important influence on the production of antibodies by B-lymphocytes and thus on the humoral immune response. The $CD4^+$ T-cell group also consists of Tregs, which are T-cells that suppress potentially deleterious activities of other T-cells. They are crucial in the maintenance of self-tolerance and in the prevention of autoimmune diseases. Tregs are CD25- and Foxp3- positive and express high amounts of CD5 and cytotoxic T lymphocyte antigen 4 (CTLA4) [50]. Finally, there is a T-cell population referred to as natural killer (NK) cells, which recognize CD1 rather than MHC I or II molecules [47]. The CD1 family is able to present lipid antigens, including mycobacterial cell wall contents such as phospholipids and lipopeptides. T-cells which are able to recognize antigen presentation via CD1 secrete Th1-like cytokines such as IFN-γ [51].

Every individual has a multitude of T-cells that can detect foreign MHC- or CD1-peptide complexes with single molecule sensitivity, which makes it nearly impossible for pathogens to escape the adaptive immune system [47].

1.4 Immune response to *Helicobacter pylori*

As mentioned above, *H. pylori* is an active stimulator of both the innate and the acquired immune system. It is the cause of humoral and cellular immune responses with local and systemic antibody responses and an intense immune cell recruitment. However, the human immune system is not capable of *H. pylori* eradication.

Due do its special properties *H. pylori* moves quickly through the gastric mucus layer, where it adheres to the gastric epithelium or stays close to it, preferably in the pyloric antrum [52]. This recruits monocytes and phagocytic cells to the gastric mucosa [53].

Recognition of bacterial molecules by the innate immune system is mediated inter alia through TLRs, which are expressed on APCs. In general, this contact leads to the secretion of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8. It also leads to the surface expression of co-stimulatory molecules that are essential for the induction of the adaptive immune response such as CD80 and CD86. However, TLRs, which are typically able to recognize bacterial LPS (TLR 4) or flagellin (TLR 5), do not recognize these products in *H. pylori* infection at all or recognize them only poorly. E.g. recombinant flagellin of *H. pylori* induces much lower concentrations of IL-8 through TLR 5 signaling than recombinant flagellin of *Salmonella typhimurium.* Also, *H. pylori* does not shed flagellin to medium as *Salmonella typhimurium* does, in order to evade TLR 5 signaling [54]. As for TLR 4 signaling, *H. pylori* LPS has a lower biological activity compared to LPS from other gram-negative bacteria. Phosphorylation of lipid A of LPS might play a role in this [55, 56]. Fucosylation of the O side chain of *H. pylori* LPS mimics human Lewis molecules and other blood-group antigens, which might help to evade activation of the innate immune system [57]. Nevertheless, infection with *H. pylori* is associated with an increase in cytokines and chemokines which stimulate the migration of granulocytes, monocytes and lymphocytes into the inflamed mucosa [27]. Other *H. pylori* components such as urease or bacterial DNA are involved in increased IFN-γ and IL-12 production and are thus shifting a Th1 response [58].

Bacteria that cross the epithelial border are immediately detected by phagocytes and subsequently engulfed. Although *H. pylori* is ingested by these phagocytes, it seems to be resistant to phagocytic killing. Additionally, its phagocytosis is delayed and the formed phagosomes are different from normal phagosomes. This effect is only seen in type I *H. pylori* strains, so it is likely that CagA and VacA are involved [59, 60]. But, there are also studies that do not support an involvement of either VacA or the cag-PAI in prolonged intracellular survival, although *H. pylori*-induced homotypic fusions in human monocytes were confirmed [61, 62]. The involvement of urease in delayed phagocytosis has also been reported [63, 64].

Phagocytes generate nitric oxide (NO) through an inducible nitric oxide synthetase (iNOS). NO is an important component of the innate immune response in order to kill bacteria. It is highly

reactive, so to avoid being killed by it *H. pylori* produces an arginase which converts L-arginine to urea and L-ornithine. L-arginine is needed to produce NO by iNOS, so *H. pylori* arginase competes with iNOS for their common substrate. This regulates NO synthesis and, in this way improves bacterial survival [7]. The gene encoding for arginase is rocF, and as expected, rocF mutants are effectively killed in an NO-dependent manner [27]. Although the relevance of NO and iNOS in humans compared to rodents is not fully affirmed, Kroencke et al. reviewed that NO is involved in infectious diseases in humans, e.g. as in vitro killing of bacteria by NO or iNOS production in *E. coli*- or *Staphylococcus aureus*-infections of humans and in diseases of chronic inflammation in humans [65], so it is likely to be involved in *H. pylori* infection of humans as both situations apply for *H. pylori* infection. Also, Thomas and Mattila reviewed that due to different conditions and differences in the experimental setting, the presence of iNOS and/or NO varies a lot in different studies of human macrophages [66].

Several studies have validated that *H. pylori* inhibits T-cell proliferation [23, 25, 67, 68]. Gerhard et al. showed that a decreased proliferation of lymphocytes is caused by a cell-cycle arrest in the G1 phase. They used different *H. pylori* strains for co-incubation with T- and B-lymphocytes isolated from *H. pylori*-infected patients. In their study, the cell-cycle arrest was neither cag-PAI- nor VacA-dependent, but due to a low-molecular-weight protein which was secreted by almost all bacteria strains. Although antigen-specific T-cell activation, tested through nuclear factor of activated T-cells (NFAT) -activation, IFN production, and CD25 or CD69 up-regulation, remained intact, it was not possible for T-cells to enter the S-phase of the cell-cycle [69]. Alternatively, another group showed that cell-cycle arrest, and thus inhibition of T-cell proliferation after *H. pylori* infection, can be attributed to VacA. This might be through inhibition of the calcium signaling pathway, leading to a blocked NFAT activation, which blocks IL-2 production [23]. Gebert et al. used much higher concentrations of bacterial supernatants, so it is possible that the inhibition of T-cell proliferation was seen because of a VacA-dependent induction of apoptosis in these cells. Molinari et al. suggested a selective inhibition of the Ii-dependent antigen presentation by VacA and thus leading to a partially blocked T-cell proliferation [22]. Ii-dependent antigen presentation refers to peptides presented via freshly synthesized MHC II molecules instead of MHC II molecules recycled from the cell surface. Both pathways normally complement each other. Later on it was found by Schmees et al. that the proliferation-inhibiting protein is supposed to be the γ-glutamyl transpeptidase (GGT) of *H. pylori*. The inhibition is a result of a G1 phase arrest of the cell-cycle which is characterized by

increased amounts of the Cdk inhibitor p27 as well as reduced cellular levels of cyclin molecules [25]. They found reduced levels of c-Raf phosphorylation and c-Myc signaling in cells incubated with GGT; both are cellular mediators of Ras-dependent signaling. This indicates that a disruption in the Ras-dependent signaling by GGT is responsible for the induction of the cell-cycle arrest in T-cells and the blocked lymphocyte proliferation. Remarkably, GGT did not lead to a decrease in IL-2 secretion, a cytokine, which is essential for T-cell proliferation. IFN-γ was not reduced either, nor was the effect a consequence of T-cell apoptosis. Only low concentrations of supernatant were needed do inhibit T-cell proliferation, which makes an in vivo effect more reasonable [25]. Another study discussed the possibility of cholesterol involvement in immune evasion: they reported a cholesterol dependent increased phagocytosis of *H. pylori* and a vigorous T-cell activation upon cholesterol supplement to medium of *H. pylori* infected murine APCs. Interestingly, they showed that cholesteryl α-glucosides protect *H. pylori* from phagocytosis and reported the *H. pylori* cholesterol-α-glucosyltransferase to be responsible for avoidance of T-cell responses [70]. This effect could be shown for human T-cells as well [68].

Tregs may also contribute to the inability of the immune system to clear *H. pylori* infection. They actively suppress the immune response and have been reported to modulate antibody and T-cell responses during *H. pylori* infection and thereby reduce immunopathology in *H. pylori* gastritis [17, 71].

Some tests have explored the possibility of vaccination against *H. pylori*. In mice, a variety of them were effective, leading to immunization of neonatal mice, but they only decreased the number of bacteria and led to a "post-immunization gastritis". Furthermore, the mechanisms of the effective immunization are not known. All that is known is that MHC II expression and $CD4^+$ T-cells are needed for protection in mice and that most likely neither antibody, IL-4, IL-5, IL-13, IL-12, TNF-α, nor IL-18 are involved in protective immunization [72].

1.5 Aim of the study

APCs are found in the gastric mucosa and are most likely involved in *H. pylori*-specific immune responses. The aim of this study was to investigate whether the incubation with *H. pylori* influences the antigen presentation capability of these cells.

An antigen presentation assay had to be established in which we were able to study the APC:T-cell interaction after incubation with *H. pylori* of the APC without the interference of the bacterium with phagocytosis and T-cell-proliferation.

To this end, a previously described antigen presentation assay was modified in which THP-1 cells, a human macrophage-like cell line, monocyte-derived macrophages and monocyte-derived DCs were incubated with *H. pylori* wild type and then used to stimulate a murine hybridoma T-cell line called F9A6 cells [73]. The F9A6 cells were restricted to HLA-DR1 presenting a peptide of the Ag85B protein of *M. tuberculosis*. Through the use of an *H. pylori*-independent antigen and the corresponding hybridoma cells to measure T-cell activation, we were able to avoid the influence of a potential delayed phagocytosis of *H. pylori* itself by the APCs (e.g. macrophages [59]). We were also able to avoid a potential anti-proliferative and apoptotic effect of *H. pylori* on T-cells [23, 25, 74] because the hybridoma T-cells had no direct contact to the bacteria in our assay.

The influence on antigen presentation of *H. pylori* wild type was compared to *H. pylori* mutants lacking the virulence factors VacA, GGT, or urease. Additionally, the effect of incubation with *H. pylori* was compared to the effect of *E. coli*-incubation or treatment with *E. coli* LPS.

2 MATERIAL AND METHODS

2.1 Instruments

Table 1: Instruments

Instrument	**Manufacturer**
Benchtop Centrifuge 5417C	Eppendorf, Hamburg, Germany
Benchtop Centrifuge 5417R	Eppendorf
Centrifuge Heraeus Megafuge 1.0R/2.0R	Thermo Scientific, Waltham, MA, USA
Centrifuge Heraeus Multifuge 1 S-R/2 S-R	Thermo Scientific
Incubator Thermo Forma Series II Water Jacketed CO2	Thermo Scientific
Incubator Hera Cell/Cell 150	Thermo Scientific
Microscope Olympus IX50	Olympus, Center Valley, PA, USA
FACSScan flow cytometer	BD Pharmingen, San Jose, CA, USA
FACSCalibur	BD Pharmingen
SpectraMax190 plate reader	Molecular Devices, Sunnyvale, CA, USA

2.2 Cell culture

2.2.1 Cell lines

Table 2: Cell lines

Name	**Cell type**	**Source**	**Cell culture medium**
THP-1	Human acute monocytic leukemia cell line	DSMZ: ACC 16	RPMI 1640 (Invitrogen, Gibco, CA, USA),10% heat inactivated FCS

F9A6	T-cell hybridoma cell line	Canaday *et al.*, 2003	DMEM (Gibco), 10% heat inactivated FCS, 2 mM L-glutamine (Invitrogen), β-Mercaptoethanol, 100 U/L penicillin (Invitrogen), 100 µg/L Streptomycin (Invitrogen)

2.2.1.1 THP-1 cells

THP-1 cells were collected from 75 cm^2 flasks (TPP, Trasadingen, Switzerland), $8x10^4$ cells were seeded into 96-well plates (TPP) and incubated in an H_2O saturated atmosphere with 5% CO_2 and 37°C. After overnight substitution of 1 µM PMA (Sigma-Aldrich, Saint Louis, MI, USA) it was verified microscopically that the cells were viable and attached to the bottom of the wells. Shape, viability, and adherence were monitored again on the following day after overnight treatment with 150 U/ml IFN-γ (Sigma-Aldrich) and after every following step.

2.2.1.2 F9A6 cells

The F9A6 cell line is a murine antigen-specific CD 4^+ T-cell line, which recognizes its antigen $Ag85B_{aa97-112}$ in the context of the human MHC class II molecule HLA-DR 1. Without stimulation, the cells do not produce IL-2, but once stimulated, they secrete IL-2 [73]. The cells can be stimulated nonspecifically, e.g. by substitution of PMA and ionomycin or anti-CD3, or specifically via the HLA-DR 1:$Ag85B_{aa97-112}$ complex presented by an APC. To test whether the F9A6 cells could be activated to produce IL-2, the cells were stimulated through anti-CD3 (BD Pharmingen) at 3 µg/ml in pre-coated 96-well plates. Some cells were left unstimulated or treated with cisplatin (Merck Millipore, Calbiochem, Darmstadt, Germany) at 66 pM. Cisplatin induces apoptosis and was used as a control for cell viability. Cells were incubated overnight, and high IL-2 levels were detected when F9A6 cells were stimulated with anti-CD3. In contrast, unstimulated cells were not able to produce IL-2 (Fig. 1a). Viability of cells was lost when cisplatin was added (Fig. 1b).

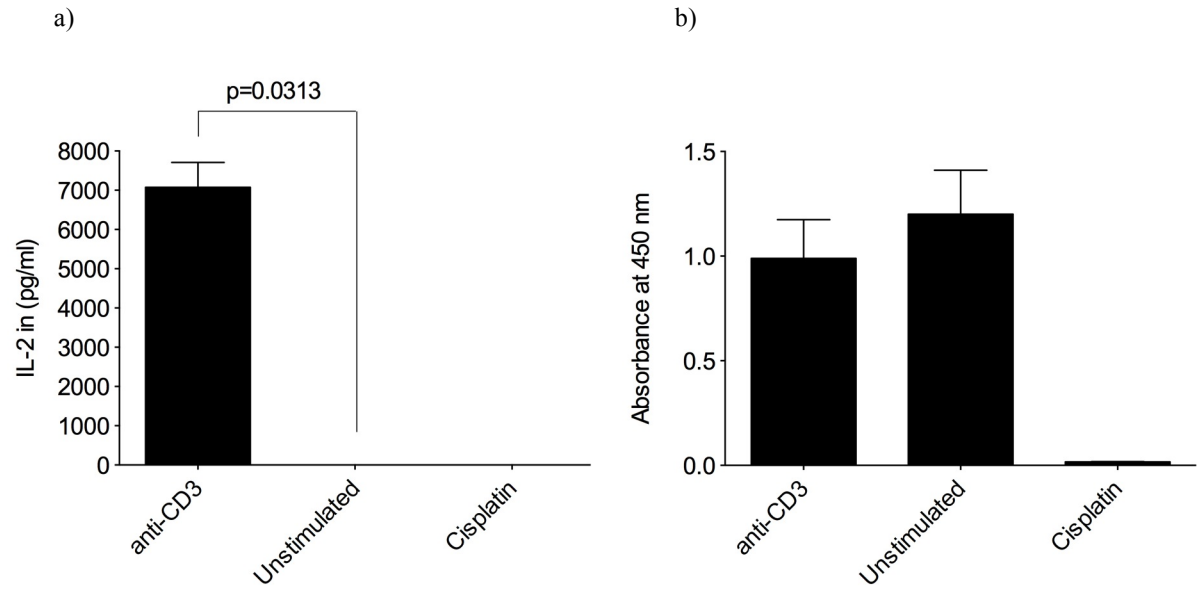

Figure 1: F9A6 cells can be stimulated via anti-CD3
F9A6 cells were stimulated with anti-CD3, left unstimulated or treated with cisplatin, cell activation was quantified by IL-2 levels in the supernatant (a) and cell viability detected via a cell viability kit (b).
Bars represent means of 3 independent experiments; error bars represent SEM. Wilcoxon matched-pairs signed rank test was used.

2.2.2 Cryoconservation of the cells

Aliquots of $1x10^6$ cells were centrifuged for 3 minutes at 800 rpm and room temperature. The pellets were suspended in 1 ml of freezing medium (90% hi FCS/ 10% DMSO) and added to pre-cooled cryotubes (Sigma, Taufkirchen, Germany). Tubes were immediately added to a cryobox, left overnight at -80°C, and then stored in liquid nitrogen.

2.2.3 Thawing and re-cultivation of the cells

Cryotubes were thawed in a water bath at 37°C immediately after withdrawal from liquid nitrogen. The cells were added slowly to 7 ml of pre-warmed heat-inactivated FCS and centrifuged for 3 minutes at 800 rpm and room temperature. The pellets were suspended in appropriate cell culture medium and transferred to 75 cm^2 flasks.

2.2.4 Human primary cells

To generate primary cells from HLA-DR 1-positive blood donors blood from potential donors was collected and analyzed by the tissue typing laboratory (HLA-Labor) of the Charité, Campus Virchow Klinikum (Berlin). Five out of 23 persons tested were positive for the expression of

HLA-DR 1. Donors confirmed their informed consent to collection of blood samples and tissue typing (vote of the ethics committee: EA1/062/11).

2.2.4.1 Isolation of lymphocytes

Human lymphocytes were isolated from peripheral blood by density centrifugation over a Ficoll-Paque^{TM}Premium gradient (GE Healthcare, NJ, USA). 20 ml of anticoagulant-treated blood was diluted 1:1 with PBS (Gibco) and layered on 10 ml Ficoll-Paque^{TM} Premium in 50 ml conical tubes (Sarstedt) and centrifuged without break at 760 x g for 20 min at 20°C. The resulting population consisted mainly of lymphocytes and monocytes called peripheral blood mononuclear cells (PBMCs). PBMCs were taken from the white cell layer of the gradient and washed with fresh, cold PBS at 625 x g for 10 min at 4°C. Following two further washing steps (4°C, 10 min, 350 x g), the cells were resuspended in 20 ml final volume of fresh complete RPMI medium and stored on ice.

2.2.4.2 Magnetic separation of CD14$^+$ monocytes

Monocytes were isolated from PBMCs using CD14$^+$ MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). After washing the PBMCs in MACS buffer (Miltenyi Biotec) at 350 x g for 10 min at 4°C, they were counted and incubated for 15 min at 4°C in the dark with 100 µl of MACS buffer and 5 µl of CD14$^+$ paramagnetic beads per 1x10^7 cells. Then, the cells were washed with MACS buffer. For cell separation, MACS LS columns (Miltenyi Biotec) were prepared as follows: they were placed into the magnetic field of a MACS midi magnet (Miltenyi Biotec) and per 2 x 10^9 cells in total, one LS column was equilibrated by rinsing with 5 ml MACS buffer. After washing, the PBMCs were resuspended in 1 ml MACS buffer per 1 x 10^8 cells and transferred into the equilibrated columns. Once a column reservoir was empty, the column was washed three times with 3 ml MACS buffer. The columns were then removed from the magnetic field and the CD14$^+$ cells were flushed out with 2 ml of MACS buffer and collected in a new 15 ml tube (Sarstedt) filled with 8 ml of fresh medium. Cells were centrifuged (room temperature, 350 x g, 10 min), resuspended in complete RPMI medium, and placed on ice. Depending on the blood donor, percentages of CD14$^+$ cells from PBMCs varied from 10 to 20%.

2.2.4.3 Generation of professional primary APCs

DCs can be generated from human $CD14^+$ monocytes in cell culture medium supplemented with human granulocyte-macrophage colony-stimulating factor (hGM-CSF) and IL-4, which leads to the expression of HLA-DR and other surface molecules such as CD11b as well as to the loss of CD14 expression [31, 41]. To generate immature monocyte-derived DCs, $3x10^6$ monocytes per well were seeded in 6-well culture plates (TPP) in complete RPMI 1640 medium containing 2 mM L-glutamine (Merck, Darmstadt, Germany, or Gibco), 10 mM HEPES (Gibco), 10% FCS, penicillin (100 U/ml) and streptomycin (100 g/ml) (Sigma) supplemented with 1000 U/ml of hGM-CSF (Bayer HealthCare Pharmaceuticals, VA, USA) and 100 U/ml of recombinant IL-4 (R&D Systems, MN, USA). They were incubated in an H_2O saturated atmosphere with 5% CO_2 and 37°C average temperature. 200 µl of fresh medium (RPMI 1640, 2 mM L-glutamine, 10 mM HEPES, 10% FCS, penicillin and streptomycin as well as hGM-CSF and IL-4) were substituted every second day for 7 days. For infection assays and FACS analysis, DCs were collected on day 7 and added to 15 ml tubes. Cells were washed once with RPMI 1640 and 10% FCS without antibiotics before counting and FACS analysis.

CD14 and CD11b surface expression of DCs was routinely monitored by FACS analysis (3.1.4.) Fig. 2 demonstrates that DCs were CD14-negative and CD11b-positive.

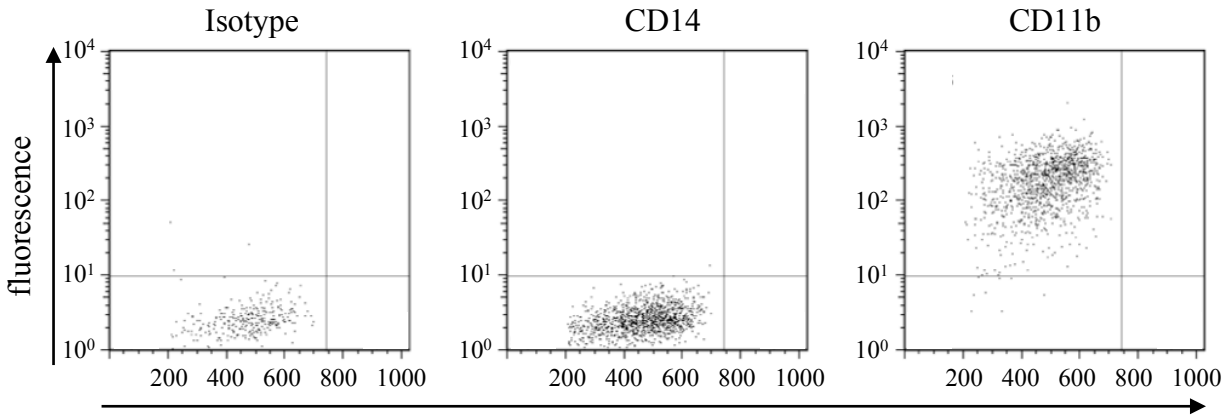

Figure 2: Phenotype of monocyte-derived DCs
CD14-positive monocytes were cultured in RPMI 1640 supplemented with 10% FCS for 7 days. On days 0, 2, 4 and 6, hGM-CSF and hIL-4 was added. DCs were stained with monoclonal antibodies against CD14 and CD11b or the appropriate isotype control. CD14 and CD11b surface expression was determined routinely. One representative experiment is shown.

M1 macrophages were differentiated from monocytes by cultivation of $3x10^6$ cells per well in 6-well culture plates in complete RPMI 1640 medium containing 2 mM L-glutamine, 10 mM HEPES, 10% FCS, penicillin (100 U/ml) and streptomycin (100 g/ml) in the presence of 50 U/ml of hGM-CSF, at 5% CO_2 and 37°C average temperature in an H_2O-saturated atmosphere for 7 days. On days 0, 2, 4 and 6, plates were centrifuged at 300 x g for 5 min at room temperature, supernatants were removed, and the medium was replaced by fresh RPMI 1640 medium containing 10% FCS, penicillin and streptomycin as well as 50 U/ml of hGM-CSF. After centrifugation and removing of the supernatants on day 7, macrophages were collected by the addition of 1 ml of 5 mM EDTA (Gibco) to each well. EDTA-covered plates were left 20 min at 37 °C to induce the detachment of the cells. Afterwards, macrophages were collected and washed three times with PBS to remove the EDTA.

2.3 Flow cytometry

CD14 and CD11b as well as HLA-DR surface expression on DCs was determined by flow cytometry. Additionally, the median of the fluorescence intensity (MFI) of HLA-DR was analyzed, which correlates with the amount of HLA-DR molecules expressed by each cell.

Counted cells were added to 96-well flat bottom plates for staining. Fluorochrome-coupled antibodies specific for CD14, CD11b, or HLA-DR or the appropriate isotype control were added in a concentration of 1:50 and incubated for 25 min in the dark at 4°C. Subsequently, the cells were washed twice with PBS and incubated in PBS with 10% formaldehyd (Sigma) for 20 min at 4°C for fixation. The cells were analyzed on day 7.

Table 3: Antibodies for flow cytometry

Name	**Labelling**	**Clone**	**Company**
Isotype	FITC/PE	X_{40}/X_{39}	BD Pharmingen
anti CD11b	PE	$ICRF_{44}$	BD Pharmingen
anti CD14	PE	MDP_9	BD Pharmingen
anti HLA-DR	FITC	L_{243}	BD Pharmingen

2.4 Cell viability assay

The Colorimetric Cell Viability Kit I (PromoKine, Heidelberg, Germany) was used to determine the cell viability following treatment with *H. pylori*. The assay was performed according to the manufacturer's instructions. As a control, some cells were left untreated (without bacteria or LPS and without Ag85B) and some cells were treated with 66 pM cisplatin (Merck Millipore) for 16 h to induce apoptosis and serve as a negative control. All measurements were done in duplicates.

2.5 ELISA

To detect the IL-2 secreted by the F9A6 cells, sandwich enzyme-linked immunosorbent assay (ELISA) were performed. The Mouse IL-2 DuoSet Kit (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's protocol.

2.6 Bacteriology

2.6.1 *H. pylori* strains and mutants

All *H. pylori* strains used in this study were obtained from the MPIIB Department of Molecular Biology's strain collection.

Table 4: *H. pylori* strains and mutants

Name	Stock number	Mutated gene locus	Antibiotic resistance
P12	P243	WT	-
P12ΔVacA	P216	VacA	Chloramphenicol
P12ΔGGT	P345	GGT	Chloramphenicol
P12ΔUreB	P400	UreB	Kanamycin

2.6.1.1 Cultivation of *H. pylori*

In advance of every experiment, bacteria were taken from a frozen glycerol stock and transferred to an agar plate according to the antibiotic resistance genes inserted in the bacteria. After two to three days of incubation in a microaerophilic environment (5% CO_2, 4% O_2 and 37°C), bacteria

were transferred to fresh plates in different dilutions. Only freshly transferred bacteria (from the day before) were used for infection assays. The bacteria were not used longer than one week after thawing.

2.6.1.2 Media and supplements of *H. pylori*

Table 5: Agar plates

Agar plates	
GC Agar Base (Remel, Thermo Scientific)	36 g
H_2O_{bidest}	ad 1 L
Following autoclaving, the following substances were added:	
Horse serum, inactivated (Biochrom, Berlin, Germany)	100 ml
Vitamin mix	10 ml
Nystatin (2 mg/ml)	500 µl
Trimethoprim (2.5 mg/ml)	2 ml
Vancomycin (10 mg/ml) (Biochrom)	1 ml
According to the bacterial strain, the following antibiotics were added:	
Chloramphenicol (4 mg/ml)	1 ml
Kanamycin (8 mg/ml)	1 ml

Table 6: Vitamin mix

Vitamin mix	
The following substances were used to gain solution 1:	
D (+) glucose	100 g
L-glutamin	10 g
L-cysteine hydrochlorid monohydrat	26 g
Cocarboxylase	100 mg
Ferric (III)-Nitrate Nanohydrate	20 mg
Thiamine hydrochlorid	3 mg
p-aminobenzoacid	13 mg
NAD	250 mg
Vitamin B12	10 mg
H_2O_{bidest}	ad 500 ml
The following substances were used to gain solution 2:	
L-cystein	1.1 g
Adenine	1 g
Guanine-Cl	30 mg
L-arginin monohydrochlorid	150 mg
Uracil	500 mg
H_2O_{bidest}	ad 300 ml
32% HCl	15 ml
Solution 1 and 2 were mixed and H_2O_{bidest} added ad 1 L.	

Table 7: media

Media	
BHI (brain heart infusion) (BD Pharmingen)	36 g in 1000 ml H_2O_{bidest}
Freezing medium	BHI medium with 20% glycerol and 10% heat inactivated FCS or skim milk

2.6.2 *E. coli* strain

The *E. coli* strain used as control was the BL21 wild type (stock number E229 of the MPIIB Department of Molecular Biology's strain collection).

2.6.2.1 Culture of *E. coli*

An aliquot of bacteria was thawed and transferred to Lurie-Bertani medium (LB medium) agar plates in different dilutions and left overnight at 37°C. Plates with single colonies were stored at 4°C for 2-4 weeks, sealed with polythene film. Before each infection experiment, a liquid culture of *E. coli* was prepared in 20 ml of LB medium in a 100-ml Erlenmeyer flask. The flasks were incubated overnight at 37°C shaking at 200 rpm.

2.6.2.2 Media and supplements for *E. coli*

Table 8: LB medium

LB medium	
Bacto-Trypton (BD Pharmingen)	10 g
Yeast extract (BD Pharmingen)	5 g
NaCl	10 g
H_2O $_{bidest}$	ad 1 L

Table 9: Agar plates

Agar plates	
LB medium	with 1,5% agar (BD Pharmingen)
Ampicillin	100 µg/L

Table 10: Freezing medium

Freezing medium	
LB medium	with 20% glycerol

2.7 Cell count

To count cells and to exclude non-viable cells TrypanBlue (Sigma) was used. 10 µl of 0.4% TrypanBlue was thoroughly mixed with 10 µl of the cell suspension, which was, depending on the cell density, diluted 1:10 to 1:100 with PBS or medium. 10 µl of the mixture was added to a Neubauer hemocytometer and the cell numbers were determined as follows:

$$\frac{number\ of\ counted\ cells}{4} x10^4 x\ dilution\ factors\ x\ cell\ solution\ volume\ in\ ml = cell\ amount$$

2.8 Incubation of cell cultures

Before each experiment, bacteria were examined microscopically for their shape and motility. *H. pylori* was taken from the plate with a swab and added to 2 ml of cell culture medium according to the cell type to be incubated (RPMI 1640, 10% heat-inactivated FCS, 2 mM L-glutamine for DC and macrophage incubation; DMEM, 10% heat-inactivated FCS, 2 mM L-glutamine for THP-1 cell incubation). For incubation with *E. coli,* 300 µl of *E. coli* in LB medium was added to 1.7 ml of medium.

The optical density of the bacterial solution was measured at 550 nm wavelengths for *H. pylori* and 600 nm for *E. coli* to determine the multiplicity of infection (MOI), indicating the ratio of bacteria per cell.

The adjusted amount of the bacterial solution was added to the prepared cells in either 96-well plates or 12-well plates (TPP). Each condition was set up in duplicates. For synchronization, plates were centrifuged at 800 x g for 3 min at room temperature.

2.9 Antigen presentation assay

All APCs were transferred to 96-well plates and incubated with bacteria as indicated above or treated with 100 ng/ml LPS (Invivogen, San Diego, CA, USA). An MOI of 10 was used, because MOIs >20 induced apoptosis of the cells. After the addition of bacteria or LPS, the cells were incubated at 5% CO_2 supply, 37°C and H_2O saturation for one hour. Plates were then centrifuged at 350 x g and room temperature for 5 min. Supernatants were removed and fresh medium containing penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (100 µg/ml) and Ag85B (Lionex, Braunschweig, Germany) at 12 µg/ml was added. Plates were incubated for another 6 hours. Then $1x10^5$ F9A6 cells were added to each well (100 µl per well at a concentration of 10^6 cells/ml).

F9A6 cells secrete IL-2 in proportion to the number of specific peptide:MHC complexes on the APC in the absence of other stimuli [73, 75], so we did not have to co-stimulate the F9A6 cells to produce IL-2.

The following controls were included: APCs left without incubation with bacteria, F9A6 cells and Ag85B (positive control) and APCs and F9A6 cells without Ag85B (negative control). Cells were incubated overnight. On the next day, plates were centrifuged at 350 x g for 5 min and the supernatants were collected and stored at -20°C or analyzed immediately by ELISA. Viability assays were performed while the cells remained in the plates. All conditions were set up in duplicates.

2.10 Data analysis and statistics

Surface marker expression was acquired using CellQuestPro (BD Pharmingen) and data were analyzed using CellQuestPro and FloJo (TreeStar Inc., Ashland, OR, USA) software. Median fluorescence intensities (MFIs) and the percentages of positive cells were determined after subtraction of the values for the respective isotype controls. Cytokine expression levels were interpolated from the respective established cytokine standard curves. Quantifications and statistical analysis were performed with GraphPad Prism 6 (Graphpad Software, California, USA) software by using the Wilcoxon matched-pairs signed rank test and a nonparametric

Friedman test. P values of <0.05 were considered significant.

3 RESULTS

3.1 Preliminary work

3.1.1 F9A6 cells are not affected by *H. pylori* directly

To verify that *H. pylori* does not affect F9A6 cells, we co-cultured F9A6 cells with *H. pylori*-supplemented medium with anti-CD3 pre-coated plates for 12 hours. Subsequently, the cell viability as well as IL-2 levels in the supernatants were analyzed. IL-2 concentrations in the supernatants were high and there was no statistical difference between cells in untreated medium or cells cultured in *H. pylori* wild type- or mutant-supplemented medium (Fig. 3a). This procedure was published by our group [76].

Cells not treated with anti-CD3 (unstimulated) did not produce any IL-2. The viability assay did not reveal differences in cell viability regardless of whether the cells were cultured in bacteria-supplemented medium or medium only. Unstimulated cells showed higher viability levels compared to stimulated cells, which is in agreement with the fact that hybridoma T-cells show a dose-dependent decrease in cell growth upon IL-2 production. This was observed when T-cells were activated through antigen presentation by APCs [75]. Another explanation for this phenomenon is activation-induced cell death [77]. When cells were treated with cisplatin apoptosis was induced (Fig. 3b).

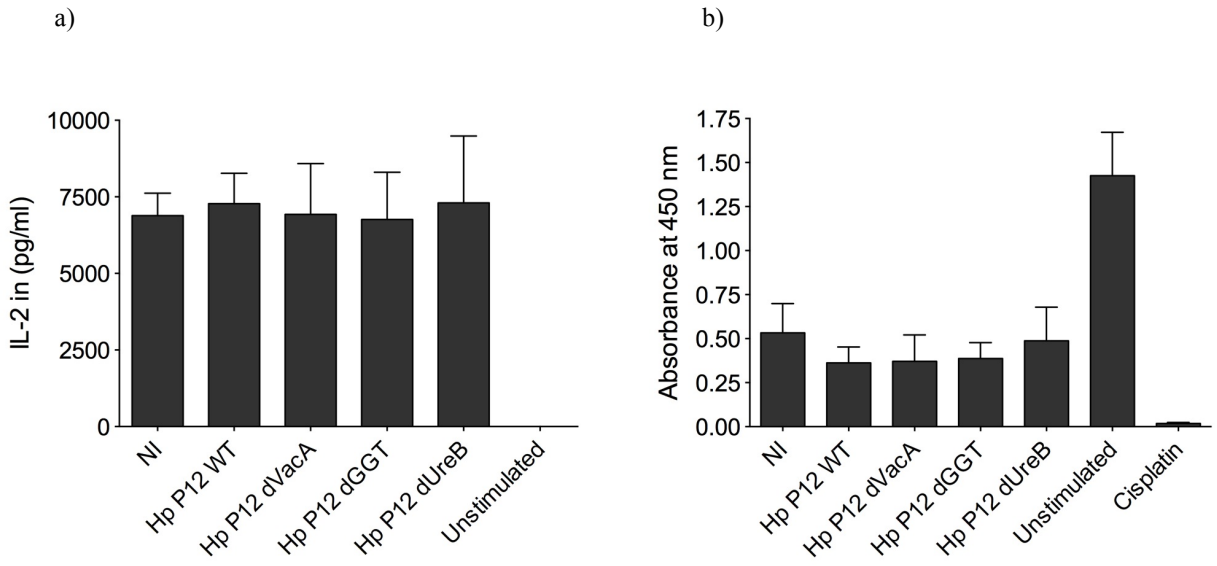

Figure 3: Based on Fehlings et al. [76] Incubation with *H. pylori*-supplemented medium does not affect F9A6 activation or cell viability.
Hybridoma cells were stimulated with anti-CD3 and *H. pylori* wild type- or mutant-supplemented medium (MOI=10) was added and left overnight. Controls included medium without bacteria (NI) and unstimulated (without anti-CD3) cells. IL-2 concentrations of supernatants were determined by ELISA (a); cellular viability by a cell viability assay (b). Bars are means of 4 independent experiments, error bars represent SEM. Data were analyzed by the Wilcoxon matched-pairs signed rank test.

3.1.2 Ag85B-pulsed DCs and macrophages from HLA-DR 1 positive blood donors induce F9A6 cell activation

Next, we wanted to know whether the APCS we used were able to induce F9A6 cell activation. Canaday et al. showed that F9A6 cells were responsive to THP-1 cells [73] . Then, we determined whether DCs and macrophages generated from peripheral blood from HLA-DR 1 positive donors were able to take up, process and present Ag85B via HLA-DR 1 to F9A6 cells.

DCs and macrophages were generated from monocytes obtained from HLA-DR 1 positive donors and seeded in 96-well plates. Ag85B-containing medium or medium only and after another 6 hours F9A6 cells were added. After overnight incubation, supernatants were collected, and the IL-2 concentrations were determined by ELISA. When F9A6 cells were added to DCs from HLA-DR 1 positive donors with Ag85B, high concentrations of IL-2 were detected in the supernatants, indicating a strong F9A6 cell activation (Fig. 4a). In contrast, when F9A6 cells were added to DCs generated from HLA-DR 1 positive donors without Ag85B, only negligible levels of IL-2 were observed ($p=0.0039$). Likewise, Ag85B-pulsed macrophages led to significantly higher IL-2 levels compared to control cells without the antigen ($p=0.0313$) (Fig.

4b). Neither DCs nor macrophages generated from PBMCs of HLA-DR 1 negative donors led to IL-2 production after pulsing with Ag85B and subsequent F9A6 cell co-culture (data not shown).

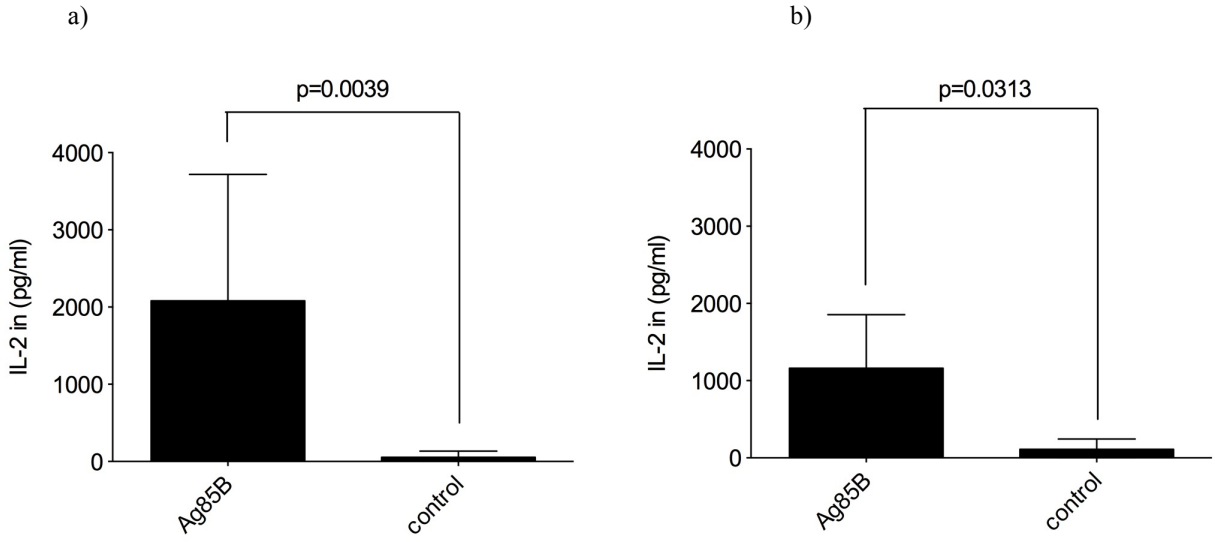

Figure 4: Monocyte-derived DCs and macrophages from HLA-DR 1^+ donors stimulate F9A6 cells.
DCs (a) or macrophages (b) were generated from PBMC. $8x10^4$ cells/well were seeded in 96-well plates and 15 µg of Ag85B or medium alone (control) were added for 1 hour. After 6 hours, 10^5 F9A6 cells/well were added to the wells. F9A6 cell activation was determined via IL-2 production measured by ELISA. Bars represent values ± SD of 5 different donors (a) or 4 different donors (b). Data were analyzed by the Wilcoxon matched-pairs signed rank test.

3.2 Effects of with *H. pylori* incubated APCs on antigen presentation

To investigate the potential interference of antigen presentation of human APCs incubated with *H. pylori*, a macrophage-like cell line (THP-1 cells) and primary monocyte-derived APCs (DCs and M1 macrophages) were incubated with a human *H. pylori* wild type strain or *H. pylori* mutants lacking virulence factors (VacA, GGT, or urease). Their potential to present an *H. pylori*-independent antigen (Ag85B of *M. tuberculosis*) to F9A6 cells was evaluated through the IL-2 production.

3.2.1 *H. pylori* does not interfere with antigen presentation of THP-1 cells

Firstly, we investigated whether THP-1 cells could be used as APCs in the antigen presentation assay. In order to do so, $8x10^4$ PMA- and IFN-γ-stimulated THP-1 cells/well were incubated with P12 *H. pylori* wild type for 1 hour or left unexposed. The medium was removed and fresh medium containing gentamicin to kill remaining bacteria and Ag85B was added. Cells were incubated at 37°C for 6 hours. $2x10^4$ F9A6 cells/well were added, and the co-cultures incubated overnight. On the next day, supernatants were harvested and IL-2 concentrations measured by

ELISA. THP-1 cells, which were not incubated with *H. pylori,* induced high IL-2 levels, indicating a strong T-cell stimulation (Fig. 5). Incubation of the cells with *H. pylori* wild type did not lead to a decrease in IL-2 production of the F9A6 cells (p=0.846). The incubation of THP-1 cells with the *H. pylori* mutants lacking the virulence factors VacA, GGT, or urease yielded similar results as the incubation with the *H. pylori* wild type (p=0.1447; data not shown). Thus, the incubation of THP-1 cells with *H. pylori* does not influence their ability to successfully present Ag85B to antigen-specific T-cell hybridoma cells (Fig. 5).

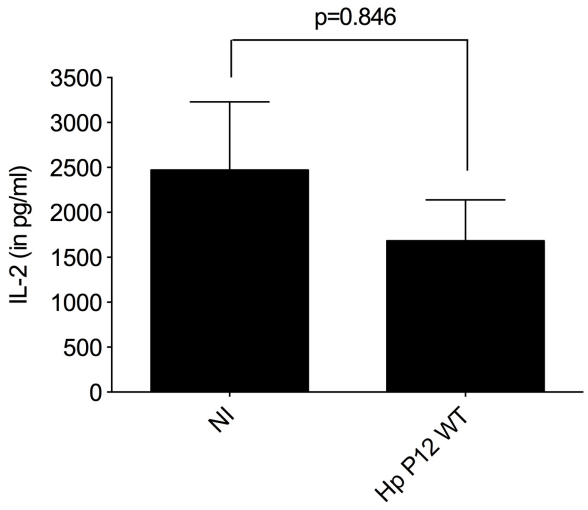

Figure 5: *H. pylori* wild type incubation of THP-1 cells did not affect their ability to activate F9A6 cells. THP-1 cells were incubated without bacteria (NI) or with *H. pylori* wild type (MOI=10). F9A6 cell activation was determined by measuring IL-2 concentrations in the supernatants. Bars represent means of 10 independent experiments, error bars show SEM. Data were analyzed by the Wilcoxon matched-pairs signed rank test.

3.2.2 *H. pylori* inhibits antigen presentation of human primary DCs

To monitor the effect of *H. pylori* incubation of human primary APCs, the established assay was adapted to monocyte-derived DCs, the prominent group of APCs [76]. DCs were differentiated from monocytes and $8x10^4$ cells/well were transferred to 96-well plates and incubated for 1 hour with *H. pylori* P12 wild type or mutants lacking VacA, GGT, or UreB. Then the cells were washed and remaining extracellular bacteria were killed by adding gentamicin. Ag85B was added at the same time. After 6 hours of incubation, $1x10^5$ F9A6 cells/well were added and the cells co-cultured overnight. On the next day, supernatants were collected for ELISA analysis and the viability of the cells was assessed.

Incubation with *H. pylori* negatively affected the ability of human DCs to activate F9A6 cells (Fig. 6a). In cells incubated with *H. pylori* wild type at an MOI of 10, antigen presentation was decreased significantly ($p=0.008$). This effect was not caused by a reduced cell viability (Fig. 6b). Experiments with MOIs of 7, 4, or 0.5 resulted in inhibition of antigen presentation at similar levels as an MOI of 10. Incubation of DCs with *H. pylori* mutants lacking VacA, GGT, or UreB led to the same decrease in antigen presentation as the incubation with wild type bacteria (for all mutants versus wild type $p> 0.5$) (Fig. 6c). In contrast, DCs without contact with *H. pylori* induced a strong IL-2 production. Antigen presentation of with *H. pylori* incubated DCs was reduced to about 10% compared to cells without incubation with *H. pylori*.

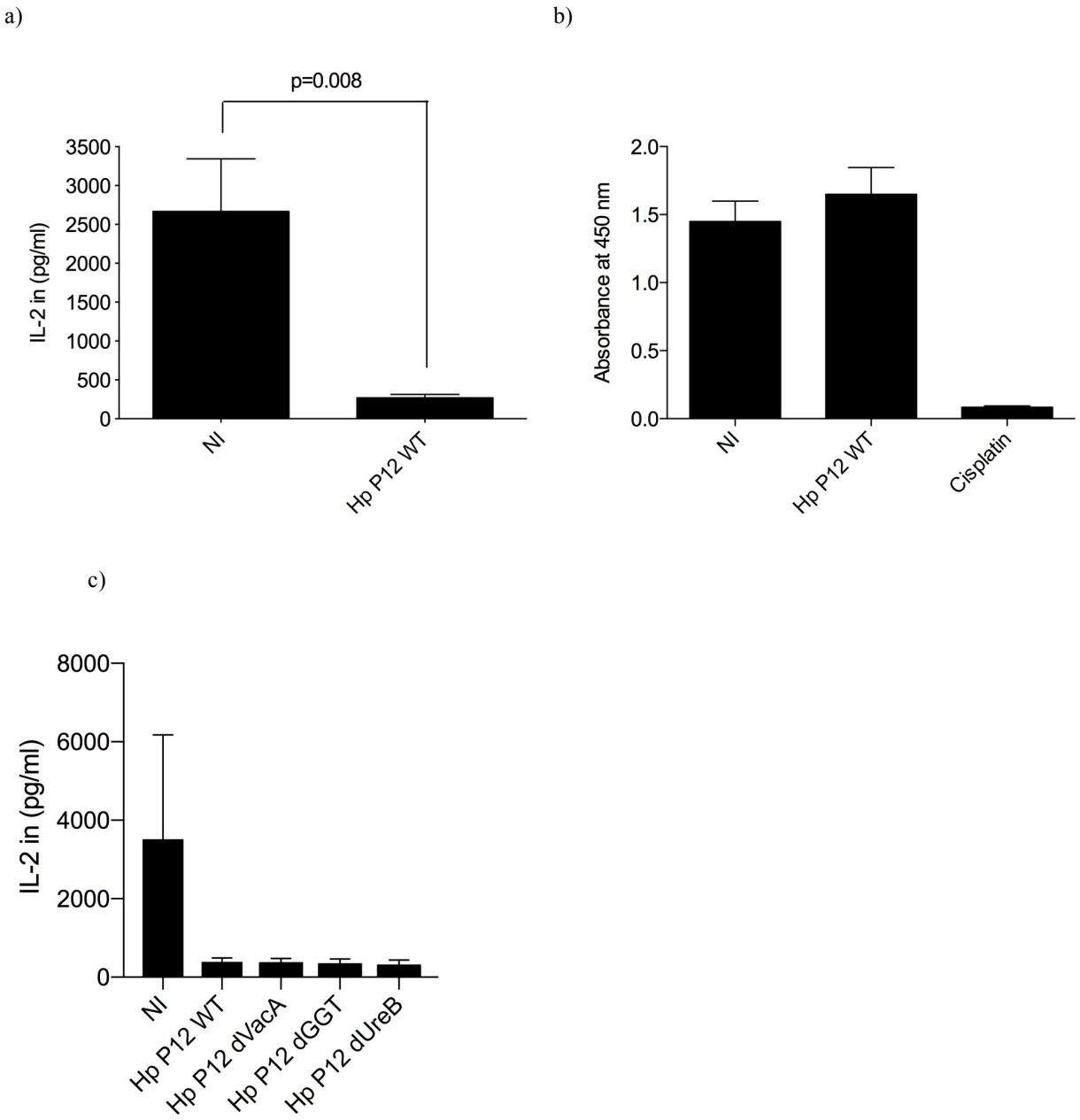

Figure 6: Based on Fehlings et al.[76] Incubation of DCs with *H. pylori* impairs their antigen presentation capacity. Monocyte-derived DCs were harvested and $8x10^4$ cells/well were transferred to 96-well-plates. They were left without incubation with *H. pylori* (NI), or incubated with *H. pylori* wild type (a), or mutants lacking VacA, GGT or UreB (MOI=10) for one hour (c). Subsequently, they were pulsed with 12 µg/ml of Ag85B and co-cultured with F9A6 cells overnight. T-cell activation was determined by measuring IL-2 concentrations in the supernatants (a and c), cell viability by colorimetric analysis of supernatants (b). Bars represent means of 8 independent experiments with 4 different donors (a) and 4 independent experiments with 3 different donors (c); error bars represent SEM. Wilcoxon matched-pairs signed rank test (a and b) and Friedman test (c) were performed.

3.2.3 *H. pylori* also interferes with antigen presentation of human macrophages

Next, we investigated whether monocyte-derived macrophages were equally affected by *H. pylori* as DCs, or whether they would remain unaffected as the macrophage-like cell line THP-1.

Similar to what we previously observed for DCs, also macrophages incubated with *H. pylori* were less able to activate F9A6 cells than macrophages without contact with *H. pylori*. Compared to DCs, the reduction of IL-2 secretion was not as strong, i.e. macrophages incubated with *H. pylori* induced around 50% less IL-2 than untreated cells (Fig. 7a).

Cell viability was not affected by incubation with *H. pylori* (Fig. 7b). As observed before for DCs, incubation of macrophages with *H. pylori* mutants lacking VacA-, GGT-, or UreB led to the same decrease in antigen presentation as the incubation with wild type bacteria ($p>0.5$) (Fig. 7c).

Thus, the inhibitory effect observed previously in DCs incubated with *H. pylori* most likely was not due to DC maturation, which often is accompanied by a reduction of antigen uptake.

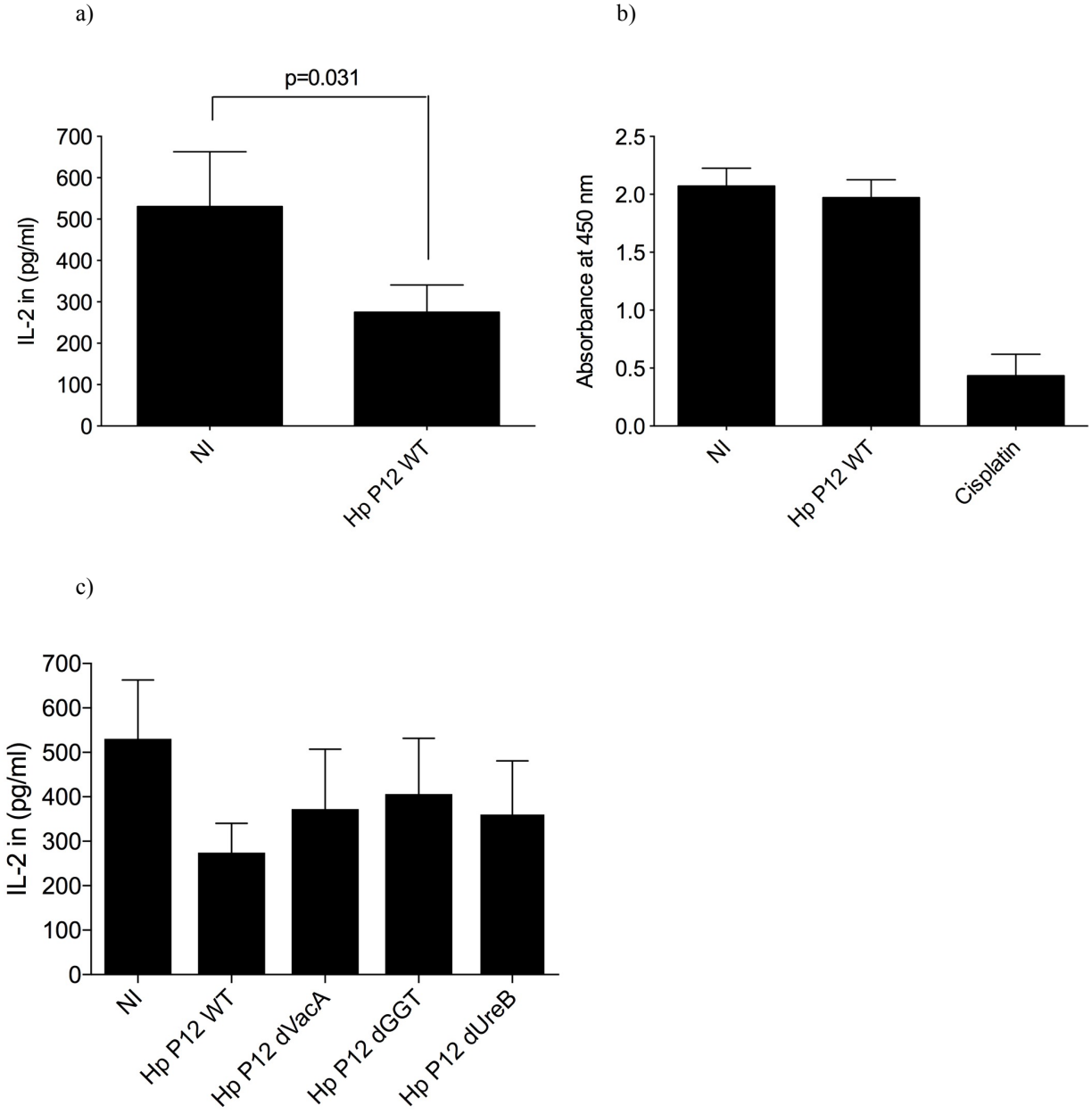

Figure 7: Incubation of human macrophages with *H. pylori* inhibits F9A6 activation.
Macrophages were generated from human PBMCs, harvested and $8x10^4$ cells/well were transferred to 96-well-plates. Cells without *H. pylori* incubation (NI), or incubation with *H. pylori* wild type (Hp P12 WT), or with mutants lacking VacA (Hp P12 dVacA), GGT (Hp P12 dGGT) or UreB (Hp P12 dUreB) (MOI=10) were washed, pulsed with Ag85B and co-cultured with F9A6 cells overnight. T-cell activation was determined by measuring IL-2 concentrations in supernatants (a and c), cell viability by colorimetric analysis of supernatants (b). Bars represent means of 6 independent experiments from 4 different donors (a) and 3 independent experiments with 2 different donors (c); error bars represent SEM. Wilcoxon matched-pairs signed rank test (a and b) and Friedman test (c) were used.

3.2.4 Incubation of DCs with *E. coli* and treatment with LPS also impair the presentation of Ag85B to F9A6 cells

To study whether another gram-negative bacterium or immunodominant PAMP, e.g. LPS, would have the same effect on antigen presentation as *H. pylori*, we used *E. coli* BL21 and *E. coli* LPS treatment. Similar to what we previously observed for *H. pylori*, the incubation of DCs with *E. coli* also led to a significant decrease in IL-2 production of the F9A6 cells (p=0.0078). LPS at 100 ng/ml also led to a significant decrease of IL-2 production (p=0.002) compared to untreated DCs (Fig. 8b). None of the effects could be explained by a loss of cell viability (data not shown). Similar results were obtained with macrophages (data not shown).

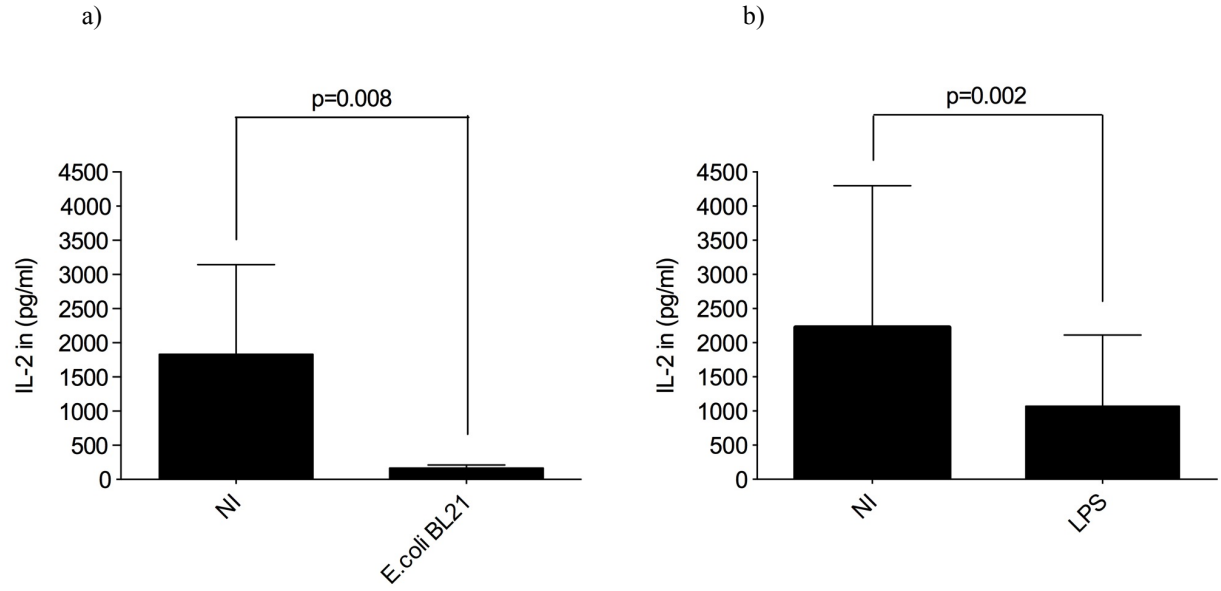

Figure 8: Incubation of DCs with *E. coli* and LPS addition leads to a decrease in antigen presentation. Monocyte-derived DCs without incubation with *E. coli* (NI) or incubated with *E. coli* BL21 (MOI=10) (a), or treated with 100 ng/ml LPS (b) before pulsing with Ag85B and co-culture with F9A6 cells. IL-2 concentrations in supernatants were analyzed by ELISA. Data represent 4 independent experiments with 3 different donors (a) and 5 independent experiments with 3 different donors (b) ±SD. Data were analyzed with the Wilcoxon matched-pairs signed rank test.

3.3 Analysis of steps, which could be involved in the *H. pylori*-induced inhibition of antigen presentation by DCs

H. pylori could interfere with antigen presentation at various steps, including antigen uptake, processing, or peptide binding to the MHC class II molecule; or during assembly, transport to the cell surface, or surface expression of MHC class II.

3.3.1 Ag85B epitope aa97-112 presentation leads to inhibited activation of F9A6 cells

To investigate whether the process of antigen degradation into peptides was affected by incubation of APCs with *H. pylori* in the experimental system studied, only the peptide aa97-112 of Ag85B, which is the epitope recognized by the F9A6 cells when bound to a HLA-DR 1 molecule, was added to DCs or macrophages instead of the whole Ag85B. Under these conditions, incubation of DCs or macrophages with *H. pylori* also resulted in a decrease of IL-2, while not with *H. pylori* incubated, peptide-pulsed cells led to high IL-2 levels in supernatants (data not shown). This indicates that *H. pylori* most likely does not interfere with protein degradation.

3.3.2 HLA-DR surface expression of DCs and macrophages

As a next step, we studied the surface expression of HLA-DR on DCs and macrophages. The HLA-DR 1 molecule binds and presents the Ag85B peptide to the F9A6 cells and an impairment of MHC class II assembly might result in fewer HLA-DR:Ag85B complexes on the surface of the cells. Therefore, DCs were incubated with *H. pylori* P12 wild type and *E. coli* BL21, treated with 100 ng/ ml *E. coli* LPS, or left untreated. After one hour the medium was removed and gentamicin-containing medium added. Cells were left overnight and stained with anti-HLA-DR antibodies. About 98% of the DCs expressed HLA-DR in all conditions (Fig. 9). There was no difference between untreated cells, cells incubated with either *H. pylori* or *E. coli*, or LPS-treated cells.

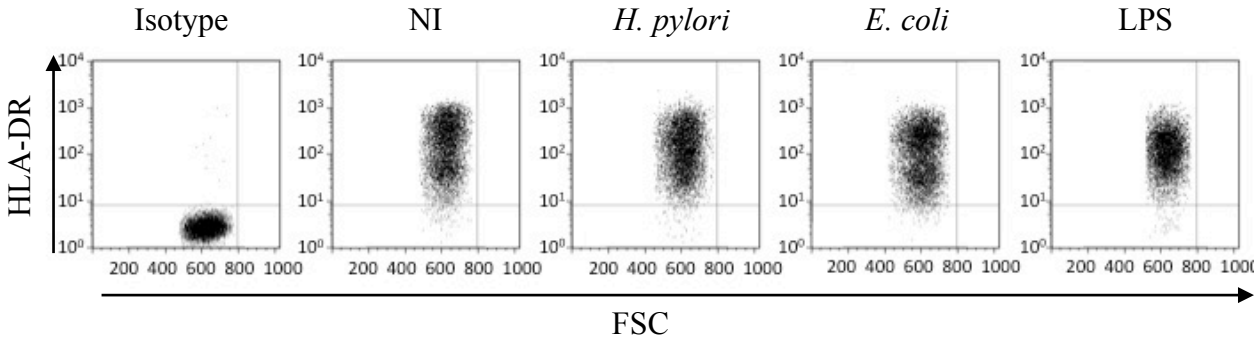

Figure 9: HLA-DR surface expression on DCs.
DCs without incubation with bacteria (NI) or infected in 12-well plates with *H. pylori* wild type or *E. coli* or treated with 100 ng/ml LPS for one hour. Afterwards, medium was aspirated and fresh, gentamicin-containing medium was added. After overnight incubation, cells were stained for FACS analysis. One of two individual experiments with comparable results is shown.

Macrophages yielded similar results. In untreated and LPS-treated macrophages, around 98% of the cells were HLA-DR positive, in with bacteria incubated cells, 91% (*H. pylori*) or 95% (*E. coli*) of the cells expressed HLA-DR (Fig. 10).

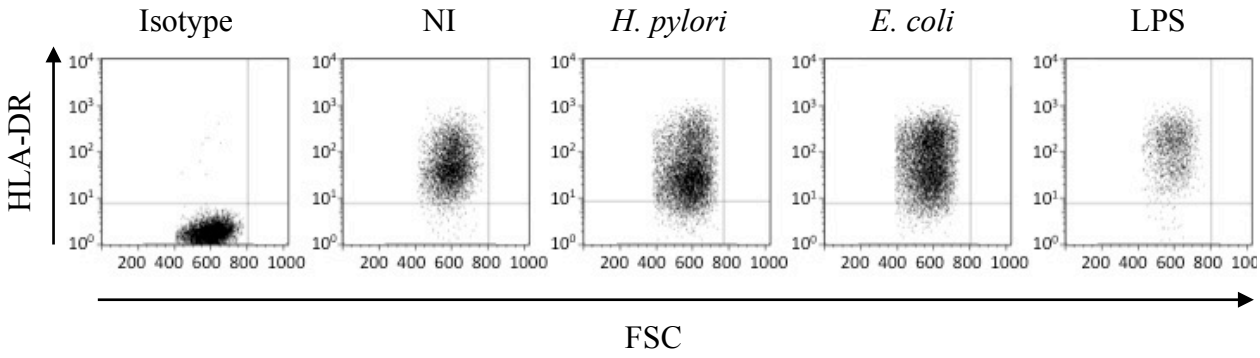

Figure 10: HLA-DR surface expression on macrophages.
Macrophages not incubated with bacteria (NI) or incubated with either *H. pylori* wild type or *E. coli* or treated with 100 ng/ml LPS. After one hour of incubation, medium was aspirated and fresh medium containing gentamicin was added. On the next day, cells were stained for FACS analysis. One of two individual experiments with comparable results is shown.

Additionally, the MFI was analyzed. No differences of the MFI were observed in DCs, in macrophages, which were untreated, or *H. pylori*- , or *E. coli*-incubated (Table 11).

Table 11: Expression of HLA-DR by DCs and macrophages upon incubation with either *H. pylori* or *E. coli*
Means of MFI of two independent experiments (MFIs of isotype controls were subtracted).

Cell type	**NI**	***H. pylori* P12**	***E. coli* BL21**
DCs	279	303	290
macrophages	76	103	113

No differences in HLA-DR expression on DCs or macrophages which were incubated with either *H. pylori* or *E. coli* were observed. Therefore, neither MHC II assembly, transport to the cell surface, nor surface expression were involved in impaired antigen presentation.

3.3.3 Early Ag85B addition enables antigen presentation of DCs despite incubation with *H. pylori*

To investigate whether an earlier addition of Ag85B could preserve the antigen presentation capacity of DCs in our assay, the antigen was added at various points in time prior to the incubation of the DCs with *H. pylori* [76]. DCs were seeded in 96-well-plates and the antigen was added 48 hours or 24 hours before incubation with *H. pylori* wild type, or at the same time. F9A6 cells were added immediately after the addition of bacteria. Antigen presentation of DCs was not inhibited and comparable to cells without contact to *H. pylori* when Ag85B was added 48 hours prior to incubation with *H. pylori* (Fig. 11). In contrast, Ag85B addition to the DCs 24 hours before incubation with *H. pylori* led to significant lower IL-2 levels than induced by cells without incubation with *H. pylori* (p=0.0313). When Ag85B was added at the same time as the *H. pylori* incubation of the DCs, their capacity to present the antigen to F9A6 cells decreased further.

Thus, *H. pylori* incubation of DCs impairs their antigen presentation capacity. But when the cells take up antigen more than 24 hours prior to bacterial incubation of the DC, the impaired antigen presentation can be preserved.

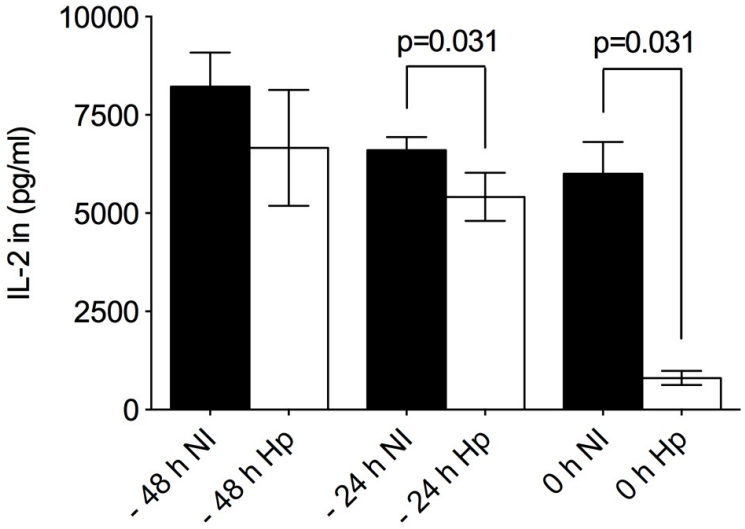

Figure 11: Based on Fehlings et al. [76] Early antigen addition preserves antigen presentation ability of DCs. $8x10^4$ monocyte-derived DCs were seeded in 96-well-plates and Ag85B was added 48 hours or 24 hours before DCs were incubated with *H. pylori* wild type (MOI=10), or at the same time and co-cultured with $1x10^5$ F9A6 cells. Cells without incubation with *H. pylori* were used as control (NI). Bars represent values±SD of 3 different experiments with 3 different donors. Wilcoxon matched-pairs signed rank test was performed.

3.3.4 Incubation of DCs with *H. pylori* still inhibits antigen presentation after 48 hours

To investigate whether the inhibition of antigen presentation was only transient, F9A6 cell were added at different points in time, i.e. 12, 24, and 48 hours after the incubation of the DCs with *H. pylori*. The cells were incubated overnight and supernatants were collected and analyzed by ELISA. Control cells were treated with LPS or maintained in medium alone [76].

When the F9A6 cells were added 12 (Fig. 12a), 24 (Fig. 12b), or 48 hours (Fig. 12c) after *H. pylori* incubation of DCs, the IL-2 levels were as low as when F9A6 cells were added after 6 hours. In contrast, LPS treatment resulted in inhibited antigen presentation only when F9A6 cells were added after 12 hours (Fig. 12a), but not after 24 or 48 hours (Fig. 12b and c).

These results demonstrate that incubation of DCs with *H. pylori* leads to a long term inhibition of antigen presentation in the experimental system studied, whereas LPS treatment inhibited antigen presentation only for a shorter time range.

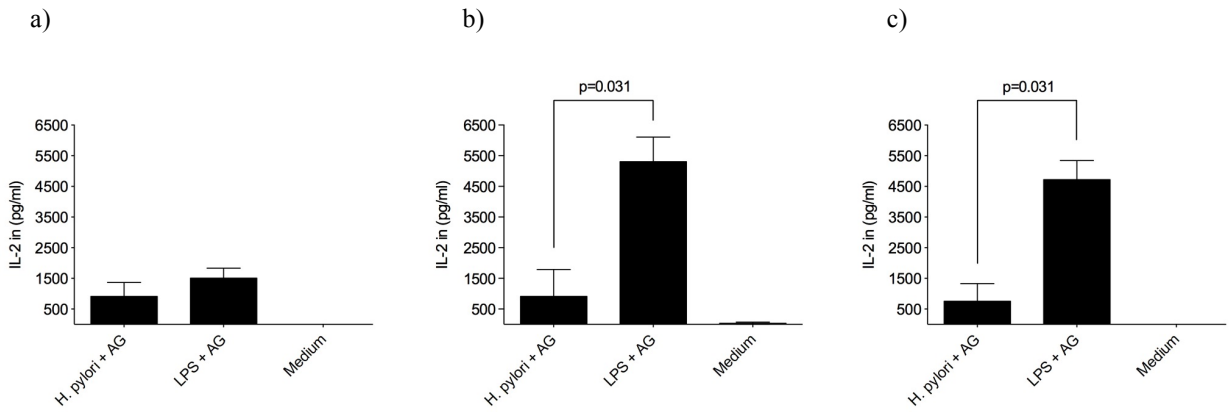

Figure 12: Based on Fehlings et al. [76] Incubation with *H. pylori* impairs antigen presentation of DCs even after 48 hours.
$8x10^4$ monocyte-derived DCs were seeded in 96-well-plates and Ag85B was added at the time of addition of *H. pylori* wild type (MOI=10), or the addition of 100 ng/ml LPS. $1x10^5$ F9A6 cells were added after 12 (a), 24 (b), or 48 hours (c). After overnight incubation, supernatants were collected and IL-2 concentrations determined by ELISA. Bars represent mean±SD of 6 individual experiments with 3 different donors. Wilcoxon matched-pairs signed rank test was performed.

4 DISCUSSION

The exact mechanisms underlying the persistent colonization of the gastric mucosa by *H. pylori* and the bacterial immune evasion still remain elusive. Increased numbers of APCs are found within the gastric mucosa of infected individuals [78-80] and DCs become activated upon *H. pylori* infection [81]. Since the bacterium is able to escape, defects in APC functions may lead to the induction of an inadequate, i.e., non-protective immune response.

Limited information is available regarding the ability of *H. pylori*-infected APCs to present antigen to naïve T-cells. Previous studies mainly focused on effects of *H. pylori* on phagocytosis [53, 82] or direct bacterial effects on T-lymphocytes [23, 68, 83], and these effects may hamper studies regarding the direct impact of *H. pylori* on antigen processing and presentation by APCs. Through the adaptation of an assay involving human APCs and murine T-cells to the *H. pylori* system, we were able to circumvent this obstacle and investigate the ability of with *H. pylori* incubated APCs, i.e., THP-1 cells, human monocyte-derived macrophages, and DCs, to present antigen to T-cells. Since various virulence factors of *H. pylori* have been discussed to be involved in its immunomodulatory effects [22, 26, 84], we included isogenic mutants lacking VacA, GGT, or an effective urease in our study.

4.1 Differences between primary APCs and THP1-cells regarding their interaction with *H. pylori*

The APCs used in this study were primary macrophages and DCs, derived from monocytes by incubation with hGM-CSF or hGM-CSF and IL-4, respectively, and THP-1 cells, a monocytic cell-line originally derived from a leukemia patient. We observed that the incubation of primary macrophages and DCs with *H. pylori* led to an inhibition of their capacity to present a complementary antigen to specific hybridoma T-cells. In contrast, the ability to present antigens was not impaired in THP-1 cells incubated with *H. pylori*.

Even though activated THP-1 cells possess properties of macrophages and are often used as a model cell line for APCs [85], they still differ in various aspects from primary cells. THP-1 cells bind *Borrelia burgdorferi* but are unable to internalize the bacteria, whereas monocytes are able to do both [86]. Lambrechts et al. [87] have reported considerable discrepancies between the

transcriptional responses of various genes in THP-1-derived monocytes, PMA-induced THP-1 macrophages and bone marrow or cord blood ($CD34^+$) progenitor-derived DCs. The bacterial effects on THP-1 cells may be species-specific, as Barrionuevo et al. [88] observed an inhibited MHC II expression and antigen presentation of Ag85B by THP-1 cells upon treatment with *Brucella abortus* or a lipoprotein derived from *B. abortus* but not by *B. abortus* LPS, even though *B. abortus* LPS inhibits T-cell activation through primary murine macrophages [89].

4.2 Inhibition of antigen presentation by *H. pylori*

H. pylori incubation of human primary APCs considerably reduced their antigen presentation ability. In order to elucidate the mechanism underlying the reduced T-cell activation, we first excluded possible disturbances within the experimental system. Since *H. pylori* may directly inhibit IL-2 production and proliferation of human $CD4^+$ T-cells [23, 68, 83], we investigated potential effects of *H. pylori* on the viability and IL-2 secretion of the F9A6 hybridoma cells, even though these cells are of murine origin. Upon incubation of F9A6 cells with *H. pylori* wild type- and mutant-containing medium, the cells did not differ regarding their IL-2 secretion or cell viability from F9A6 cells kept in medium alone. This also is supported by the results of the experimental setup with the THP-1 cells (where antigen presentation and IL-2 secretion were not inhibited). Hence, the F9A6 cells provided suitable read-out cells for our experiments.

In our initial experiments, we incubated the APCs for one hour with *H. pylori*, removed the bacteria, and added Ag85B-containing medium. Then the cells were incubated for 6 hours before the F9A6 cells were added. The decreased levels of IL-2 indicated a decrease in antigen presentation of *H. pylori*-infected APCs. One possible explanation for this finding could be the maturation of the DCs. Exposure of immature DCs to bacterial stimuli induces their maturation, which is accompanied by a reduced ability to phagocytize exogenous antigen. We therefore altered in additional experiments the time between Ag85B addition and incubation of the cells with the bacteria.

Similar to our experiments with the addition of antigen one hour after incubation of the cells with *H. pylori*, however, Ag85B addition at the time of or even 24 hours before incubation still resulted in inhibition of antigen presentation. This excluded DC maturation as a possible reason for the inhibitory effect, which is furthermore supported by our observation of similar effects of *H. pylori* on macrophages. Only when Ag85B was added 48 hours prior to the incubation of the

cells with *H. pylori*, were the DCs able to present the antigen to the same degree as control cells without bacterial contact.

Next, we added the exogenous antigen at the time of *H. pylori* addition but altered the time between that and the addition of the responder cells. Here, we observed the inhibition of antigen presentation for up to 48 hours after incubation with *H. pylori*. Interestingly, treatment with LPS instead of incubation with live bacteria resulted in an inhibited antigen presentation of up to 12 hours post infection only. When we added F9A6 cells after 24 or 48 hours, LPS no longer inhibited antigen presentation by DCs. It needs to be determined whether higher doses of LPS might cause a longer-lasting inhibitory effect in this system. Also it could be of interest to investigate for how long before bacterial treatment LPS can inhibit antigen presentation. Short et al. saw a reduced antigen presentation of *Influenza A virus* infected macrophages until 4 hours after LPS-treatment with the high point of inhibition at 24 hours of LPS-priming prior to APC:T-cell interaction. They also saw reduced infection rates of macrophages upon LPS-priming. The reason for this might be impaired function of the macrophage as an APC. They used human primary macrophages, infected them with *influenza A virus* and presented afterwards an independent antigen to CD8+ T-cells [90]. Another example of impaired antigen presentation due to LPS-treatment of the APC showed Mizuno et al.. They observed a reduced uptake of soluble antigen by young, LPS-primed mouse-macrophages [91]. In DCs maturation might play a role in inhibited antigen presentation, as well. LPS induces DC-maturation, which leads to a reduced antigen uptake [92, 93]. The time span is difficult to predict, due to differences in experimental setup and differences between humans and rodents, but it might be a reason for inhibited antigen presentation and the recovery from it after LPS-treatment in our experiment.

Virulence factors of *H. pylori* may have various effects on the host's immune system. Therefore, we investigated the possible role of three major virulence factors, i.e., VacA, GGT, and urease, in our system. However, we did not observe differences between wild type- or mutant-incubated APCs. In contrast, others have reported virulence factor-specific effects on phagocytic cells, e.g. DCs [22, 63, 84, 94, 95]. Our observation, however, is supported by our findings of live *E. coli* or LPS being able to interfere with antigen presentation in our experimental system in a manner similar to that of *H. pylori*.

Consistent with our data, LPS-dependent inhibition of antigen presentation of macrophages to antigen-specific $CD4^+$ T-cells has also been reported by Forestier et al. [89]. They used LPS of

B. abortus (and *Shigella flexneri*) and observed clustering of LPS and MHC class II molecules on the surface of murine macrophages, leading to impaired antigen presentation of hen egg lysozyme (HEL) to T-cells. This effect was not due to alteration of the level of total MHC class II surface molecules or a deficient uptake or processing of the antigen. In contrast, Svensson et al. [96] did not observe an inhibition of ovalbumin or HEL-specific T-cell stimulation when they infected murine DCs with ovalbumin or HEL-expressing *E. coli*. These discrepancies might be due to differences in the experimental systems used.

4.3 Does the nature of antigen in the experimental system determine whether *H. pylori* inhibits antigen presentation?

We did not observe differences between DCs or macrophages treated with wild type *H. pylori*, mutant bacteria lacking different virulence factors, *E. coli,* or *E. coli* LPS when the stimuli were added at the time of antigen addition. All of these stimuli induced the inhibition of antigen presentation. Thus, the observed inhibitory effect is not *H. pylori*-specific.

Since Forestier et al. [89], who also observed a reduced T-cell activation by infected APCs also used exogenous antigen in their experiments, it is intriguing to speculate that the origin of the antigen determines whether *H. pylori* (and the other stimuli tested in the present study) exerts an inhibitory effect on the antigen presentation of professional APCs. In fact, this hypothesis is supported by other studies. Svensson et al. [96] did not observe an inhibition of T-cell responses when they introduced the antigens (HEL or ovalbumin) through recombinant *E. coli,* whereas presentation of ovalbumin by murine DCs is inhibited upon infection of the cells with *Salmonella enterica* serovar Typhimurium [97]. The same bacteria, however, are unable to inhibit this process if the exogenous antigen consists of externally added peptides that can bind directly to MHC-II molecules [98]. This is in contrast to our preliminary results of inhibition of antigen presentation by *H. pylori* even when we added peptides to the APCs. Thus, the way of introducing the antigen most likely does not affect antigen presentation and its inhibition by *H. pylori* in our system.

Still, our data possibly mirror the self/non-self discrimination of antigens that has been shown for murine bone marrow-derived and splenic DCs [99]. This study provided first evidence that depending on the presence of a TLR ligand (the authors used the TLR4 ligand LPS), phagosomal contents derived from microbial pathogens are preferentially presented by DCs as compared to apoptotic self-antigens. This discrimination was uncoupled from DC maturation or antigen

processing, which implies that the presentation of a particular antigen also is determined by its origin. One explanation for this could be that the progressive proteolysis of Ii to CLIP might be a TLR-regulated step that contributes to antigen selection for presentation. If this also held true for our experimental system, presentation of exogenous protein coupled to a TLR ligand should not (or less) be inhibited by *H. pylori*.

4.4 Our assay can be helpful for further investigation of alterations in antigen presentation by *H. pylori*

Further investigation is needed to explore the mechanisms of altered antigen presentation of *H. pylori*-incubated professional APCs.

The present data demonstrate that neither a direct toxic or proliferation-inhibiting effect of *H. pylori* on T-cell hybridoma cells nor an impaired internalization and degradation of protein antigens account for the reduced antigen presentation of DCs or macrophages incubated with *H. pylori*. Additionally, the reduced antigen-presenting capacity cannot be the consequence of reduced amounts of MHC II molecules on the surface of infected APCs, as we have demonstrated earlier an increase of MHC II expression by with *H. pylori* incubated DCs [53]. T-cell hybridomas do not require co-stimulatory signals to be activated, therefore, differences in co-stimulation do not play a role in the reduced antigen presentation either. Thus, our experimental setup allows us to study the antigen-presenting capacity of *H. pylori*-incubated APCs independently of accessory steps in APC-T-cell-interactions, which could also be modified by infection.

Therefore, questions raised about inhibited antigen presentation of professional APCs incubated with *H. pylori* can be answered with our assay. Further experiments looking at various times of infection could help to define the exact time span of inhibition. Experiments set up at those critical points in time could elicit molecular changes within the APC:T-cell interaction during infection. Once the critical points in time are characterized, it would be of interest to identify ligands, which could be coupled to peptides or protein in order to potentially overcome the inhibition of antigen presentation.

In conclusion, *H. pylori*-incubated human PBMC-derived DCs and macrophages but not THP-1 cells show an inhibited capacity to present exogenous antigen to allogeneic T-hybridoma cells. The same results were obtained, however, when the cells were pre-treated with live *E. coli* or

LPS of *E. coli*. Therefore, this property is not a *H. pylori*-specific effect, which might facilitate in immune evasion. It might, however, be related to the potential of professional APCs to discriminate between potentially harmful inflammatory antigens and non-inflammatory antigens, e.g., autoantigens. The assays established should be helpful to gain additional information on inhibited antigen presentation upon incubation of DCs and macrophages with *H. pylori*. The major advantage of this read-out system is its independence of directly impairing or toxic effects on antigen uptake and T-cells.

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„Ich, Lea Drobbe, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Inhibition of antigen presentation by primary antigen presenting cells through *Helicobacter pylori*“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Anteilserklärung an etwaigen erfolgten Publikationen

Lea Drobbe hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Fehlings M, Drobbe L, Beigier-Bompadre M, Viveros PR, Moos V, Schneider T, Meyer TF, Aebischer T, Ignatius R, Usage of Murine T-cell Hybridoma Cells as Responder Cells Reveals Interference of Helicobacter Pylori with Human Dendritic Cell-mediated Antigen Presentation., European Journal of Microbiology and Immunology, 2016
Beitrag im Einzelnen: Mitarbeit bei der Konzeption des Forschungsansatzes; Planung, Datenerhebung, Datenanalyse und -interpretation zu Figure 1 und Figure 2; Mitarbeit bei der Planung, Datenerhebung, Datenanalyse und -interpretation zu Figure 3 A-C

Unterschrift des Doktoranden/der Doktorandin

CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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