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Identification of cellular mechanisms interfering with the function of the *Helicobacter pylori* *cag* type IV secretion system

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II Zusammenfassung

Helicobacter pylori (*H. pylori*) ist ein gramnegatives, stabförmiges Bakterium, das den menschlichen Magen kolonisiert. Es weist eine hohe Assoziation mit der Genese einiger Magenerkrankungen, im schwersten Fall dem Magenkarzinom, auf. Nach Bindung an die Zellen der Magenschleimhaut nutzt *H. pylori* sein *zytotoxin-assoziiertes Antigen* Typ IV Sekretionssystem (*cag* T4SS), um das zytotoxin-assoziierte Antigen A (CagA) Protein aus seinem Zytoplasma in das Zytoplasma der Wirtszelle zu injizieren. Dort greift das CagA Protein erheblich in die zellulären Prozesse ein. Doch nur Typ I *H. pylori* sind fähig zur CagA Translokation, während dem Typ II *H. pylori* das CagA Protein fehlt. Nach Kontakt mit dem *cag* T4SS, produziert und sekretiert die Wirtszelle Interleukin-8 (IL-8). 2013 beschrieben Jiménez-Soto et al. (Jiménez-Soto LF et al., 2013) erstmals das Phänomen, dass Wirtszellen, die mit *H. pylori* vorinfiziert wurden, einen Widerstand gegen Zweitinfektionen mit *H. pylori* aufbauen, was zu einer signifikanten Reduktion der CagA Translokation führt. Die folgende Doktorarbeit präsentiert neue Einblicke zur Ausbildung dieses zellulären Widerstands.

Zunächst wurde die semi-quantitative Analyse von Western Blots zur Quantifizierung translozierten CagAs optimiert, indem eine Normalisierung mittels „oberer Spur“ (ULN), sowie eine Normalisierung mittels „einzelner Bande“ (SBN) etabliert wurde. Um den Einfluss der Proteine von *H. pylori* zu eliminieren werden nur zelluläre Proteine genutzt, die größer als die meisten Proteine von *H. pylori* sind (>100kDa). Bei Zelllinien mit hoher Produktion von Proteinen größer 100kDa wird der obere Abschnitt des Western Blots genutzt, während bei Zelllinien mit niedriger Proteinexpression in diesem Bereich eine einzelne starke Bande identifiziert wird (SBN).

Mittels dieser Optimierung wird das Phänomen des aktiven zellulären Widerstands gegen die CagA Translokation, das durch Vorinfektion ausgelöst wird, weiter untersucht und es zeigte sich abhängig vom Allel des *Helicobacter pylori* Außenmembranproteins Q (HopQ). Das *hopQ* Allel I wird mit stärkerem Widerstand der Wirtszellen assoziiert als das *hopQ* Allel II. Durch die Verwendung myeloischer Zelllinien, wie z.B. primäre Leukozyten von freiwilligen Blutspendern, liefert diese Doktorarbeit die ersten Hinweise, dass der zelluläre Widerstand für Zelllinien unterschiedlichen Ursprungs sowie unter physiologischen Bedingungen gültig sein könnte. Außerdem offenbart diese Studie, dass eine Vorinfektion nicht nur zu einem Widerstand gegen CagA Translokation führt, sondern zu einer Einschränkung aller Funktionen des *cag* T4SS. Darüber hinaus kann erstmals gezeigt werden, dass das

Widerstandsphänomen biphasisch ist, mit einer schnellen Abwehr in den ersten 5 Minuten und einer langsameren, aber stärkeren nach 1 Stunde. Eine Suche basierend auf diesen Erkenntnissen nach beteiligten zellulären Mechanismen ergibt, dass die zelluläre Proteinneusynthese und der Einfluss des Zellzyklus ausgeschlossen werden können. Eine Unabhängigkeit von Schwankungen in intrazellulären Kalzium Konzentrationen scheint gegeben zu sein, muss jedoch noch weiter validiert werden.

Mit diesen neuen Erkenntnissen trägt diese Doktorarbeit dazu bei, die zelluläre Beeinträchtigung des *cag* T4SS von *H. pylori* besser zu verstehen. Verschiedene Kombinationen an *H. pylori* Stämmen führen zu stärkerer Einschränkung des *cag* T4SS und dadurch geringerer Entzündung. Folglich weisen diese Ergebnisse auf einen vorteilhaften Effekt von Infektionen mit multiplen Stämmen hin, welcher der Grund für asymptomatische Infektionen des Menschen sein könnte.

III Summary

Helicobacter pylori (*H. pylori*) is a gramnegative, rod-shaped bacterium, which colonizes the human stomach. It is strongly associated with the genesis of several gastric pathologies, the worst being gastric cancer. After binding to the gastric mucosa cells *H. pylori* uses its *cytotoxin-associated antigen* type IV secretion system (*cag* T4SS) to inject the cytotoxin-associated antigen A (CagA) protein from its cytoplasm into the cytoplasm of its host cells, where the CagA protein severely interferes with the cellular processes. But only Type I *H. pylor*i are capable of CagA translocation, while Type II *H. pylori* lack the CagA protein. Additionally, the host cells produce and secrete interleukin-8 (IL-8) after contact with the *cag* T4SS. In 2013 Jiménez-Soto et al. (Jiménez-Soto LF et al., 2013) first described the phenomenon that host cells pre-infected with *H. pylori* build up a resistance against secondary infecting *H. pylori* strains, leading to a significant reduction of CagA translocation. The following dissertation presents new insights into the formation of this cellular resistance.

First, the semi-quantitative analysis of Western blots to quantify translocated CagA was optimized by establishing Upper Lane Normalization (ULN) and Single Band Normalization (SBN). In order to eliminate the interference of *H. pylori* proteins, only cellular proteins bigger than most *H. pylori* proteins are used for normalization (> 100kDa). For cell lines with high protein expression bigger than 100kDa the upper part of the western blot is used (ULN), while for cell lines with low protein expression in this range a single strong band is identified (SBN).

With these improvements the phenomenon of an active cellular resistance against CagA translocation caused by pre-infection is further explored and it is found to be dependent on the allele of *Helicobacter pylori* outer membrane protein Q (HopQ). The *hopQ* allele I is associated with stronger resistance of the host cells than *hopQ* allele II. Using myeloic cell lines, like e.g. primary leukocytes from voluntary blood donors, this dissertation provides first evidence that the cellular resistance may be valid for cell lines from several origins and under physiological conditions. Apart from that, this study reveals that pre-infection does not only lead to resistance against CagA translocation but an impairment of the whole functionality of the *cag* T4SS. Furthermore, the investigation discovers the resistance phenomenon to be biphasic with a fast resistance within the first 5 minutes and a slower and stronger resistance after 1 hour. Searching for the involved cellular mechanisms based on this finding cellular protein *de novo* synthesis and an influence of the cell cycle is ruled out, while an

independency of changes in intracellular calcium concentration seems to be given, but still needs to be validated further.

With these unprecedented discoveries this dissertation contributes to a better understanding of the cellular interference with *H. pylori*'s *cag* T4SS. Several combinations of *H. pylori* strains lead to increased impairment of the *cag* T4SS and accordingly less inflammation. Therefore, these results suggest a beneficial effect of multiple *H. pylori* strain infections, which may be the reason for asymptomatic infections in human beings.

IV Introduction

Helicobacter pylori (*H. pylori*) is a gramnegative bacterium, which was first identified when examining patients with gastritis and gastric or duodenal ulcers (Marshall BJ and Warren JR, 1984). It is usually found in the human stomach, where it seems to survive and thrive the best. The presence of *H. pylori* in the stomach is strongly associated with the genesis of several gastric pathologies (EHSG, 2012). Detailed research on the interaction of *H. pylori* with its host is essential. This dissertation contributes with a new insight into the interference of the host cells with one of *H. pylori's* virulence factors, the *cag* Type IV secretion system.

IV.1. Helicobacter pylori

The survival of microorganisms in the human stomach was thought to be impossible due to the highly acidic environment (Otero LL et al., 2014). Repeated descriptions of a spiral bacteria in this organ were heavily doubted for over a hundred years until Robin Warren and Barry Marshall successfully cultured it from a patient's stomach in 1982 (Marshall BJ and Warren JR, 1984). This gramnegative epsilon-proteobacterium was finally named *Helicobacter pylori*.

IV.1.1. Clinical relevance

H. pylori can be found in about 50% of the world's population. Its prevalence varies, presenting higher incidences in Africa and Asia than in America and Western Europe (Eusebi LH et al., 2014). The transmission of this infection is still unclear. There is evidence for acquisition in early childhood by horizontal transmission from the parents, most likely in an oral-oral or faecal-oral manner (Roma E et al., 2009) (Elitsur Y et al., 1999).

H. pylori infection naturally persists lifelong and causes gastric inflammation in all cases (Atherton JC 2006). Most infected individuals stay asymptomatic and therefore undetected. Only 10% develop symptoms and get diagnosed with gastric or duodenal ulcers (Otero LL et al., 2014) (Atherton JC 2006). The clinically most important discovery was, that *H. pylori* was found in the stomach of around 60% of gastric adenocarcinoma patients and around 90% of patients with gastric lymphoma (Parsonnet J et al., 1991). This high correlation suggests that it plays a role in the development of cancer and led WHO to classify *H. pylori* as class 1 carcinogen (Møller H et al., 1995).

Causing 1% of all deaths in Germany in 2013/2014, gastric cancer still has one of the worst survival rates. The German Centre for Cancer Registry data calculated a relative 5-year-survival rate for women in Germany of about 33% and 30% for men (RKI 2017). The implication of bacteria as a cause of gastric cancer opened the unique possibility of cancer prevention by antibiotic treatment. Therapy schemes to eradicate *H. pylori* infection were developed. A combination of proton pump inhibitors and two different antibiotics became standard first-line therapy. This triple therapy can be extended by bismuth to a quadruple therapy (EHSG, 2017).

After the treatment, patients were free of gastric disorders and indeed, gastric cancer incidences dropped (Asaka M et al., 2010). Especially mucosa-associated lymphoid tissue lymphoma (MALT lymphoma), a rare subtype of gastric cancer, showed a high association with *H. pylori* infection. After eradication therapy, complete disease remission could be seen in 50-90% of the patients (Nakamura S et al., 2013). A dream came true – cancer prevention and therapy was possible by simple antibiotic treatment. Soon, *H. pylori* eradication therapy was integrated into medical guidelines.

However, at the same time, studies started to pile up reporting of new disorders after eradication therapy. Gastro-oesophageal reflux disease with its complications, Barrett's oesophagus and oesophageal adenocarcinoma, have been correlated with *H. pylori* eradication (Labenz J et al., 1997). Correspondingly, epidemiological studies have shown negative association of *H. pylori* infection with Barrett's oesophagus, erosive oesophagitis and oesophageal carcinoma (Rokkas T et al., 2007) (Fischbach LA et al., 2012). This means that having *H. pylori* might protect from oesophageal diseases. Additionally, inverse correlations of the development of obesity, asthma and other atopic disorders with *H. pylori* colonization have been made in several studies (Francois F et al., 2011) (Chen Y et al., 2008) (Chen C et al., 2017).

Research seems to have focused on two approaches on this gastric bacterium in the last years. The first one searches for the most effective infection eradication to prevent further gastric diseases. The second approach roots in the peculiarity that the infection rate among humans is so high, yet the ratio of patients developing symptoms is so much lower (Atherton JC 2006) and therefore questions a life without *H. pylori* to be better. This approach has strongly been boosted when reports of other microorganisms in the human stomach have been published forming the construct of a gastric microbiota (Bike et al., 2006) (Delgado S et al., 2013). Additionally, many patients infected with *H. pylori* host more than one strain. Multi-strain

infections are a majority and alter the severity of disease (Romo-González C et al., 2009) (Kim YS et al., 2009) (Secka O et al., 2011).

IV.1.2. Colonization and virulence factors

IV.1.2.1. Flagella and Urease

*H. pylor*i is a rod-shaped bacteria, which is highly motile due to two to six flagella at one location (Marshall BJ and Warren JR, 1984). It is almost exclusively found in the human stomach, in spite of the highly acidic environment there. In order to resist this aggressive surrounding, it hides in the mucus layer above the gastric epithelium. The gastric epithelium is covered by a bicarbonate-buffered mucus layer, which protects it from its own acidic juice. On *H. pylori's* way through the acidic juice in the gastric lumen to this mucus layer it is capable of reducing the pH of its surroundings by expressing urease (Dunn BE et al., 1990). Urease is an enzyme which converts urea into carbon dioxide and ammonia by hydrolysis. The ammonia neutralizes the main component of the acidic gastric juice, hydrochloric acid, and allows short term survival in this extreme environment (Dunn BE et al., 1998).

IV.1.2.2. Gene and genome diversity

In the attempt to understand the pathogenicity of *H. pylori* better, its genetic material was and is studied intensively. One extraordinary finding was, that *H. pylori's* strain variety is vast (Akopyanz N. et al., 1992). Even in a single patient, over time, a high DNA sequence variability can be observed. One patient can be infected by several strains at once, but moreover the composition of these strains change over time (Morelli G. et al., 2010). A reason for this is *H. pylori's* high rate of mutation, recombination and horizontal transfer (Suerbaum S et al., 2007). It alters its genome by point mutations as well as by insertion of DNA fragments, which it receives from others, or exclusion of genes, which it does not need. This capability enables *H. pylori* to adapt to its host quickly and maintain a longterm colonization (Blaser MJ et al., 2004).

IV.1.2.3. Cag pathogenicity island

One main virulence factor is a 40-kb DNA segment called *cytotoxin-associated antigen* pathogenicity island (*cag* PAI). In view of the low GC-content compared to the rest of the *H. pylori* genome it was most likely horizontally transferred from another species (Hacker J et al., 1997) (Tomb JF et al., 1997). The *cag* PAI contains 27 to 31 genes. Most of these genes

encode the components of a type IV secretion system (T4SS) known from *Agrobacterium tumefaciens* and *Bordetella pertussis*. The components build a complex syringe-like apparatus, which sits in the bacterial membrane. With this apparatus DNA or proteins can be transported from the bacterial cytoplasm out of the cell or from the surroundings into the cell. Most microorganisms, as *H. pylori,* use a T4SS to inject an effector protein or DNA segment into a host cell (Christie PJ et al., 2005). The only effector protein of the *cag* T4SS is the cytotoxin-associated antigen A (CagA), encoded on the *cag* PAI (Odenbreit S et al., 2000). Besides the *cag* T4SS and its effector protein, the proteins encoded on the *cag* PAI seem to support the apparatus or the translocation mechanism (Fischer W et al., 2001).

The presence of the *cagA* gene as a representative of the *cag* PAI divides *H. pylori* species into two groups: Type I and Type II *Helicobacter pylori*. Type I strains encode a *cagA* gene, whereas Type II strains do not (Xiang Z et al., 1995) (Tummuru MKR et al., 1993). This classification gained its importance when the pathogenic power of CagA was discovered. Epidemiological data shows, that it is far more likely to isolate a Type I strain or Type I and II strains from a patient with gastric disorders due to *H. pylori* infections than Type II strains alone (Azuma T et al., 2004). Correspondingly, asymptomatic individuals with *H. pylori* infection are more likely to host Type II strains than Type I *H. pylori* (Xiang Z et al., 1995) (Covacci A et al., 1997). Additionally, CagA's interference with essential intracellular mechanisms of its target cells (see chapter IV.2.) supports the idea of CagA as a main cause of gastric diseases.

IV.1.2.4. Outer membrane proteins

A systematic search of *H. pylori's* genome for outer membrane proteins (OMPs) revealed a large number of possible proteins, identified by a C-terminal sequence characteristic for OMPs. Almost 4% of the strain's whole coding potential contains OMPs (Alm RA et al., 2000). On the basis of gene sequence homology all OMPs were clustered in groups. The biggest family is divided into two subgroups: the ***H****elicobacter pylori* **o**uter membrane **p**roteins (Hops) and the **Ho**p **r**elated proteins (Hors). This family consists of 33 OMPs with a strongly conserved C terminus. Hops are identified by a specific N-terminal motif, whereas Hors have a similar overall gene sequence like the Hops, but do not possess the Hop motif. Most that is known about the structure and the function of these OMPs so far, is deduced from their gene sequence. It is expected, that most of the OMPs built adhesins, some also porins. To this moment, only a few Hops were explored further. The most studied are the blood group

antigen-binding adhesin (BabA), the sialic acid binding adhesin (SabA), the outer inflammatory protein A(OipA) and the *Helicobacter pylori* outer membrane protein Q (HopQ) (Königer V et al., 2016).

BabA binds to the fucosylated blood group antigen Lewis b building a strong adhesion of *H. pylori* to the epithelial cells of the gastric mucosa. It is highly associated with Type I *H. pylori*. In 73% of Type I strains BabA can be detected, while only 5% of Type II strains show BabA expression (Ilver D et al., 1998).

SabA was shown to be an adhesin, like BabA. By binding to sialylated glycoconjugates it mediates adhesion of *H. pylori* to inflamed tissue and human neutrophilic leukocytes (Mahdavi J et al., 2002) (Unemo M et al., 2005). The latter are subsequently activated, which results in a nonopsonic neutrophil oxidative burst and phagocytosis of the bacteria. Interestingly, *H. pylori* expresses SabA irregularly, able to bind sialylated glycoconjugates in the one moment and losing this ability in the next. This inconsistency is suggested to be due to phase variation (Mahdavi J et al., 2002), enabling *H. pylori* to express a specific profile of OMPs, adapted to its environment.

The OMP OipA was detected when examining the ability of *H. pylori* to induce the proinflammatory chemokine interleukin-8 (IL-8) production in its host cell. Similar to *cag* PAI, it triggers IL-8 production in the host cell, although it uses another signal cascade in the cell than *cag* PAI (Yamaoka Y et al., 2000) (Yamaoka Y et al., 2004). This way IL-8 levels increase up to three times, if the Type I *H. pylori* additionally possesses a functional OipA compared to Type I strains without functional OipA. It is therefore not surprising that about 95% of CagA producing *H. pylori* also express the OipA protein (Odenbreit S et al., 2009). However, it was also shown that several *H. pylori* strains had the ability to regulate their OipA expression via the slipped-strand repair mechanism (Yamaoka Y et al., 2000). Like it was seen for the expression of SabA, *H. pylori* seems to be able to alter its outer membrane profile if needed.

The last outer membrane protein mentioned is the HopQ. As BabA and SabA, HopQ is an adhesin (Loh JT et al., 2008). Its binding partners were recently discovered to belong to the human carcinoembryonic antigen-related cell adhesion molecule family (CEACAM). HopQ binds directly to the N-terminal domains of CEACAM1 and CEACAM5 on the cell surface (Königer V et al., 2016). This adhesion is required by the *cag* T4SS to translocate CagA into the host cell and induce IL-8 secretion (Belogolova E et al., 2013). It also is essential for the mechanism which prohibits CagA translocation by a following *H. pylori* strain into the same

host cell (see chapter IV.3.) (Jiménez-Soto LF et al., 2013). Additionally, a sequence analysis of *hopQ* genes of several *H. pylori* strains revealed that they can be classified into two groups: HopQ allele I and allele II. Interestingly, HopQ allele I is strongly associated with Type I *H. pylori* and HopQ allele II with Type II *H. pylori* (Cao P et al., 2002). Correspondingly, HopQ allele I is correlated with more severe gastric pathologies, especially gastric cancer. Even though, HopQ allele II was also found in patients with gastric disorders and in 46% of the isolated strains both alleles of HopQ were present (Yakoob J et al., 2015) (Leylabadlo HE et al., 2016) (Cao P et al., 2005).

Intriguingly, all these outer membrane proteins showed a high consistency between unrelated strains with a nucleotide identity of 80-96% (Alm RA et al., 2000). This homology between orthologs is higher than in many other species and suggests a preservation for a specific functional reason. On the other hand the high number of proteins, that are related to each other so closely, is a sign for high diversity and continuing adaption to the surroundings (Odenbreit S et al., 2009) (Alm RA et al., 2000).

IV.2. Interaction of *H. pylor*i with its host

H. pylori colonizes the human stomach and interacts with the gastric epithelial cells on the one hand and with immune cells on the other. Under *in vitro* lab conditions cell lines of epithelial or immune cell origin experience injection of the effector protein CagA into their cytoplasm and an induction of cytokine release by the bacteria. These two interactions of *H. pylori* with its human host cells are main subject of research in this dissertation.

IV.2.1. Mechanism of CagA translocation

In vitro experiments with *H. pylori* require specific cell lines which are susceptible to CagA translocation. Intriguingly, *H. pylori* is not able to inject CagA in any cell line, which strongly indicates specific characteristics of *H. pylori's* host cells. So far, only two proteins on the host cell surface were discovered to play a role in CagA translocation: the human carcinoembryonic antigen-related cell adhesion molecule family (CEACAMs) and β1 integrin.

Integrins are transmembrane proteins, expressed on almost all types of cell lines and interact naturally with other cells and extracellular proteins. Originally, it was found that CagA translocation by *H. pylori* is limited, if the host cells do not express β1 integrin on their surface (Kwok T et al., 2007) (Jiménez-Soto LF et al., 2009). The current model proposes that

during infection with *H. pylori* β1 integrin is thought to cluster on the host cell surface and to organise lipid rafts. Then it binds to the *cag* T4SS components CagA, CagI and CagY. More precise, it is only the extracellular domain of β1 integrin which is required for successful translocation of CagA. This β1 integrin-*cag* T4SS interaction revealed several abnormalities. Instead of binding to the RGD motif, the natural binding site of integrin ligands, *cag* T4SS-β1 integrin interaction was RGD independent (Kaplan-Türköz B et al., 2012). Secondly, the outside-in signalling pathway after ligand binding was shown to be irrelevant. And finally, CagA translocation did not occur, when β1 integrin on the cell surface was fixated in an activated/extended state (Jiménez-Soto LF et al., 2009). Transformation from an activated/extended into an inactivated/bent conformation was shown to be crucial for CagA insertion. It is suggested, that this change in conformation pulls *H. pylori* even closer to the surface of its host cell, which allows the *cag* T4SS to inject the CagA through the cell membrane into the cytoplasm of its host. Intriguingly, recent findings of Zhao et al. questioned the necessity of β1 integrin for CagA translocation leading the focus to the CEACAM family (Zhao Q et al., 2018).

The carcinoembryonic antigen-related cell adhesion molecule family (CEACAMs) was recently discovered to interact with *H. pylori* (Königer V et al., 2016). Especially CEACAM1, CEACAM3, CEACAM5 and CEACAM6 were identified as specific binding partners of *H. pylori's* outer membrane protein HopQ. HopQ binds to the N-terminal domain of CEACAM, clusters it on the host cell surface and through this mediates a tight adhesion. This is specific to the human CEACAMs and does not occur with murine, bovine or canine CEACAMs. *In vitro* experiments showed that the more CEACAM is available on the cell membrane, the more CagA is injected into the host cell. Additionally, a genetic knockout of CEACAM1, CEACAM5 and CEACAM6 lead to almost complete elimination of CagA translocation (Zhao Q et al., 2018). Therefore, host cell expression of CEACAMs is an essential requirement for CagA translocation.

After translocation into the host cell CagA accumulates on the inner surface of the plasma membrane. There, it undergoes tyrosine phosphorylation by Src and Abl family kinases on their Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs (Stein M et al., 2002) (Poppe M et al., 2007). The EPIYA motifs are localized on the C-terminal end of CagA. Each EPIYA motif with its flanking sequences forms an EPIYA segment. These segments can be divided by their flanking sequences into 4 types: EPIYA-A, -B, -C and -D (Higashi H et al., PNAS 2002). CagA of clinical isolates in Western countries (Europe, America, Australia) primarily

possesses EPIYA-A, -B and -C segments. But isolates of the Eastern Asian countries (Japan, Korea, China) prefer the combination of EPIYA-A, -B and -D. Combined with the epidemiological data of more severe gastric disorders in East Asia, the East Asian CagA with the EPIYA-D segment is considered to be more virulent (Azuma T et al., 2004) (Hatakeyama M, 2006).

Phosphorylation at its EPIYA motifs enables CagA to interact with several cell signalling proteins. One way is by binding to the cytoplasmic protein tyrosine phosphatase SHP-2 and forming a CagA-SHP-2 complex (Higashi H et al., Science 2002). This complex interferes with the regulation of cell proliferation, morphogenesis and motility, which can be seen in an impressive morphologic transformation of the cells called the hummingbird phenotype. The cytoskeleton is drastically rearranged to form an elongated cell with increased mobility and the integrity of cell-cell contacts is disturbed (Higashi H et al., Science 2002).

Apart from that, phosphorylation is not required for CagA to be biologically active. In an phosphorylation-independent manner CagA binds Grb2 adaptor protein, for example. The CagA-Grb2 complex activates the Ras-MAP kinase pathway, which is assumed to cause several tumors (Mimuro H et al., 2002). Furthermore, CagA directly interacts with tight junction proteins, such as ZO-1 and JAM (Amieva MR et al., 2003). It disrupts the cell-cell adhesion so the cells lose their polarity. As a result the cells can develop pseudopodia, leave the cell structure and undergo epithelial-to-mesenchymal transition.

IV.2.2. Inflammatory host response

Gastric pathologies caused by *H. pylori* always start as inflammation of the gastric mucosa. The infection causes an increase of cytokines in the tissue, which mediate the inflammatory process (Lindholm C et al., 1998) (Yamaoka Y et al., 1997). Leukocytes are attracted to the site of inflammation and activated. So far, very little is known about the direct influence of *H. pylori* on the inflammation process.

Beside gastric epithelial cells, *H. pylori* also interacts with immune cells. Especially dendritic cells were found to come into contact with *H. pylori* by squeezing their dendrites through the mucosa into the gastric lumen in order to trap antigens. *H. pylori* can inject CagA into their cytoplasm and alter their behaviour (Busch B et al., 2015). CagA paralyses the cells and inhibits the regular immune response. Additionally, the *H. pylori* outer membrane adhesin, SabA, was identified as an activator of neutrophilic granulocytes (see chapter IV.1.2.4.) (Unemo M et al., 2005).

The release of cytokines is also altered by *H. pylori.* Bacterial and cellular factors, which modulate cytokine secretion, are best known for interleukin-8 (IL-8). Two independent initiators were identified to induce the production of IL-8 in the host cells: OipA and *cag* T4SS. OipA binds as an adhesin (see chapter IV.1.2.4.) and leads to phosphorylation of the signal transducers and activators of transcription 1 (STAT1) in eukaryotic cells (Yamaoka Y et al., 2004). STAT1 binds to an interferon regulatory factor (IRF)-1, which then induces IL-8 transcription via the interferon-stimulated responsive element (ISRE)-like element in the IL-8 promotor. *Cag* T4SS, on the other hand, activates the ISRE-like element through the NFκB pathway (Yamaoka Y et al., 2004). For this activation the *cag* T4SS has to be fully functional in its ability to translocate CagA into the host cell (Fischer W et al., 2001). There is no increment in IL-8 without CagA injection, although CagA itself is not responsible for IL-8 induction (Sokolova O et al., 2013).

The immune response to *H pylori* infection can be very diverse in individual patients. Proinflammatory polymorphisms have been identified as risk factors for gastric cancer in *H. pylori* infected patients. Interleukin-1 gene cluster polymorphisms and polymorphisms of the tumor necrosis factor α gene are associated with a higher risk for gastric cancer than infected patients without these polymorphisms (El-Omar EM et al., 2000) (Machado JC et al., 2003). Like the bacteria can be classified in high- and low-risk genotypes (see chapter IV.1.2.), the patient also displays factors, which can characterise the individual as a high- or low-risk genotype. This might be one of the reasons behind why some infected people develop severe gastric pathologies and others do not.

IV.3. Resistance phenomenon

In 2013 Jimenez-Soto et al. published an intriguing phenomenon, which was observed during multiple strain infection *in vitro* (Jiménez-Soto LF et al., 2013). It seems that the eukaryotic host cells built up some kind of resistance against the *cag* T4SS after they came in contact with *H. pylori* bacteria.

Two different multiple strain infection setups were used: co- and pre-infection. Co-infection describes an infection of host cells with a suspension containing two *H. pylori* strains. Pre-infection means that the host cells are infected with one strain before getting infected again by a second strain later. Both, the primary and the secondary infecting strain, have been analysed in their ability to translocate CagA into the host cells and have been found to be inhibited. This inhibition has been shown to be independent of a competition for receptor binding sites,

but rather due to a resistance of the host cell to the *cag* T4SS. The first *H. pylori* bacteria to reach the host cells trigger a cellular reaction, which restrains the following *H. pylori* bacteria to inject CagA into the host cells. The outer membrane proteins AlpAB, HopI and HopQ have been identified to be essential for the resistance phenomenon. Apart from that, one cellular requirement for CagA translocation, β1 integrin (Jiménez-Soto LF et al., 2009), could be ruled out to play a role in the resistance phenomenon.

In conclusion, Jimenez-Soto et al suggest that a primary infecting *H. pylori* strain triggers a resistance in its host cells against secondary infecting strains (Jiménez-Soto LF et al., 2013). This resistance phenomenon in eukaryotic host cells is subject of research in this dissertation.

IV.4. Semi-quantitative analysis of translocated CagA

This dissertation explores interference with the translocation of the effector protein CagA. The interference is evaluated by the amount of CagA in the host cells, which therefore needs to be quantified as exactly as possible. Quantitative analysis of CagA, that has been translocated into the host cell, is tricky, because it has to be distinguished from the CagA still present in the bacteria or excreted into the extracellular space. The supernatant of infection settings can be removed, to take the bacteria and the extracellular fluids away. But secure elimination of all bacteria, even the ones attached to the cells, is extremely difficult (Jiménez-Soto LF et al., 2016). Luckily, CagA gets phosphorylated at its tyrosine residues after translocation into the host cell, which sets it apart from not-translocated CagA proteins (Odenbreit S et al., 2000). This tyrosine-phosphorylated form of CagA can be detected by the use of antibodies.

The best-established method so far to detect phosphorylated CagA is Western blot. This method offers even more than just verification of the existence of a protein, but a semi-quantitative analysis of this protein (Heidebrecht F et al., 2009). One obstacle is, that for reliable quantification the protein concentration and the sample load would have to be exactly the same in every sample. To overcome this obstacle normalization signals are necessary. Normalization signals show the amount of protein on the western blot for each sample, so the signal of interest can be put in relation to its total protein load. Two ways of normalization commonly used are the housekeeping protein normalization and the total protein normalization (Gürtler A et al., 2013) (Colella AD et al., 2012).

Housekeeping proteins were intended to be generally and constantly expressed in most cell lines, like tubulin or actin. Therefore the amount of total protein could be estimated according

to the amount of that housekeeping protein in the sample. Unfortunately, several studies have shown, that in many cases the investigated treatment of the cells interacted with the expression of the housekeeping proteins (Li R et al., 2013). As a result, the signal of the housekeeping protein did not reflect the amount of total protein of the sample anymore.

The most reliable, most robust and most sensitive quantifications are provided by the Stain-Free technology (Gürtler A et al., 2013). Stain-Free technology is a method for total protein normalization. The idea is to stain all proteins at the same time, so the total protein amount per sample can be estimated. Stain-Free technology uses trichlorethanol. This method is based on the use of ultraviolet (UV) light to cross-link chlorine molecules from the trichlorethanol to the tryptophan residues in the proteins. These tryptophan residues then show an increased fluorescence, which can be detected at wavelengths of 300 nm (Ladner CL et al., 2004). Compared with the common staining method Coomassie brilliant blue the protein pattern with trichlorethanol is the same. But with its dependency on tryptophan residues trichlorethanol was shown to be more sensitive for low amounts of proteins than Comassie brilliant blue, especially for low amounts of peripheral membrane proteins (Ladner CL et al., 2004).

Nevertheless, in the field of infection biology, additional difficulties disturb the quantification process. The presence of bacteria, viruses or parasites may alter normalization signals. Any cellular protein expression of the host, especially the expression of housekeeping proteins, can be changed. Because of this, the use of Stain-Free technology as a standard for semi-quantitative analysis of CagA translocation is evaluated in the beginning of this dissertation.

V Material and Methods

V.1. Materials

V.1.1. Cell lines

AGS	ATCC CRL 1739a, human gastric adenocarcinoma epithelial cells
ST3051	human gastric adenocarcinoma epithelial cell line
THP1	ATCC TIB-202, human acute monocytic leukaemia cell line
Primary leukocytes	Isolate from blood of volunteers, see chapter V.2.2.2.

V.1.2. Bacterial strains

P12	Clinical isolate	(Schmitt et al., 1994)
P217	Clinical isolate	(Jiménez-Soto LF et al., 2009)
P145	Clinical isolate	
26659	Genome sequenced wildtype	(Tomb JF et al., 1997)
G27	Clinical isolate	(Covacci A et al., 1993)
Tx30a	Clinical isolate	(Atherton JC et al., 1995)
X47	Mouse adapted strain from Australia	(Handt LK et al., 1995)
P12ΔT4SSs	ΔcagPAI, ΔTfs3, ΔTfs4, ΔflaA, ΔflaB, ΔcomB	(Jiménez-Soto LF et al., 2013)
P12ΔhopQ	ΔhopQ, $Strep^R$	(Königer V et al., 2016)

V.1.3. Plasmids and Primers

LJ168: 5'-CATGGCGGCCGCTTAATACGCGAACACATAATTGAG-3'

LJ177: 5'-CTTTAAGGAACATCTTATGAAAAAAACGAAAAAAACGATTCTGC-3'

LJ180: 5'-CAAACACCCCTTCATGGCAAACA-3'

V.1.4. Reagents and solutions

V.1.4.1. Reagents

HIFI-Polymerase (Thermo Scientific), DreamTaq DNA Polymerase (Fermentas), dNTP-Set (PanBiotech), HBSS buffer (+Ca2+, +Mg2+, -phenol red, Gibco), FLIPR Calcium 6 (Molecular Devices), Ionomycin (Sigma), Cycloheximide (ready made, Sigma-Aldrich), Rotiphorese NF-Acrylamid / Bis-Lösung 30% (Roth), Ammonium Persulfate (Sigma), Tetramethylethylenediamine (TEMED, Sigma), 2,2,2-Trichlorethanol (Aldrich), TMB substrate (BD-Biosciences), Immobilon Western developing solution (Millipore), Vectastain ABC HRP Kit (Vector Laboratories), Tween-20 (Roth), Phenylmethylsulfonyl fluoride (PMSF, in Isopropanol, Sigma), Leupeptin (in H2O, Sigma), Pepstatin A (in Methanol, Sigma), Ortho-Vanadate (in H2O, Sigma), DNAse I from bovine pancreas grade II (Roche), L-Serine (Roth), Glycin (MP Biomedicals), L-Asparagin-monohydrat (Sigma), Ethanol (Roth), propidium iodid (10µg/ml, MACS Miltenyl Biotech), QIAmp DNA Mini Kit (250) (Qiagen), Sodiumchlorid >99,5% (Roth), Precision Plus Protein All Blue Standard (BioRad), Bovin Serum Albumin, Fraction V (BSA, Biomol), Methanol (Roth), Sodium-Dodecylsulfate in pellets (SDS, Serva), Glycerol >99,5% (Roth), Ethylenediamine-tetraaceticacid (EDTA, Roth); Sulfuric acid (H2SO4, Merck), Tris(hydroxymethyl)-aminomethan (TRIS, MP Biomedicals), Hydrochloric acid (Roth), Sodium dihydrogen phosphate (NaH2PO4, Sigma), Nonylphenylpolyethylene glycol (NP-40, Fluka), Sodium deoxycholate (Sigma), Bromphenol-blue (Serva), 6-Amino-n-caproic acid (Roth), D-Glucose (Roth), L-Glutamine >99% (Roth), L-Cystein >99% (Roth), Thiamine pyrophosphate (Merck), Iron(III)-nitrate (Sigma), Thiamin (Sigma), 4-Aminobenzoic acid (Sigma), Nicotinamide adenine dinucleotide (NAD, Merck), Vitamin B12 (Merck), L-Cysteine (Merck), Adenine (Merck), Guanine (Merck), L-Arginine (Merck), Uracil (Fluka).

V.1.4.2. Solutions

10x PBS	2 g/l KCl, 80 g/l NaCl, 2 g/l KH2PO4, 14,4 g/l Na2HPO4 * 2H2O, 28,9 g/l Na2HPO4 *12H2O

For DNA extraction

T10E1	10 mM TRIS-HCl pH 7,5, 1 mM EDTA

For infection assay

PBS*	PBS, 1 mM PMSF, 1 µM Leupeptin, 1 µl Pepstatin, 1 mM Ortho-Vanadat
RIPA	50 mM TRIS-HCl pH 7,4, 150 mM NaCl2, 1 mM EDTA, 1 % (v/v) NP-40, 0,25 % (w/v) Sodium-deoxycholate
RIPA*	RIPA, 1 mM; PMSF, 1 µM Leupeptin, 1 µl Pepstatin, 1 mM Ortho-Vanadat, DNAse

For Western blotting

6% Singel gel (1mm)	1,2 ml 30% Bis-Acrylamide, 3, 1 ml 2x Single gel buffer, 60 µl Ammonium Persulfate, 50 µl TEMED, 1,6 ml water (Ahn T et al., 2001)
2x Single gel buffer	152 mM TRIS-HCl pH 7,4, 0,2 M L-Serine, 0,2 M Glycine, 0,2 M L-Asparagine
2x SDS-loading buffer	100 mM TRIS-HCl pH 6,8, 4 % SDS, 0,2 % Bromophenol-blue, 20 % Glycerol
5x Running buffer	250 mM Glycine, 0,1 % SDS, 25 mM TRIS-HCl pH 8,3
Anode I buffer	0,3 M TRIS-HCl pH 10,4, 10 % Methanol
Anode II buffer	25 mM TRIS-HCl pH 10,4, 10 % Methanol
Cathode buffer	25 mM TRIS-HCl, 40 mM 6-amino-n-Caproic acid (or glycine), 10 % Methanol, final pH 9,4
TBS	150 mM NaCl, 20 mM Tris/HCl pH 7,5
Washing buffer	TBS, 0,075 % Tween-20
Blocking buffer	TBS, 0,075 % Tween-20, 3 % BSA
10x Stripping solution	250 mM Glycine, 10 % (w/v) SDS, pH 2

For ELISA

Coating buffer	100 mM Na2HPO4 pH 9,6
Washing buffer	PBS, 0,05 % (v/v) Tween-20
ELISA buffer	50 mM TRIS-HCl pH 7,6

Blocking buffer	PBS, 10 % (v/v) FBS
Stopping solution	1 M H2SO4

V.1.5. Antibodies

α-P-tyr (4G10)	Mouse monoclonal antibody against tyrosine phosphorylated proteins (Upstate)
α-CagA (AK 268)	Rabbit polyclonal antibody against the N-terminal part of CagA of *Helicobacter pylori*
α-tubulin	Mouse monoclonal antibody against human and mouse tubulin alpha subunit (Upstate)
AK 257	Rabbit polyclonal antibody against the C-terminal part of CagA of *Helicobacter pylori*
α-IL-8 (Coating antibody)	Mouse monoclonal antibody against human IL-8 (BD-Biosciences)
α-IL-8 biotin (Detecting antibody)	Mouse monoclonal antibody against human IL-8, biotin conjugated (BD-Biosciences)
α-mouse IgG POX	Goat monoclonal antibody, horseradish peroxidase conjugated (Sigma)
α-rabbit IgG POX	Goat monoclonal antibody, horseradish peroxidase conjugated (Sigma)

V.1.6. Cell culture solutions and additives

Trypan blue (Sigma), EDTA (2 mM), Penicillin/Steptomycin (Life Technologies), Trypsin/EDTA (TE) (Life Technologies), RPMI 1640 (Gibco), Fetal Bovine Serum (FBS) (Gibco), PercollTM plus (GE Healthcare Life Science), Heparin (Sigma), Dulbecco´s Phosphate Bufferd Saline (PBS, -Ca2+, -Mg2+, Gibco).

Complete media (CM)	RPMI 1640, 10 % (v/v) FBS

V.1.7. Bacterial culture solutions and additives

GC-Agar base (Thermo Scientific), Brucella Broth (BD), Cholesterol 250 x (Life Technologies), Kanamycin (Sigma).

Freezing media for *Helicobacter pylori*	10 % FBS, 20 % Glycerol, 70 % Brucella Broth, sterile filtrated
Agar plates	36 g/l GC-Agar base, 1 % Vitamin Mix, 0,4 % Cholesterol (Jiménez-Soto LF et al., 2012), (8 mg/l Kanamycin)
Vitamin Mix	100 g/l D-Glucose, 10 g/l L-Glutamine, 26 g/l L-Cystein, 0,1 g/l Thiamine pyrophosphate, 20 mg/l Iron(III)-Nitrat, 3 mg/l Thiamin, 13 mg/l p-Aminobenzin acid, 250 mg/l Nicotinamid-adenine dinucleotide (NAD), 10 mg/l Vitamine B12, 1,1 g/l L-Cystine, 1 g/l Adenine, 30 mg/l Guanine, 0,15 g/l L-Arginine, 0,5 % Uracil

V.1.8. Consumables

96-deepwell plate, 2 ml, non-sterile (NUNC); 96-well plate, immune-absorbant (NUNC); cell culture treated bottles with filter, 75 cm^2 (BD Falcon); Cell scraper (BD Falcon); Cell-culture treated plates, 6-well (Costar); Cotton swaps, sterile (deltalab); Cuvettes, disposable (1 cm, Brand); Falcon, 15-50 ml (Sarstedt); Freezing tubes, 2 ml (Nalgene); glas pasteur pipettes, stuffed, disposable (Poulten & Graph GmbH); Hypodermic needle, disposable, 24 G (Braun); Membrane adapter (Sarstedt); PCR tubes, 0,2 ml, Strips of 8 with attached caps (Brand); Petri Dish plates, 35 mm with 14 mm Microwell (MatTek); Pipette tips, 10-1000 µl, unsterile (Sarstedt); Polyvinylidenfluorid (PVDF) membranes 0,2 µm (BioRad); Reaction tubes, 1,5 ml (Eppendorf); Safty-Multifly-needle, 21 G (Sarstedt); Stripettes, 5, 10, 20, 50 ml (Costar); Syringe, 20 ml (Braun); Syringe-driven filters, 0,22 µm (Millipore); Transparent foil (Kaut-Bullinger).

V.1.9. Equipment

Absorbance Reader Sunrise (TECAN systems) and Magellan3 software; BD FACSCanto II flow cytometer (BD Biosciences, Heidelberg) with FlowJo® software; Centrifuge 5424R (Eppendorf); Centrifuge Megafuge 3.0R and Megafuge 16R (Heraeus, Thermo Scientific); Centrifuge Mikro 20 (Hettich); GelDoc Station (BioRad) with ImageLab 4.1 software; Incubator Heracell 150i (Thermo Scientific); Incubator Microincubator MI22N (Scholzen); Incubator (WTB Binder); Incubator Ultima (Revco); Inverted Microscope Wilovert S (Hund);Magnetic stirrer MR 3001 (Heidolph); Mini-Protean II glass spacer plates (0,75 mm, 1 mm) and glass short plates with casting frame, casting stand and 10-15-well comb

(BioRad); Neubauer chamber, 0,0025 mm2 (Assistant); PCR Thermocycler peqSTAR (VWR Peqlab); PCR Thermocycler Mastercycler personal (Eppendorf), PCR Thermocycler ThermoHybaid Px2 (Thermo Scientific); Pipette controller accu-jet® pro (Brand);Pipette Finnpipette 20-200 µl (Thermo Scientific); Pipette Transferpette-8 (0,5-10 µl, 10-100 µl, 20-200 µl, Brand); Pipettes pipetman, 20-1000 µl (Gilson); Rolling Mixer RM5 (Assistant); Scales (Fischer Biotech); Scales BP61S (Sartorius); Semi-dry blot transfer system (Fischer Biotech); Spectrophotometer DR 2000 (Hach); Spinning disc confocal microscope () and Volocity software; Sterile hood (BDK); Voltage Units Power Pac300 and Power Pac1000 (BioRad); Vortex Genie 2 (Scientific Industries); Water bath (GFL).

V.2. Methods

V.2.1. Bacteria

V.2.1.1. Culture

*H. pylor*i was grown on agar plates at 37 °C, 85 % N_2, 10 % CO_2, and 5 % O_2 for 24 hours.

V.2.1.2. Freezing

H. pylori, grown overnight on plates, is collected by a sterile cotton swap, suspended in 1 ml Freezing Media and immediately put on ice until storage at -70 °C.

V.2.1.3. Calculating the Multiplicity of Infection

Bacteria were collected from the plate by a sterile cotton swab and suspended in PBS. For OD550 measurement a dilution of 1:100 is made by transfer of 10 µl bacteria suspension into a cuvette with 0,99 ml PBS. Best results for CagA translocation are achieved by a multiplicity of infection of 60 bacteria per cell (MOI 60). Knowing that an OD550 of 0,1 represents 2-3x10^7 cfu/ml the appropriate volume of bacteria suspension can be calculated.

V.2.1.4. Chromosomal DNA extraction

For chromosomal DNA extraction bacteria, grown overnight on plates, were collected by a sterile cotton swap and suspended in 500 µl PBS. They were centrifuged at 4000 rpm for 10 minutes and the supernatant was discarded. The extraction was done with the QIAmp DNA Mini Kit by Qiagen with some modifications. The pellet was added 180 µl of ATL buffer and 20 µl proteinase K, vortexed and incubated at 56 °C under intermittent shaking.

When the pellet was lysed, the solution was assembled by short centrifugation and 200 µl AL buffer was added. After mixing by pulse-vortexing the sample was incubated at 70 °C for 10 minutes. Then 200 µl ethanol was mixed in by pulse-vortexing and the solution was assembled by short centrifugation. The sample was transferred to a Mini spin column and centrifuged at 8000 rpm for 1 minute. The tube with the filtrate was replaced by a new tube. After the addition of 500 µl AW1 buffer the sample was centrifuged at 8000 rpm for 1 minute and the tube with the filtrate discarded. This was repeated with a new tube and 500 µl AW2 buffer at 14000 rpm for 3 minutes. The filtrate in the tube was discarded and the tube reused for centrifugation at 14000 rpm for 1 minute. Then the column was put in a microcentrifuge tube and 200 µl of T10E1 was added. After incubation at room temperature for 1 minute and centrifugation at 8000 rpm for 1 minute 200 µl T10E1 was added and incubated at room temperature for 5 minutes. The extracted DNA was stored at -20 °C after centrifugation at 800 rpm for 1 minute.

V.2.1.5. DNA sequencing

For sequencing the *hopQ* gene of *H. pylori* the gene was amplified by PCR using the forward primer LJ177 and the reverse primer LJ168. The PCR mixture was made according to the manual of the DreamTaq DNA Polymerase with 0,2 pmol/µl of each primer and 0,5 µl Template DNA solution of unknown concentration. The initial denaturation was done at 94 °C for 3 minutes. Then 30 seconds of denaturation at 94 °C, 30 seconds of annealing at 51 °C and 2 minutes of elongation at 72 °C was repeated 30 times. The final elongation at 72 °C was done for 10 minutes. The PCR product was sent to GATC Biotec for Sanger sequencing the same day with the primer LJ177 for the first 800 base pairs and the primer LJ180 for the second part of the *hopQ* gene. Junction and analysis of the sequencing results was done in CLC DNA Workbench 6.0.2. Protein sequence was analysed for secondary structure prediction using Phyre 1.0 server.

V.2.2. Cells

V.2.2.1. Culture

Each cell line was grown in complete media at 37 °C, 5 % CO_2. Every two to three days they were split as soon as they reached values close to 90% confluency .

Adherent cells

Cells were washed with PBS, before detaching by Trypsin/EDTA for 5 minutes at 37 °C, 5 % CO_2. In cases where protease treatment would have compromised the fast adhesion of cells to the culture surface, cells were detached using PBS/EDTA solution instead of Trypsin/EDTA. The reaction was stopped by addition of complete media. Then the desired volume of cell suspension was transferred to a new bottle and complemented by complete media.

Non-adherent cells

THP1 cell suspension was centrifuged at 900 rpm for 5 minutes. The supernatant was removed and the pellet suspended in media depending on the splitting factor. Once a week the cells were first suspended in PBS and washed by gentle up and down pipetting before being centrifuged again and split in new media.

Estimating the number of viable cells

For plating a specific number of cells per well the number of viable cells was estimated. After cells were put in solution a sample of 10 µl cell suspension and 90 µl trypan blue was loaded onto a Neubauer Chamber. The unstained cells were counted and the number of cells estimated as followed: number of cells x dilution factor x 10^4 cells/ml.

V.2.2.2. Leukocyte isolation from whole blood

Leukocyte isolation was performed as described by Zeitler et al (Zeitler AF et al., protcols.io, 2017/2). Isotone Percoll® was prepared by addition of 0,135 g NaCl to 15 ml Percoll® and diluted with PBS to a Percoll® 55 % solution. Whole blood was drawn from human volunteers by a syringe charged with at least 20 u/ml Heparin. In a falcon 12 ml whole blood and 25 ml PBS / 2 mM EDTA were mixed by inversion. Slowly 35 ml blood suspension was added onto 15 ml Percoll® 55 % solution. Mixing of cells and Percoll® was avoided. The gradient was centrifuged at 400 g for 20 minutes at 16 °C without brake, so 4 layers became visible (Serum + Platelets / leucocytes / Ficoll + PMNs / erythrocytes). With a stuffed Pasteur pipette leukocytes were collected and diluted in at least 20 ml PBS. Leukocyte solution was centrifuged at 200 g for 5 minutes and suspended in 1 ml complete media.

V.2.2.3. Synchronization of cell cycle

For CagA translocation *H. pylori* uses cellular proteins expressed depending on the cell cycle. Maximal CagA translocation is achieved when cells are synchronized by serum starvation.

The cells are incubated overnight in medium free of FBS. 1 hour before infection media is changed to complete media, so cells start into G1 phase and are receptive for CagA translocation.

V.2.2.4. Inhibition of protein de novo synthesis in eukaryotic cells

Cycloheximide was added to the cell medium 30 minutes before the infection assay. The final concentration in the medium was 2,5 mM. The medium containing cycloheximide was not removed during the experiment. Effective inhibition of protein synthesis was proven by insignificant secretion of IL-8 by the host cells.

V.2.2.5. General pre-infection assay

The infection assays were done in 6-well plates in complete media as described by Jiménez-Soto (Jiménez-Soto LF, protocols.io, 2017) (Jimenez-Soto LF, protocols.io, 2017/2). The cells were incubated for 1-3 days to reach a confluence of 80 - 100 %, corresponding to around 1 x 10^6 cells per well. If the cells were supposed to be synchronized in their cell cycle, the media was changed 16-18 hours before infection. If not described differently, the cells

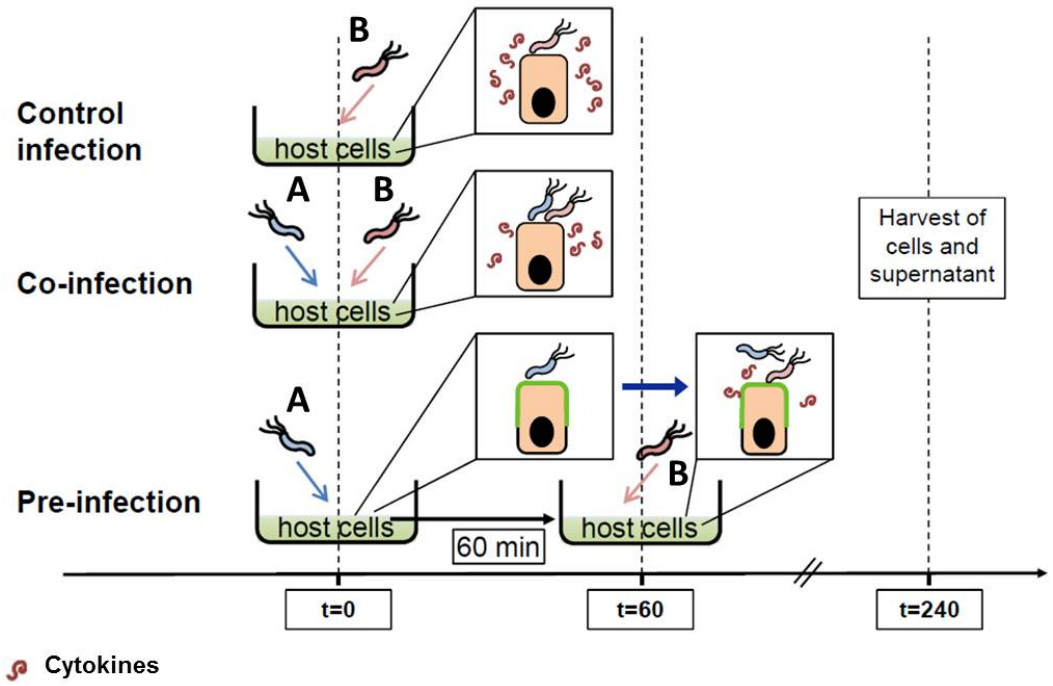


Figure V.1: Experimental setup of co- and pre-infection assay.
Host cells were treated in three different ways: control, co- and pre-infection treatment. Control infections were infections with the secondary infecting strain (strain B) alone. Infections with a suspension of the primary (strain A) and secondary infecting strain (strain B) are called co-infections. For pre-infection conditions the primary infecting strain was incubated with the host cells for 60 minutes, if not described differently, before infections with the secondary infecting strain. Cells and supernatant was harvested 3 hours after the first infection. Reference: (Zeitler AF et al., 2017)

were infected with the first strain (pre-infection) with an MOI of 60 and incubated for 1 hour at 37 °C, 5 %CO_2, before infection with the second strain with an MOI of 60. No media was changed in between. After another 3 hours of incubation at 37 °C, 5 % CO_2 the reaction was stopped by putting the cells on ice.

V.2.2.6. Harvest of infected cells

The harvest is continuously performed on ice to stop the infection. PBS*/RIPA* is prepared freshly and kept on ice. After harvest samples were prepared for Western blot by boiling with 30 µl of 2 x SDS loading buffer at 95 °C for 10 minutes. After boiling they were put on ice immediately and stored at -20 °C.

Harvest of adherent cells

Supernatant was collected for IL-8 ELISA and stored at -20 °C. Then 1 ml PBS* was added and the cells were scrapped off the surface in cold conditions. The cell suspension was centrifuged at 500 g, 4 °C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 20 µl PBS*.

Harvest of non-adherent cells

Cell suspension was collected and centrifuged at 300 g, 4 °C for 10 minutes. The Supernatant was collected for IL-8 ELISA and stored at -20 °C. The pellet was resuspended in 30 µl RIPA* buffer.

V.2.2.7. Analysis of cell cycle by propidium iodide staining

Cells were incubated in 500 µl TE for 10 minutes at 37 °C, 5 % CO_2 to harvest. The TE was neutralized with 500 µl RPMI Media and the cell suspension was centrifuged at 200 g for 6 minutes. The pellet was suspended in 250 µl cold PBS and transferred drop by drop into 2,5 ml ethanol 70 % with a Pasteur pipette. Shaking while dropping prevented cell clumping during fixation. The cells were incubated for 30 minutes on ice, then centrifuged at 200 g for 10 minutes. Ethanol was decanted and the pellet washed in 1 ml PBS. After centrifugation at 200 g for 10 minutes the pellet was suspended in 250 µl PBS and stored at 4 °C. 15 minutes before measurement propidium iodide was added to a final concentration of 2 - 3 µg/ml and briefly mixed with a vortex. The argon laser of the flow cytometer was set at 488 nm for excitation. Emission was measured at 620 nm. Statistical analysis was done with Flow-Jo Software.

V.2.2.8. Calcium assay by live cell microscopy

ST3051 cells were suspended in PBS/EDTA and plated on MatTek plates to reach a confluency of around 60-70 %. For calcium imaging the FLIPR Calcium 6 Assay Kit by Molecular Devices was used. The cells were washed with HBSS buffer and incubated with 50 µl HBSS buffer and 50 µl loading dye for 2 hours at 37 °C, 5 % CO_2. After washing the cells with 1 ml HBSS buffer they were incubated for 1 hour with 1 ml of complete media, stored at 37 °C, 5 % CO_2 overnight. Cells were imaged at the spinning-disk confocal microscope at 37 °C, 5 % CO_2 using the 488 nm laser. Addition of bacteria or reagents was done during imaging in a 500 µl solution with complete media, stored at 37 °C, 5 % CO_2. Shortly before infection bacteria were measured their OD550, calculated to reach a final MOI of 60 and stored at 37 °C, 5 % CO_2. As a positive control 0,5 µl of 1 mM ionomycin was used. This calcium ionophore was found to rapidly increase intracellular calcium levels by increasing the permeability of the cellular membrane for the ion (Beeler TJ et al., 1979).

V.2.3. Proteins

V.2.3.1. Separation of proteins and blotting

For immune detection of proteins, the proteins of the samples have to be separated by SDS-PAGE, as described by Jiménez-Soto (Jiménez Soto LF, protocols.io, 2016). Depending on the molecular weight of the desired proteins a 6 % or 7 % bis-acrylamide-gel with trichlorethanol was prepared, inserted into an electrophorese chamber with running buffer and loaded with samples. The samples were run through the gel at 100 V for 15 minutes and at 140 V for 65 - 70 minutes. Then the separated proteins were blotted onto PVDF membranes by a semi-dry electric transfer chamber at 1,2 mA / cm^2 for 75 minutes using Anode I, Anode II and Cathode buffer as transfer buffers. Dried membranes were stored at 4 °C.

V.2.3.2. Immune-detection by Western blot

PVDF membranes were activated in a methanol bath for 30 seconds, put in a tube and blocked with 5 ml Blocking buffer for 1 hour on a rolling machine. Blocking buffer was removed and 5 ml TBS with the first antibody in 1:10 000 dilution was added for 1 hour on the rolling machine. Then the membrane was washed four times by rolling 10 minutes with 5 ml Washing buffer. The second antibody was added with 5 ml Washing buffer in 1:10 000 dilution and incubated for 45 minutes on the rolling machine. After washing the membrane four times by rolling 10 minutes with 5 ml Washing buffer the tubes with the membranes

were inverted on paper towel to remove all Washing buffer and 1 ml Immobilon Western developing solution A and B were added 1 ml each. The developing solution was incubated for 30 - 60 seconds rolling before putting the membrane into a transparent film for imaging. The membrane was imaged with GelDoc Station by BioRad with a multichannel protocol using Colorimetric and ChemiHighResolution. Background and overexposure was avoided for all documentation.

For storage or for analysis with another antibody the membrane was stripped with 5 ml Stripping solution for 1 hour. Before storage in Washing buffer at 4 °C the membrane was washed with Washing buffer two to three times by inversion.

V.2.3.3. Semi-quantitative analysis of Western blots

Semi-quantitative analysis was performed as described by Zeitler et al (Zeitler AF et al., protocols.io, 2017). Amount of phosphorylated CagA was quantified relative to control infections. Multichannel images of the Western blots were processed in ImageLab 4.1 software by accurate detection of the lanes and bands by hand in the ChemiHighResolution channel. In the lane profile window the band was adjusted to its peak to minimize background signals. Control infection was marked for the software to measure relative band quantities. Comparability was certified by normalization with either tubulin as a housekeeping protein or Stain-Free technology. Normalization factors were multiplied by the relative band quantity in Excel.

Normalization with a housekeeping protein

In the normalization mode with housekeeping protein of the ImageLab 4.1 software the image of the tubulin Western blot was added to the image of the P-CagA Western blot as a second channel. Lanes were detected by hand as thorough as possible in both images and bands were marked in the tubulin Western blot. The software measured the normalization factors for each marked band.

Normalization with stain-free technology

The trichlorethanol in the electrophorese gels was activated by UV-light for 1 minute before blotting. Its chloride residues reacted with the tryptophan residues of the proteins and the proteins became fluorescent. The fluorescent signal was imaged in the GelDoc Station. In the normalization mode with total protein of the ImageLab 4.1 software the image of the fluorescent proteins was added to the image of the P-CagA Western blot as a second channel.

Lanes were detected in both images - in the image with the fluorescent proteins by hand as thorough as possible and without the accumulated proteins in the pockets and the bottom of the gel - and bands were marked in the P-CagA Western blot. The software measured the normalization factors for each marked band.

V.2.3.4. ELISA for IL-8

Sandwich ELISA was performed as described by Odenbreit et al (Odenbreit S et al., protocols.io, 2017). A 96-well plate was coated with 50 µl/well of a solution of 5 ml Coating buffer and 15 µg coating antibody and incubated at 4 °C overnight. The unbound antibody was removed by washing twice with 200 µl/well Washing buffer. For blocking the uncoated parts of the wells 100 µl/well Blocking buffer was added and incubated for 1 hour at 37 °C. Blocking solution was removed by washing twice with 200 µl/well Washing buffer. The standard was made of solutions with 800 pg/ml, 400 pg/ml, 200 pg/ml, 100 pg/ml, 50 pg/ml, 25 pg/ml, 12,5 pg/ml and 0 pg/ml IL-8 in complete media, which was added 100 µl/well. The samples were diluted with complete media at least 1:2 depending on the expected amount of IL-8 and applied 100 µl/well. They were incubated overnight at 4 °C. After removing the samples by washing six times with 200 µl/well Washing buffer a solution of 5 µg biotinylated IL-8 antibody in 10 ml Washing buffer with 10 % FCS was added 100 µl/well and incubated for 45 minutes at 37 °C. Unbound antibody was removed by washing six times with 200 µl/well Washing buffer. Streptavidin-peroxidase complex solution, prepared 30 minutes before by mixing 1,5 µl POX-streptavidin-solution A, 1,5 µl POX-streptavidin-solution B and 200 µl ELISA buffer, was diluted in 10 ml of ELISA buffer and applied 100 µl/well. After 45 minutes of incubation at room temperature the unbound complex was removed by washing six times with 200 µl/well Washing buffer. TMB substrate was used mixing 5 ml TMB-solution A and 5 ml TMB-solution B and applying 100 µl/well of the mixture. The reaction with streptavidin-peroxidase was stopped by addition of 50 µl Stopping solution when the 200 pg IL-8-standard turned blue. The signal was measured as described in TMB substrate instructions at 450 nm wavelength (reference wavelength 570 nm). Signals were analysed by Magellan3 software.

V.2.4. Statistical Analysis

Statistical analysis was performed with GraphPad Prism Version 5.0. If not described differently, all values were analysed with one-way ANOVA and Turkey post-tests to determine the relevance between treatments. P-CagA levels after Co- and pre-infection treatment were compared with a Student t-test.

VI Results

Infections always have two main players: bacteria and cells. The following experiments aim to better understand the cellular resistance against CagA translocation and IL-8 induction by *Helicobacter pylori* (*H. pylori*) during multiple infection. The results presented have been published in the following articles:

1.) **Optimized semi-quantitative blot analysis in infection assays using the Stain-Free technology**. Zeitler AF, Gerrer KH, Haas R, Jiménez-Soto LF. J Microbiol Methods, 2016, dx.doi.org/10.1016/j.mimet.2016.04.016

2.) **Host cell resistance to CagA translocation is as variable as *Helicobacter pylori*.** Zeitler AF, Gerrer KH, Haas R, Jiménez-Soto LF. Matters, 2017, dx.doi.org/10.19185/matters.201706000006

VI.1. Establishing Stain-Free technology for infection assays

Western bots are a common method to evaluate proteins not only in quality but also in quantity. To compare protein quantity in different samples the sample load has to be measured first. This dissertation uses semi-quantitative analysis of Western blots, which has two established strategies to evaluate the sample load: Total protein normalization (TPN) and housekeeping protein normalization (HKP). The most reliable normalization is achieved by Stain-Free technology, a method for TPN (see chapter IV.4). Still, interferences of treatment, especially infections, with normalization methods have to be considered at all times.

The amount of CagA translocated by *H. pylori* into eukaryotic cells is mainly measured by semi-quantitative analysis of Western blots. After CagA is translocated its tyrosine residues at its C-terminal end get phosphorylated. The amount of phosphorylated CagA (P-CagA) corresponds to the amount of translocated CagA and can be detected in Western blots by anti-phosphotyrosine antibodies. The signal of the phosphotyrosine is put in relation to the protein load of each sample. For this a normalization method is required, which is independent of proteins influenced by infection, easy to handle and has a high functional range. Although these criteria are met by Stain-Free technology, we encountered two challenges during our experiments.

VI.1.1. Influence of bacterial proteins on the Stain-Free technology

Samples of infection experiments often contain not only host cells but also bacterial components, which can influence further examination. *H. pylori* binds strongly to the cells during infection and contaminates the cell lysates with bacterial proteins. This interference needs to be addressed by an adaption of the Stain-Free read-out.

In Figure VI.1A Stain-Free signal patterns of several cell samples can be observed. Infected cell samples demonstrated repeatedly an altered protein pattern compared to uninfected cell samples. Close observation of the signal pattern showed that the load of tryptophan-rich proteins between 25kDa and 100kDa of infected cells was higher than the one of uninfected cells. The reason for this was revealed when examining a Stain-Free blot of bacterial lysates. In Figure VI.1B the pattern of bacterial proteins is shown. As examples for *H. pylor*i the strains P12, P217 and 26695 were used and compared to

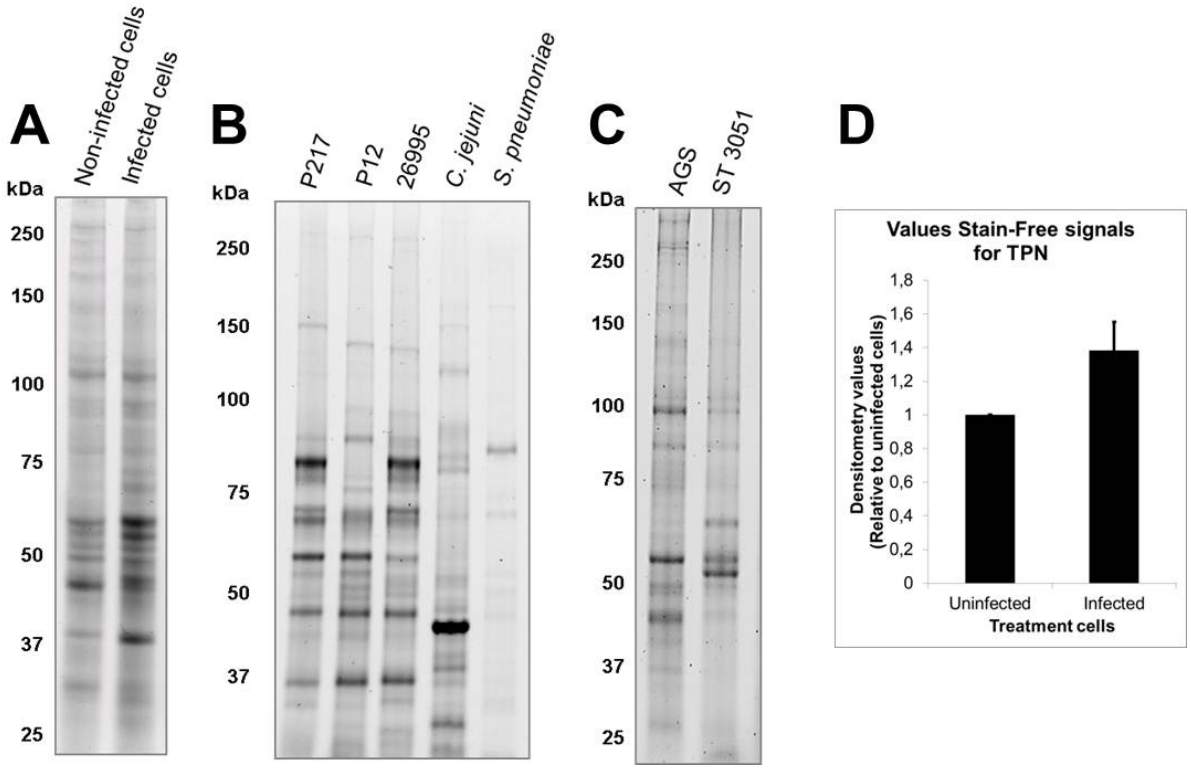


Figure VI.1: Stain-Free signals of different eukaryotic and bacterial cell lysates.

Cell lysates in 6% polyacrylamide single gel (A) Comparison of non-infected and with *H. pylori* infected AGS cells. Stain-Free signals of proteins <100kDa are stronger in the infected cell sample. (B) Comparison of different bacterial cell lysates. Strains P217, P12 and 26695 are *H. pylori* strains. Non-*H. pylori* strains are *C. jejuni* and *S. pneumoniae*. (C) Comparison of two eukaryotic epithelial cell line lysates, AGS and ST-3051. (D) Quantification of differences on Stain-Free signals between uninfected and infected cells. Stain-Free images were analysed using the volume feature from ImageLab 4.1 software. Lanes corresponding to the uninfected cells were selected and set as standard volume, while comparing with cells that have been infected with *H. pylori* strain P12 at a MOI of 60 for 4 hours (n = 4). Reference: (Zeitler AF et al., 2016)

Campylobacter jejuni and *Streptococcus pneumoniae*. Most of their tryptophan-rich proteins were smaller than 100kDa. In contrast the lysates of the two epithelial cell lines AGS and ST 3051 (Figure VI.1C) demonstrated strong signals at all sizes.

Comparing bacterial and cellular lysates, only the lower half of the gel of cellular proteins (<100kDa) might interfere with bacterial proteins.

VI.1.2. Validating the new approach in Stain-Free technology

Interference of bacterial proteins during normalization has to be minimized for reliable and stable results. Figure VI.1 showed that *H. pylori* mainly interferes with signals below 100kDa. The upper half of the gel of infected cells (>100kDa) is therefore unlikely to interact with bacterial proteins influencing the normalization with Stain-Free technology. By using only Stain-Free protein signals above 100kDa normalization should become more reliable. This new approach needed to be validated against other methods used so far, e.g. tubulin as a housekeeping protein or Stain-Free technology.

ImageLab 4.1 software (Bio-Rad) gives the opportunity to adapt lanes of a Stain-Free blot during total protein normalization manually. This way the Stain-Free read-out area could be adjusted to the upper half of the lane (>100kDa). The red line in Figure VI.2A shows the cut-off between most of the bacterial proteins and the upper half of the cellular proteins. The area above the red line was used for the modified normalization method. In Figure VI.2B-C the upper lane normalization (ULN) was applied to blots of AGS cells infected with *H. pylori* strains P12 and P217. Additionally, phosphorylated CagA was measured and normalized by the two known methods housekeeping protein normalization and Stain-Free normalization.

The three normalization methods show very similar results confirming the new approach as a valid method. In contrary to the two known methods the Upper Lane Normalization (ULN) presented with less variability and therefore more reliability (Data not shown). Therefore, Western blots of AGS cells in this dissertation were semi-quantitatively analysed with the ULN.

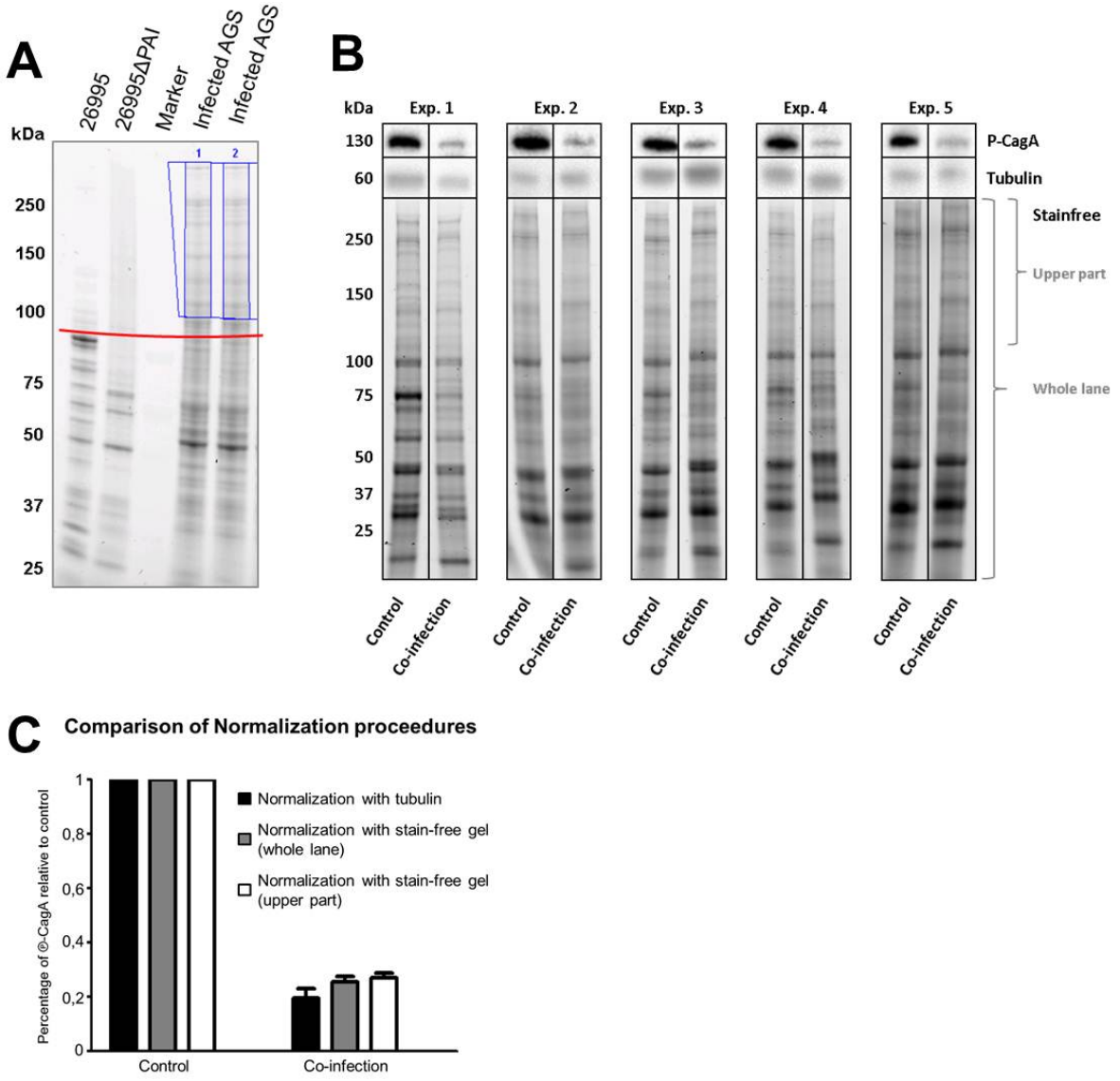


Figure VI.2: Validation of Stain-Free normalization using only the upper part of the lane (ULN).
(A) Imaging area of Stain-Free signal chosen for normalization. Signals from wild type strain 26695 and its mutant lacking 29–32 proteins encoded in the Cag pathogenicity island (26695ΔPAI) were used as criteria to define the upper lane region in AGS cells. (B) Stain-Free, phosphorylated CagA (P-CagA) and tubulin signals of five experiments. Lysates are from AGS cells co-infected with H. pylori strains P12 and P217. Signals were used for the analysis of phosphorylated CagA. (C) Results of the semi-quantitative western blot analysis of Figure VI.2B. Quantification results using either HKP (tubulin), total protein (whole lane) or signals from proteins above 100 kDa (upper part) as normalization parameters for the phosphorylated CagA signals validate upper lane normalization (ULN). Reference: (Zeitler AF et al., 2016)

VI.1.3. Adapting Stain-Free technology on immune cell infections

When applying the new method of Upper Lane Normalization (ULN) on the samples of infected immune cells a new challenge arose. The tryptophan-rich protein patterns of primary leukocytes and the monocytic leukaemia cell line THP1 differ from the one of epithelial cells. As it can be seen in Figure VI.3, above the size of 75kDa the Stain-Free signal of these cells

was a lot weaker than the one of the epithelial cell line AGS. It was too weak to be measured as valid normalization factor. Although single strong bands can be seen, the overall signal strength was not enough to stay clear of confounding. Unfortunately, the setting of the infection assay did not allow us to increase the total protein load of the samples easily, so the signals could not be enhanced. ULN needed to be adapted to this new situation.

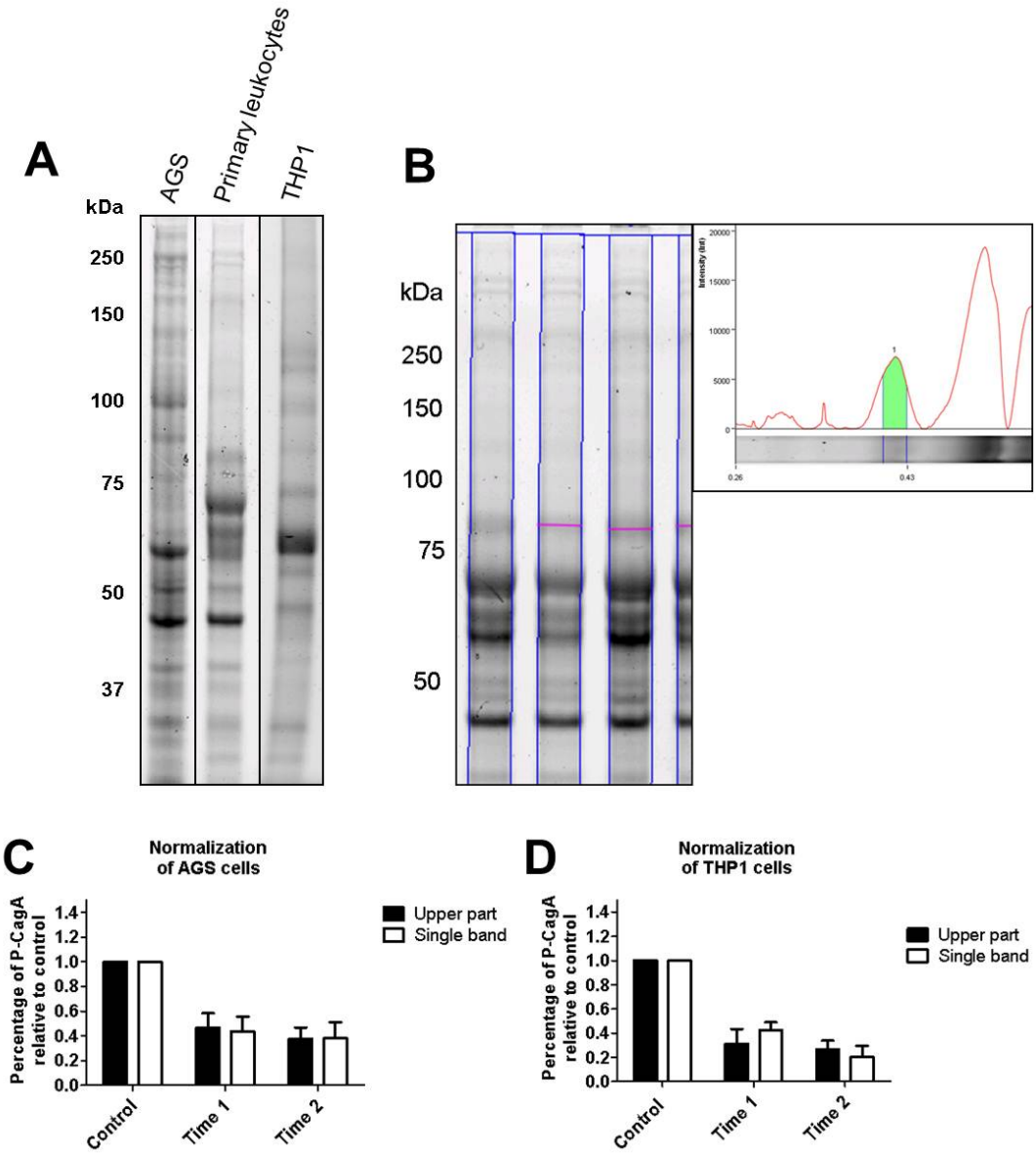


Figure VI.3: Normalization using a single band of the Stain-Free signal.

(A) Stain-Free signal of two immune cell line lysates compared to the epithelial cell line AGS in a 7% polyacrylamide single gel. (B) Definition of the single band using the ImageLab 4.1. software. (C) Validation of the single band normalization (SBN) method in comparison to the upper lane normalization (UPN) method on AGS cells pre-infected with a P12 mutant and infected with P12 wild type strain at different time points (n = 4). (D) Comparison of the ULN vs SBN in THP1 cells pre-infected with a P12 mutant and infected with P12 wild type strain at different time points (n = 3). Reference: (Zeitler AF et al., 2016)

The strong single bands above 100kDa of the immune cells met all criteria for a valid normalization factor: (i) strong enough to be distinguishable from other bands, (ii) constant and independent of infection and (iii) no overlap in size with bacterial proteins. Therefore we chose one of those bands and measured it like the band of a housekeeping protein. ImageLab 4.1. software offers a mode specific for housekeeping protein normalization with the possibility to define the band very precisely in the lane profile window. In Figure VI.3B it is illustrated how the boundaries of each band were set by hand to the points of highest slope in signal intensity to minimize background noise. AGS and THP1 cells, which were pre-infected with a P12 mutant and infected with P12 wild type strain at different time points, were analysed with ULN and the new single band normalization (SBN) to validate the adapted method. The results are given in Figure VI.3 and show stable and reliable measurements. All infected immune cells in this dissertation were semi-quantitatively analysed with the SBN.

VI.2. Analysis of resistance phenomenon

Although the colonisation rate of humanity with *H. pylor*i is high, pathologies associated with its infection are much less. The severity of the infection and its associated diseases are correlated with pathogenicity mechanisms like the cytotoxin associated gene A protein (CagA) and its corresponding *cag* Type IV Secretion System (*cag* T4SS). The *cag* T4SS injects CagA into eukaryotic cells and induces secretion of the chemokine Interleukin-8 (IL-8). Recent research shows, that the ability of *H. pylori* to translocate the CagA toxin into epithelial cells is diminished when cells are pre-infected with *H. pylori* (see chapter IV.3.). This effect is due to a resistance of the cells induced by the first infection.

To simulate this multiple infection, co- and pre-infection setup were followed as previously published (Jiménez-Soto LF et al., 2013). It is illustrated in Figure V.1. Cells were either pre-infected for 1 hour, co- infected with a bacteria suspension of two strains or infected with a single strain alone. The latter was considered as the control, which every sample was put in relation to. For pre-infection an *H. pylori* strain was chosen, which cannot translocate CagA (CagA(-) strain) to have no interference with the CagA of the secondary infecting strain. The secondary infecting strain was a CagA(+) strain and the same as the one used for control infection. The investigation of conditions, influencing factors and characteristics of the resistance phenomenon are presented here.

VI.2.1. Cellular response depending on the *Helicobacter pylori* strain

The resistance phenomenon is a complex consequence of interaction between *H. pylori*'s outer membrane proteins (OMP) and the cellular surface. It was described by Jiménez-Soto et al. (Jiménez-Soto LF et al., 2013), who discovered the reduction of phosphorylated CagA (P-CagA) in co- and pre-infection set-ups. But so far, different strain combinations or a possible inhibition of IL-8 secretion has never been observed and analysed. Variations in the amount of P-CagA and secreted IL-8 between strain combinations give hints to factors influencing the resistance. One of these factors was already identified as the HopQ protein and is examined here further.

VI.2.1.1. Resistance against CagA translocation depends on the pre-infecting strain

A test of different combinations of *H. pylori* strains determines variations in the ability to trigger or to overcome the cellular resistance against CagA translocation. For this assay the CagA(+) strains P12, P145, P217, 26695 and G27 were examined in their ability to translocate CagA during secondary infections. For the sake of analysing only the CagA translocated by the second strain, the CagA(-) strains X45 and Tx30a were used for pre-infection. P12, 26695, G27 and X47 were chosen because their genome has already been sequenced. P147 and P217 are unique in their size of CagA. They have the largest CagA protein known so far. Whereas X47 and Tx30a are strains with no *cagA* gene at all. Further, X47 is adapted to mouse infections and might therefore help transferring the experimental set-up into a mouse model. Additionally, the mutant P12ΔT4SSs was used for pre-infection. It does not have any type IV secretion systems, which could inject CagA, interact with the cells or compete with the secondary infecting strain. The human adenogastric carcinoma cell line AGS performed as host cell line.

The amount of P-CagA was measured by semi-quantitative analysis of Western blots relative to the control infection with the secondary infecting strain alone, using the previous established protocol (see chapter VI.1.). Figure VI.4 displays the results of all strain combinations.

Surprisingly, the decline of P-CagA by pre-infection was considerably different depending on the combination of strains. Especially P217 showed significant differences in P-CagA levels (compare Figure VI.4D). It was either inhibited in CagA translocation heavily by pre-infection with P12ΔT4SSs (average P-CagA level of 17 %), or rarely affected at all by pre-

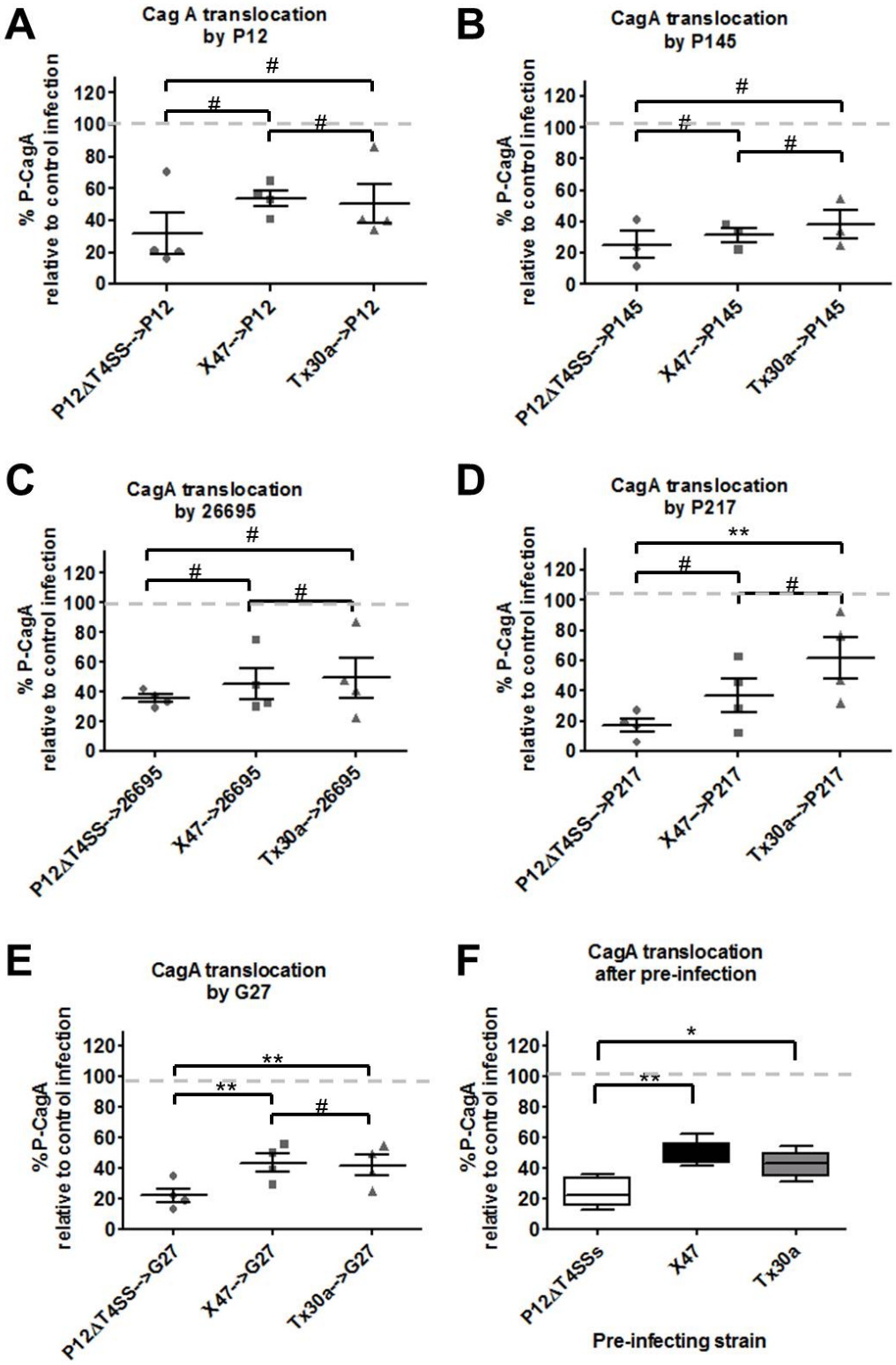


Figure VI.4: CagA translocation of *H. pylori* Type I strains after cell treatment with pre-infecting strains.
Pre-infection for 60 minutes with P12ΔT4SSs, X47 or Tx30a, followed by secondary infection with P12, P145, G27, P217 or 26695 (n=20). (A)-(E) Amount of phosphorylated CagA relative to the control single strain infection with each Type I strain tested (n=4). Comparison of the three pre-infecting strains for each secondary infecting strain. Scatter dot plots (mean and SEM). (F) Summary of the effect of pre-infecting strains on CagA translocation by all of the secondary infecting strains. Boxes (median and quartiles) with whiskers (min-max values). Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #. Reference: (Zeitler AF et al., 2017)

infection with Tx30a (average P-CagA level of 62 %). In the same way, G27 faced strong resistance in cells, which were pre-infected with P12ΔT4SSs, but less in the ones, which were treated with X47 or Tx30a. It seems, as if the choice of the pre-infecting strain is of great importance for the strength of the cellular resistance.

This is confirmed, when comparing the pre-infecting strains independently from the secondary infecting strain, displayed in Figure VI.4F. Pre-infections with the mutant P12ΔT4SSs constantly caused the lowest CagA translocation rates, compared to X47 and Tx30a. On the other hand the highest P-CagA levels were frequently seen with Tx30a as a pre-infecting strain, apparently rarely able to trigger a cellular response. The choice of the secondary infecting strain, however, has only little effect on the final amount of P-CagA.

VI.2.1.2. Pre-infection compromises whole functionality of cag type IV secretion system

Binding of the *cag* T4SS to the infected cell and injection of CagA activates the cellular NFκB pathway ending in IL-8 synthesis and secretion (see chapter IV.2.2.). This way the cell signals to the surrounding tissue that an infection is present and an inflammatory response should start. As described before, the *cag* T4SS is inhibited in its ability to translocate CagA, if cells are pre-infected. But it is unknown whether this inhibition is limited to the injection process or due to a complete loss of function. The functionality of the *cag* T4SS is directly represented by the secretion of IL-8 (Fischer W et al., 2001) and therefore measurable by enzyme-linked immunosorbent assays (ELISAs).

In order to investigate the functionality of the *cag* T4SS on pre-infected cells, the supernatant of the previous experiment (see chapter VI.2.1.1.) was examined by IL-8-ELISA. IL-8 concentration of cells infected with the secondary infecting strain alone were considered as the positive control showing full functionality of the *cag* T4SS.

Unfortunately, there are additional triggers of IL-8 secretion beside a functional *cag* T4SS, that have to be taken out of the equation first. One trigger is the change of media during the cell synchronization process, which results in higher IL-8 levels. Another stimulus for IL-8 secretion are *cag* T4SS-independent virulence factors of *H. pylori*, which are also expressed by the pre-infecting strains (see chapter IV.2.2.). In order to see the effect of pre-infection on the IL-8 induction by secondary infection, the data was processed as followed: The amount of IL-8 triggered by the pre-infecting strain alone was subtracted from the IL-8 level of the multiple infected cells. This way, the amount of IL-8 could be calculated, which is induced

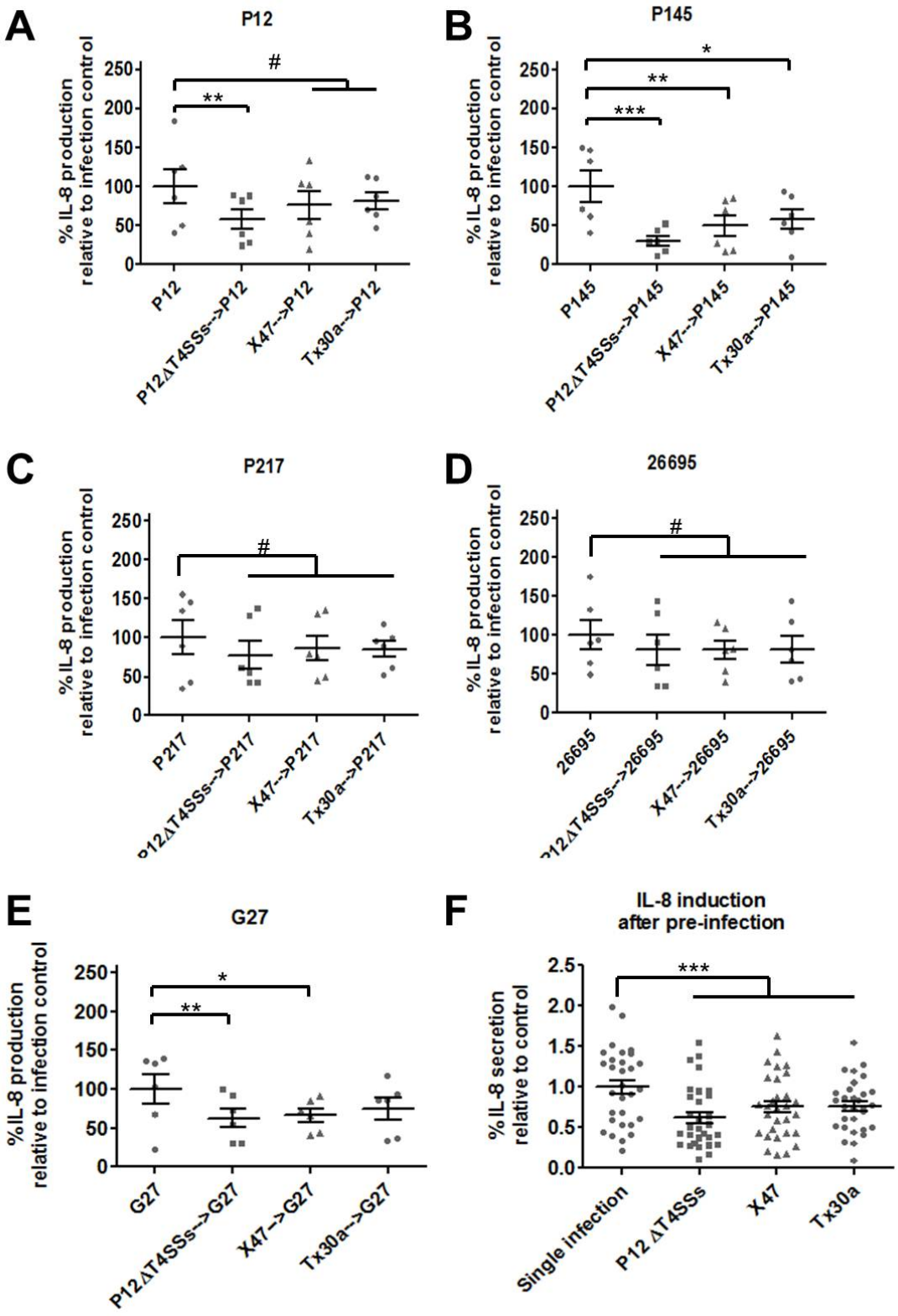


Figure VI.5: IL-8 secretion after pre-infection experiments with different strain combinations.

(A)-(E) IL-8 production by AGS cells after pre-infection with P12ΔT4SSs , X47 or Tx30a for 1 hour and secondary infection with P12, P145, G27, P217 or 26695. Amount of IL-8 relative to the single infection with each secondary infecting strain. (n=6) (F) Comparison of the effect of pre-infecting strains to single infection independent of the secondary strain used. Scatter dot blot (mean and SEM). Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #. Reference: (Zeitler AF et al., 2017)

due to a secondary infection after pre-infection. These results were then put in relation to the amount of IL-8 secreted by control infections with the secondary infecting strains alone. Now the IL-8 induction by the secondary infecting strain in pre-infected cells was visible in comparison to single infection, which is displayed in Figure VI.5.

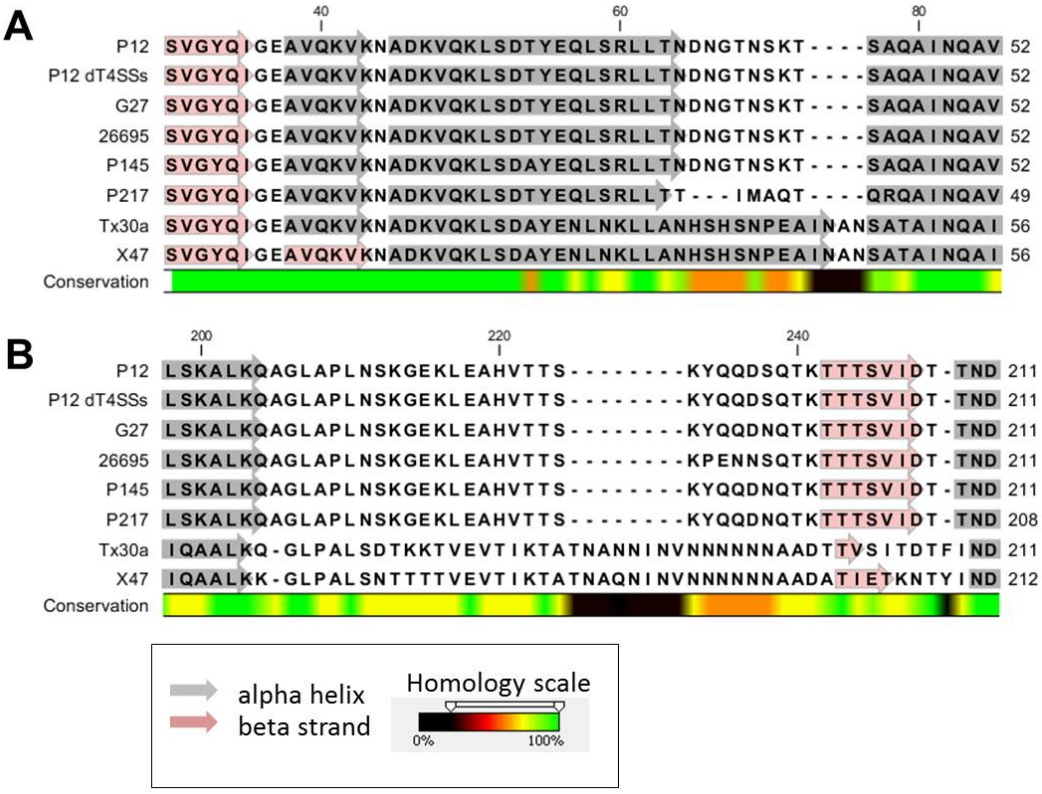
Confirming the results of CagA translocation in the previous chapter, IL-8 induction by *H. pylori* was reduced, when cells were pre-infected. Although standard deviation was bigger here, due to the high sensitivity of this assay, the average IL-8 levels showed only slight differences (Figure VI.5A-E) to the CagA levels before (Figure VI.4A-E). The mutant P12ΔT4SSs again triggered the strongest resistance among the pre-infecting strains (Figure VI.5F).

Further, IL-8 levels induced by P145 are drastically lower with an average difference to the other secondary infecting strains of (32,0 – 40,4) %. This suggests, that P145 is remarkably weaker than all other secondary infecting strains in the ability to overcame the cellular resistance.

Overall these results prove a dysfunction of the whole *cag* T4SS after pre-infection. It is not only the translocation process, which is impaired. This means, that the cells might even resist against the binding of the *cag* T4SS, not only the injection manoeuvre. These insights into the strength of resistance in terms of strain combination are more or less in line with the former discoveries in P-CagA levels. It seems, therefore, as if the choice of the pre-infecting strain is important in regard to reduced IL-8 as well as P-CagA levels. In contrast to the previous results, the choice of the secondary infecting strain seems to be of greater significance for the functionality of the *cag* T4SS.

VI.2.1.3. Variations in the hopQ gene in different Helicobacter pylori strains

The two experiments before showed a strong dependence of the cellular response on the strains used for pre-infection. The strong resistance induced by P12ΔT4SSs faces the minimal effect of X47 or Tx30a. As it is known, that *H. pylori* has a high variability in its genome (Akopyanz N. et al., 1992), this genome diversity might cause differences in the strength of cellular resistance. One of the outer membrane proteins to be essential for the cellular resistance is HopQ (Jiménez-Soto LF et al., 2013). It is already known to exist as two different alleles (Cao P et al., 2002). Variations in the *hopQ* gene of all strains used in the previous experiments are analysed in the following.



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Strain	CagA translocation	IL-8 induction Cag PAI dependent	HopQ allele
P12	+	+	Allele I
P12dT4SS	-	-	Allele I
26695	+	+	Allele I
P217	+	+	Allele I
P145	+	+	Allele I
G27	+	+	Allele I
Tx30a	-	-	Allele II
X47	-	-	Allele II

Figure VI.6: Analysis of the *hopQ* gene of each *H. pylori* strain used for the pre-infection assays.
After translation of the ORF of each *hopQ* gene, protein sequence was analysed for secondary structure prediction using Phyre 1.0 server and categorized into HopQ allele I or allele II. (A) Exemplary N-terminal region with variations corresponding to allele I (P12, P12ΔT4SSs, G27, 26695, P145 and P217) and II (Tx30a and X47). (B) Exemplary amino acid region between approx. 198 and 212 of HopQ. Red arrows behind sequence signalize predicted beta-strands, while grey arrows highlight regions predicted to present alpha helices. The colour scale below the graphics represents the conservation levels of the aligned amino acid sequences. (C) Table of sequenced *H. pylori* strains and their capability to translocate CagA and induce IL-8. Reference: (Zeitler AF et al., 2017)

After sequencing the *hopQ* alleles of each primary infecting and secondary infecting strain, the sequences were translated into protein sequences. These amino acid sequences were aligned to sample sequences of HopQ allele I and HopQ allele II in CLC DNA Workbench 6.0.2. Exemplary regions, which characterize the differences between HopQ allele I and allele II, are displayed in Figure VI.6A-B. As published by Cao et al. (Cao P et al., 2002)

before, all Type I strains showed the highest similarity to the HopQ allele I sequence, including P12ΔT4SSs. The HopQ sequences of X47 and Tx30a correlated with the HopQ allele II. It seems as if the allele of HopQ might correlated with the different abilities of pre-infecting strains to trigger cellular resistance.

VI.2.2. Cell response depending on the host cell line

So far *H. pylori* has been shown to trigger a cellular resistance to its own CagA translocation only in epithelial cell lines, like the human gastric adenocarcinoma cell lines AGS or ST3051 (Jiménez-Soto LF et al., 2013). But its physiological target cells are not only gastric epithelial cells. It has also been found to inject CagA into immune cells, like dendritic cells reaching in the gastric lumen (see chapter IV.2.2.). Dendritic cells, responsible for initiating the fight against infections with bacteria like *H. pylori*, might also be able to resist CagA injection after being infected in advance. Therefore, the resistance of epithelial cells to CagA translocation during secondary infection also needs to be evaluated in immune cells.

VI.2.2.1. Strong resistance in THP1 cells

The THP1 cell line is a well established host cell line to represent immune cells during infection assays with *H. pylori.* It originated from the peripheral blood of a 1 year old, male infant suffering from acute monocytic leukaemia, and therefore consists of undifferentiated monocytes. Furthermore, it is known to be susceptible to CagA translocation in *in vitro* experiments, meeting the main requirement for our infection assays.

For the co- and pre-infection treatments strains from our previous experiments were chosen to compare the resistance phenomenon in different cell lines independent of the *H. pylori* strains (see chapter VI.2.1.). P12ΔT4SSs as a strain with no functional *cag* T4SS and therefore incapability of CagA translocation represented the primary infecting strains. P12 wildtype performed the secondary infection, translocating CagA into the host cells after primary infection. This strain combination with an incubation time of 1 hour for pre-infection showed the strongest cellular resistance with the lowest P-CagA and IL-8 levels in adenogastric carcinoma cells (see chapter VI.2.1.1. and VI.2.1.2.). Next, THP1 cells were tested according to the amount of translocated CagA and its ability to build up a cellular resistance after pre-infection with the very same strain combination. P-CagA signals on the Western blots are analysed semi-quantitatively by single band normalization (see chapter VI.1.3.).

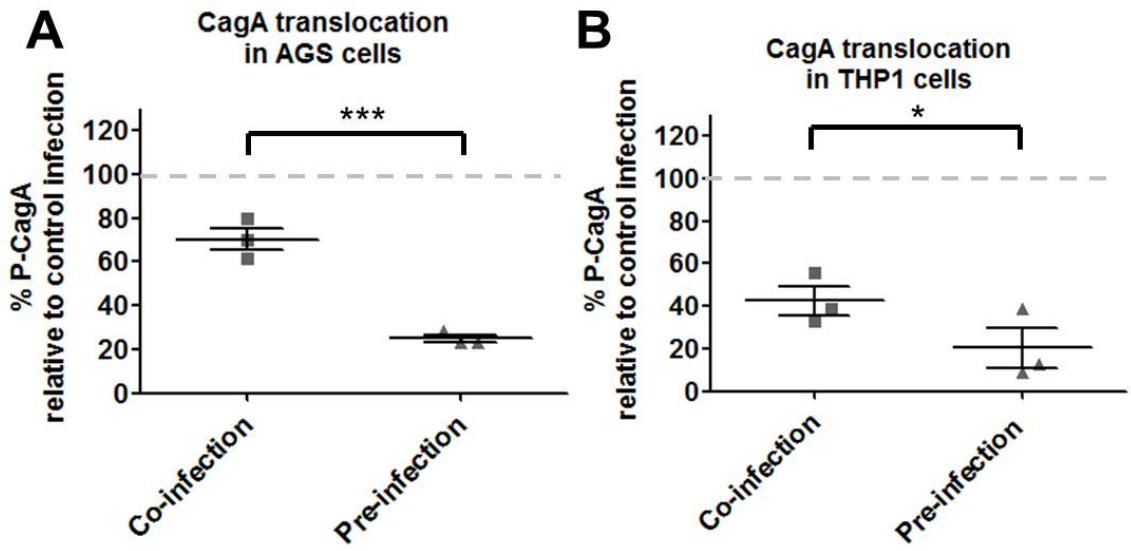

Figure VI.7: CagA translocation in AGS and THP1 cells after co- and pre-infection treatment
Co- and pre-infection with P12ΔT4SSs as primary and P12 wildtype as secondary infecting strain. P-CagA levels relative to the control infection with P12 wildtype alone. (A) CagA translocation into human epithelial cancer cell line AGS. (B) CagA translocation into human monocytic cancer cell line THP1. Scatter dot blots (mean and SEM) (n=3). Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #. Reference: (Zeitler AF et al., 2017)

THP1 cells respond to pre-infection with P12ΔT4SSs with a strong resistance against CagA translocation by P12 wildtype (Figure VI.7B). Interestingly, already in co-infecting conditions, P-CagA levels were reduced down to 40%. After pre-infection treatment, P12 wildtype was only able to inject 20% of the amount of CagA, which it was injecting in single infection conditions. Compared to AGS cells, displayed in Figure VI.7A, the THP1 cell line was capable of building the same resistance against CagA translocation after co- and pre-infection treatment.

These findings show that both, epithelial cells and immune cells, are capable of developing a resistance against CagA injection by *H. pylori*. Therefore, the mechanism behind the cellular resistance seems to be similar, if not even stronger.

VI.2.2.2. Strong resistance in primary leukocytes

So far, all cell lines tested for the resistance phenomenon were cancer cell lines. The phenomenon could be an *in vitro* cancer cell type phenomenon, insignificant for the physiological behaviour of *H. pylori* and its host. Using primary leukocytes, which were extracted from a fresh blood sample of a voluntary donor, the resistance phenomenon was tested on a host cell line close to the physiological setting. As for the THP1 cells, P12 wildtype and P12ΔT4SSs were used for the co- and the pre-infection and semiquantitative analysis of the Western blots was performed by SBN.

As expected, primary leukocytes show a strong resistance against CagA translocation by the P12 wildtype, which can be observed in Figure VI.8. It was as strong as in THP1 cells and oppressed CagA translocation in pre-infecting conditions down to 30%. Intriguingly, the co-infection effect in primary leukocytes was so strong, no significant difference could be seen between co- and pre-infection treatment. Additionally, the amount of P-CagA in co-infecting conditions was significantly different in human leukocytes from the one in AGS cells.

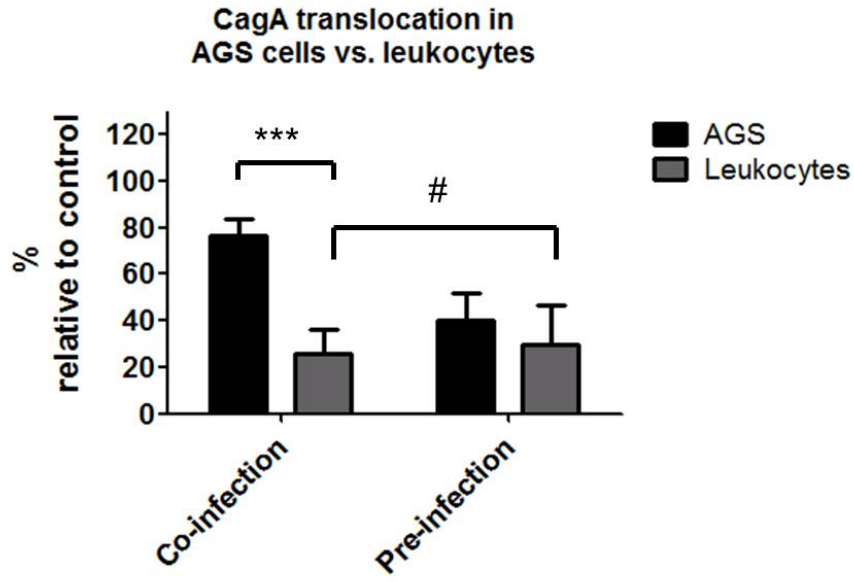

Figure VI.8: CagA translocation in AGS cells and primary leukocytes after co- and pre-infection treatment.

AGS cells and primary leukocytes were co- and pre-infected with P12ΔT4SSs as primary and P12 wildtype as secondary infecting strain. P-CagA levels are relative to the control infection with P12 wildtype alone. Columns (mean and SEM). (n=3) Statistical analysis with two-way ANOVA and Bonferroni posttests. Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #. Reference: (Zeitler AF et al., 2017)

These results confirm the findings in THP1 cells. After pre-infection leukocytes also develop a cellular resistance against CagA translocation by a secondary infecting strain. Furthermore, these findings suggest, that immune cells are even more receptive to pre-infection treatment and develop a resistance stronger than in epithelial cells.

VI.2.2.3. Cag type IV secretion system independent interleukin-8 induction in immune cells

During infection *H. pylori* induces IL-8 secretion in its host cells. Two virulence factors of *H. pylori* were found so far to be responsible for this: *cag* T4SS and OipA (see chapter IV.1.2.4.). These two factors are highly associated with Type I *H. pylori* strains. Type II *H. pylori* or any strain without *cag* T4SS or OipA is therefore considered to be incapable of inducing IL-8 secretion in eukaryotic host cells.

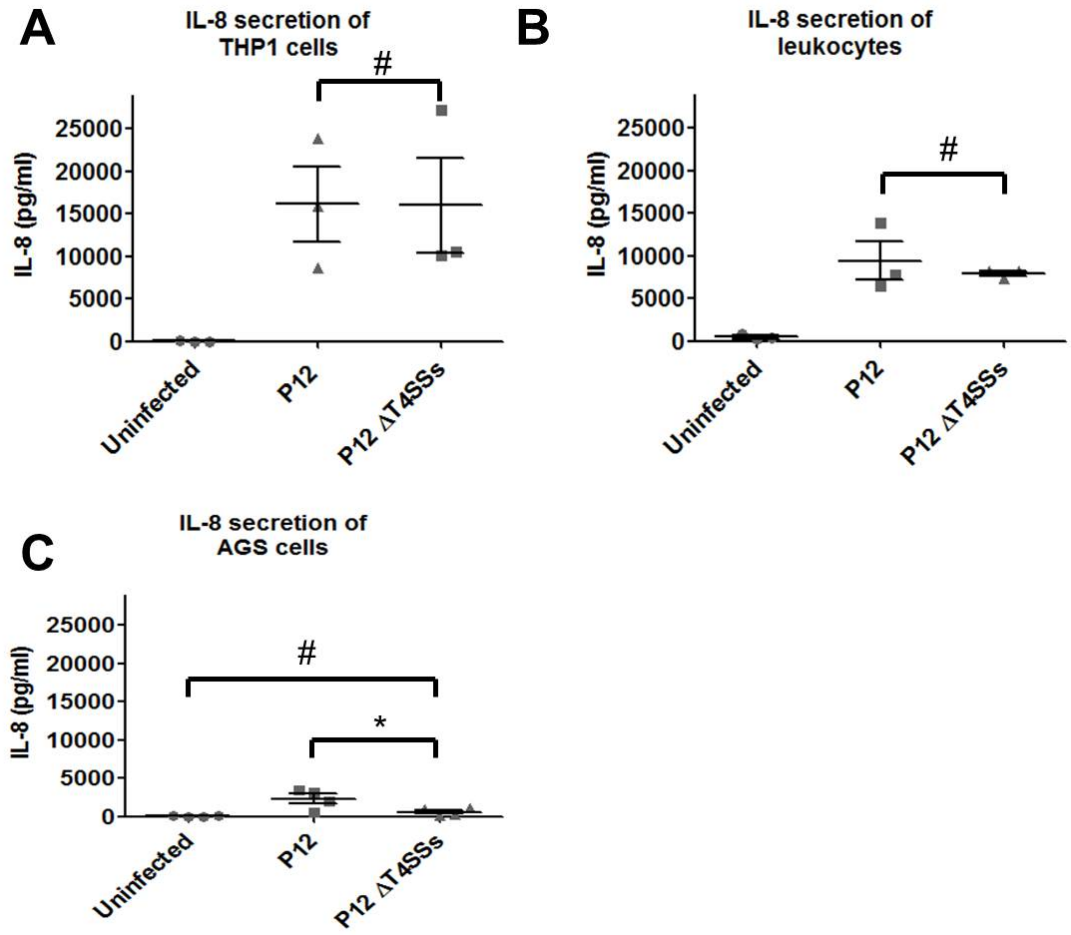

Figure VI.9: IL-8 secretion of AGS, THP1 and human blood leukocytes.
(A)-(C) Evaluation of IL-8 production by the *cag* T4SS in AGS, human blood leucocytes and THP1 using P12ΔT4SSs and P12 strains in single infections. Scatter dot blots (mean and SEM). (n=3) Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #. Reference: (Zeitler AF et al., 2017)

An inspection of the IL-8 samples in this dissertation revealed an intriguing surprise. Figure VI.9 demonstrates IL-8 induction by P12ΔT4SSs and P12 wildtype in the epithelial cell line AGS and two immune cell lines THP1 and human blood leukocytes compared to uninfected cell samples. In immune cell lines the P12 mutant, P12ΔT4SSs, was able to induce high IL-8 production. Without the *cag* T4SS the mutant triggered THP1 cells and primary leukocytes to secrete as much IL-8 as P12 wildtype. AGS cells, however, behaved as previously reported: P12 wildtype induced significantly higher IL-8 secretion than P12ΔT4SSs. Moreover, AGS cell did not secrete more IL-8 after infection with the P12 mutant than uninfected.

VI.2.3. Relevance of time for the cellular response

The cellular resistance against CagA translocation and IL-8 induction by *H. pylori* is a phenomenon of gastric epithelial cells as well as immune cells. It is triggered by a primary

infecting strain and interferes with the *cag* T4SS of a secondary infecting strain. This second strain is subsequently inhibited in its ability to inject CagA into the cytoplasm of the host cell.

The first approach to find out the cellular mechanisms of the resistance phenomenon was to explore its dynamics. How long do the cells need to be pre-infected to build up a resistance as strong as seen after 60 minutes? Is the reduction in CagA injection linear or exponential? And finally, will the reduction of CagA translocation and reduction of IL-8 secretion depend in the same way on the time of pre-infection? Answers to these questions will provide a schematic of P-CagA and IL-8 levels depending on the duration of pre-infection and give a hint to the cellular processes which lead to the resistance against CagA translocation.

VI.2.3.1. Cell resistance depends on the time of pre-infection

As seen in Figure VI.7-9 the resistance to CagA injection and IL-8 induction is significantly stronger after a pre-infection treatment of 60 minutes than in co-infection condition. But in co-infection conditions reasonably reductions of P-CagA and IL-8 levels are already detected. In order to determine if this is just a preliminary effect, which is followed by the resistance process, or if the resistance is already seen in co-infection conditions, but has its maximum at 60 minutes pre-infection treatment, various pre-infection delays are observed more closely. The time points of secondary infection chosen were 5, 10, and 15 minutes after primary infection. This way, the delay between primary and secondary infection, until the resistance phenomenon starts to build up, can be defined. From this delay the cellular signalling pathways, which are responsible for the resistance phenomenon, can be deduced. A short delay hints towards a fast signalling cascade like changes in calcium concentration, whereas a longer time span suggests a more time-consuming process like protein *de novo* synthesis.

As it can be seen in Figure VI.10A the preliminary effect of co-infection against CagA injection was stable for the first 15 minutes of pre-infection. P-CagA levels were constantly reduced down to 60% without enhancement. However, after 1 hour of pre-infection treatment the P-CagA levels dropped significantly. Pre-infection for around 60 minutes results in a strong reduction of P-CagA down to 20%. So the decline of P-CagA levels depending on the duration of pre-infection was not linear, but rather biphasic. The levels dropped already in co-infecting conditions but built a plateau for the first 15 minutes. Apart from the co-infection effect, there seems to be a second mechanism, which is active after 60 minutes and intensifies the cellular resistance against CagA translocation.

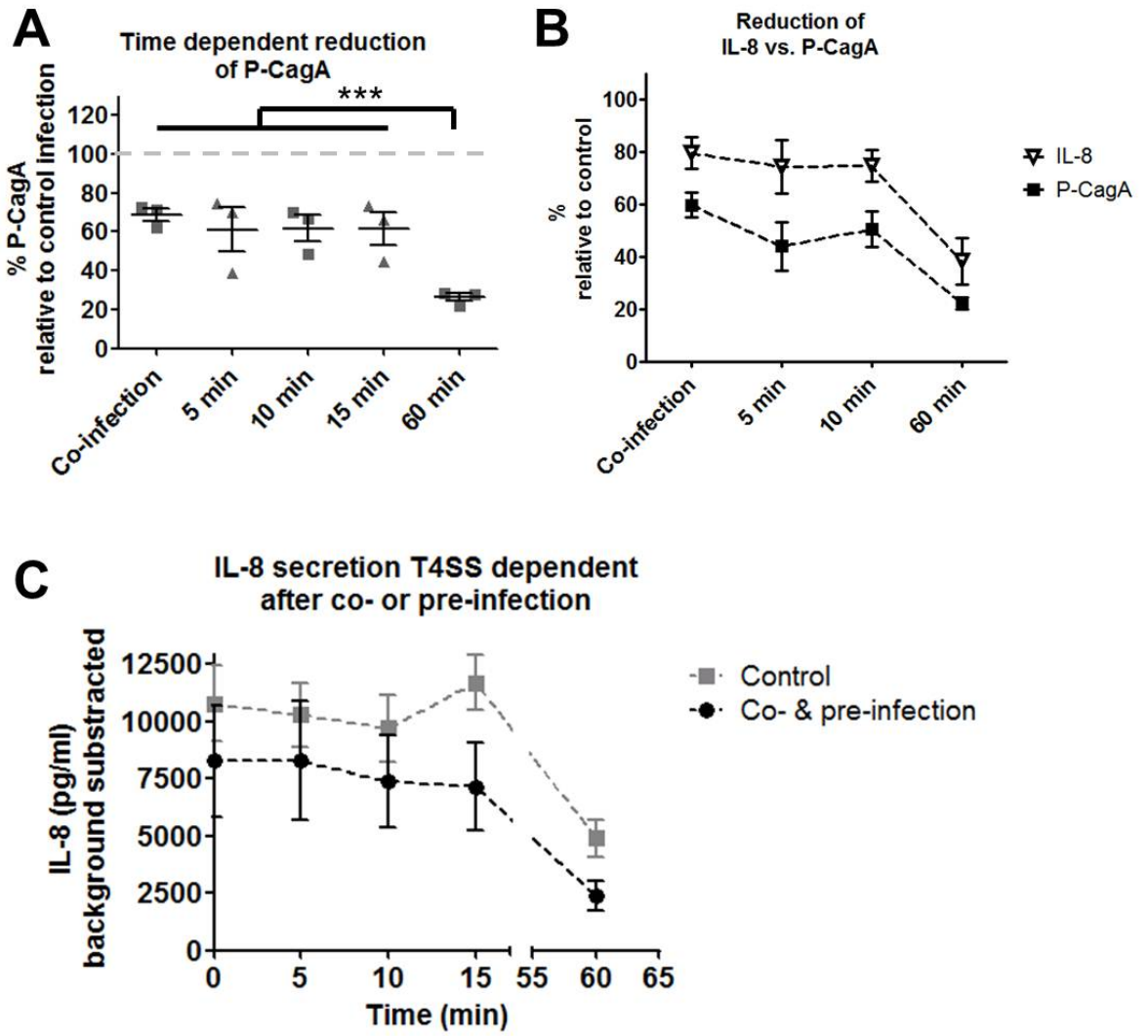

Figure VI.10: Cellular response to pre-infection for several time lapses.

AGS cells, pre-infected with P12ΔT4SSs, were secondary infected with P12 wildtype at different time points (0, 5, 10, 15, 10 minutes after pre-infection). (A) Amount of P-CagA relative to the control infection at the same time laps. Scatter dot blot (mean and SEM). Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, # (B) Comparison of CagA translocation and IL-8 secretion for the time lapses 0, 5, 10 and 60 minutes. Graph shows mean values and SEM. Statistical analysis with two-way ANOVA and Bonferroni posttests. Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #. (C) Comparison of IL-8 levels in control infection and the summary of co-/pre-infections for different time lapses. Graph shows mean values and SEM. Statistical analysis with two-way ANOVA and Bonferroni posttests. Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #. Reference: (Zeitler AF et al., 2017)

An examination of the corresponding IL-8 secretion, seen in Figure VI.10B-C support the theory of a biphasic process. The reduction of IL-8 levels during co-infection and pre-infection for up to 15 minutes showed the same consistency as the P-CagA levels at around (60-50) %. This was followed by a significant reduction down to 40% of IL-8 compared to their control after 1 hour pre-infection treatment. Thus, the same dynamic was observed in the reduction of CagA translocation and the reduction of IL-8 secretion.

Furthermore, this difference in IL-8 levels between the preliminary effect of the first 15 minutes of pre-infection and the heavy resistance after 60 minutes of pre-infection is a lot bigger than in the P-CagA levels. It can be observed in Figure VI.10B that this preliminary effect seems to play a larger role for the resistance against CagA injection than for the resistance against IL-8 induction. But the second mechanism later leads to a strong resistance against both, CagA translocation as well as IL-8 induction. Overall, it seems as if the resistance phenomenon has two phases: the first phase is a fast, but weak cellular resistance mainly against CagA translocation and less against IL-8 induction. But later, after 1 hour of pre-infection, the resistance is strong and universal against CagA injection and IL-8 induction.

VI.2.3.2. Cell resistance independent of cell cycle

All experiments on the resistance phenomenon by multiple infection have been done with cancer tissue cells, which have been synchronized in their cell cycle. Due to the assumption that CagA injection by *H. pylori* is β1 integrin dependent and β1 integrin is expressed cell cycle dependently (see chapter IV.2.1.), cell cycle synchronization is a method of increasing CagA translocation into the host cell. This way experiments measuring the amount of P-CagA are more sensitive. However, cell cycle synchronization also means, that the bacteria always interact with cells in the same status, one status exclusively. This might bias all observations on the resistance phenomenon and needs to be examined carefully.

Validation of the synchronization method

The cell cycle is a description of recurrent phases of cell metabolism, which regulate DNA duplication and cell division. The cell cycle phases G, S and M can be distinguished by their DNA content (Crowley LC et al., 2016). The method, which was used to arrest the cycle of the host cells, was serum-starvation over night. The host cells were infected 30 minutes after serum re-addition. These moments were evaluated for their cell status by a propidium iodide staining of the DNA content and compared to an unsynchronized cell population. If serum-starvation leads to a cell cycle synchronization, the DNA content of each cell in the population should be the same. Unsynchronized cells should show a diverse signal pattern of cells in different phases in the cell cycle.

In Figure VI.11 the propidium iodide signal is seen of the unsynchronized cells (gray with dotted black line), the serum starved cells (solid red line) and the serum starved cells 30 minutes after addition of complete media (solid blue line). The signal pattern of synchronized cells had a crucial difference to the one of unsynchronized cells. The serum-

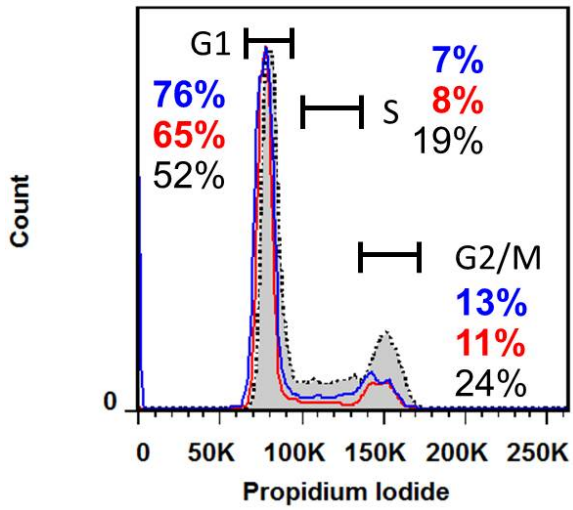

Figure VI.11: DNA staining of synchronized and unsynchronized cells.
DNA staining of AGS cells using propidium iodide after serum starvation and non-serum starvation. Grey background curve, dotted black line represents unsynchronized cells, solid red line curve are serum starved cells before serum addition and solid blue line are cells after 30 minutes addition of serum containing media after serum starvation. (n=2)

starved cell population showed a high number of cells with low DNA content (65-76% of the population). This amount is higher than in the untreated cell population (52% of the population). Accordingly, there are only about 20% of the synchronized cells with a high DNA content, compared to about 40% of the unsynchronized cells. Which means that the cells of the serum-starved population were mainly in G1 phase and rarely in G2/M or S phase. Therefore, synchronization treatment leads to an arrest of the cell cycle in G1 phase and prohibits a transition into the cell dividing process in G2/M and S phase.

Same resistance phenomenon in synchronized as in unsynchronized host cells

In order to rule out, that cell cycle related metabolism plays a role in the observed resistance of the host cells against CagA translocation, infection assays were performed without previous synchronization of the host cells. The experimental setup was exactly the same as for the experiments on the time of pre-infection (see chapter VI.2.3.1.) with the exception of cell synchronization. Thus, the results can be compared directly and any change in the capability of the host cells to resist CagA injection can be seen immediately. If the resistance phenomenon diminishes, there might be a receptor involved, which is essential for the resistance but expressed on the cell surface cell cycle dependently.

P-CagA and IL-8 levels of unsynchronized host cells were the same as the levels of host cells, which were synchronized before infection (see Figure VI.12A-C). The AGS cells showed not only the same low but fast resistance response to CagA injection and IL-8 induction in co-

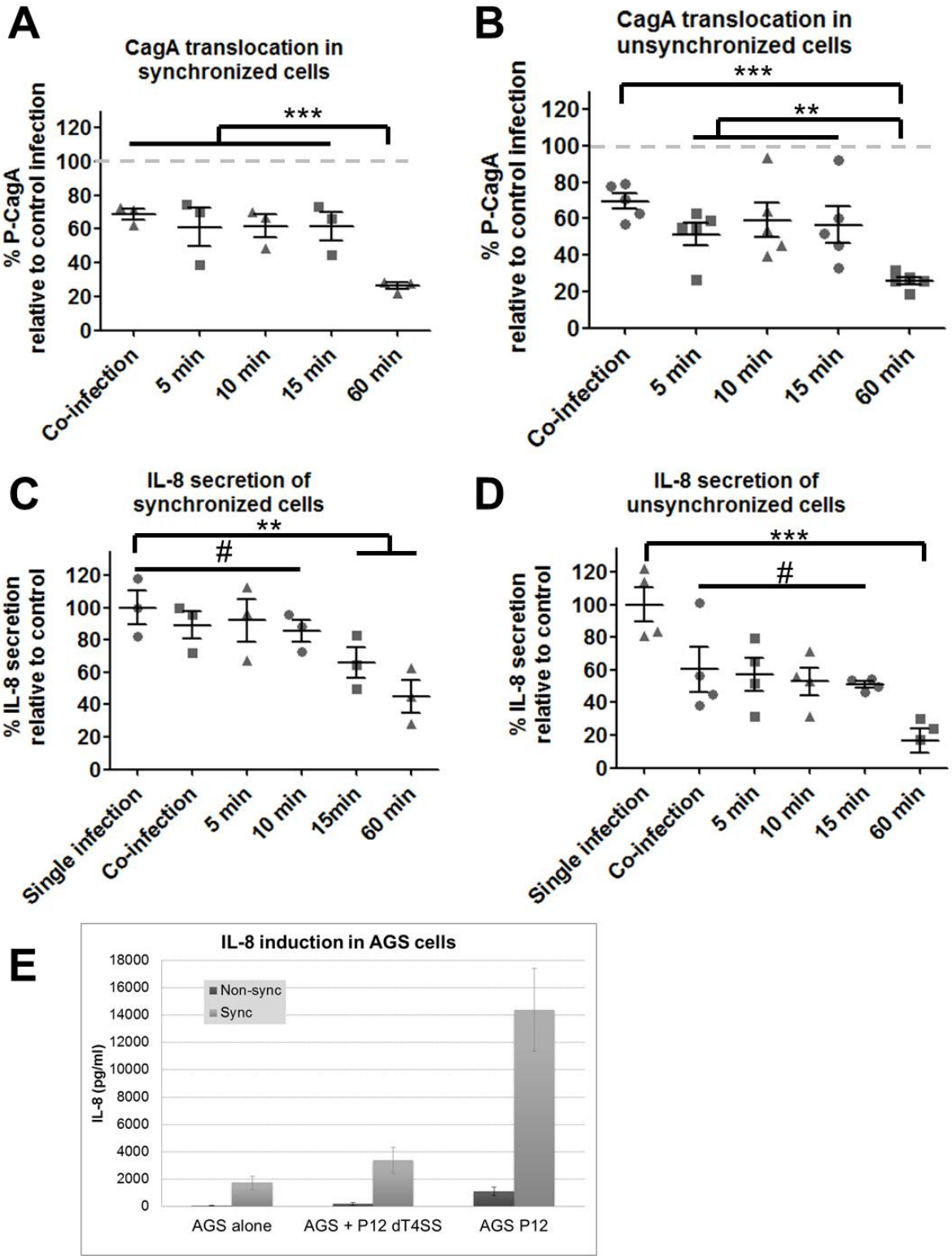

Figure VI.12: Effect of synchronization treatment of AGS cells on resistance phenomenon.

Synchronized and unsynchronized AGS cells were co- and pre-infected as in chapter VI.2.3.1. . (A)-(D) Cag-A translocation and IL-8 induction in synchronized and unsynchronized cells depending on the time of pre-infection. Scatter dot blots (mean and SEM). Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, # . (E) Effect of cell synchronization on IL-8 induction by strains lacking the *cag* T4SS (P12ΔT4SSs) compared to the wild isotype strains (P12). Reference: (Zeitler AF et al., 2017)

infecting conditions but also the strong reduction of P-CagA and IL-8 levels after 1 hour of pre-infection. Moreover, the same dynamic of the resistance developement could be observed: A similar modest P-CagA and IL-8 reduction in the 5-15 minutes pre-infection treatments resulting in a significantly stronger reduction after 1 hour of pre-infection.

Surprisingly, IL-8 secretion of synchronized AGS cells was stronger than untreated AGS cells. Figure VI.12E illustrates that not only the uninfected unsynchronized AGS cells released more IL-8 but also infection with *H. pylori* resulted in abundant IL-8 secretion. It seems like the synchronization treatment does not interfere with the resistance phenomenon itself, but it primes AGS cells and makes them more sensitive for IL-8 secretion in general. This might disturb the analysis of interleukin production in pre-infection assays and should be watched closely.

Overall, the resistance phenomenon is independent from cell cycle synchronization by serum-starvation. It is therefore independent from cyclic changes in the cell's metabolism and, more important, not an artefact of the cell pre-treatment.

VI.2.4. Protein *de novo* synthesis is not required for cell resistance

The resistance of eukaryotic cells against CagA injection by a secondary infecting *H. pylor*i strain has been shown to build up in two phases (see chapter VI.2.3.1.). The second phase takes about 30-60 minutes to be visible as a significant reduction in P-CagA. One of the cellular processes that could be induced during the 30-60 minutes upon infection would be a *de novo* protein synthesis. The pre-infection treatment of the host cells could trigger the synthesis of a protein responsible for the resistance phenomenon. Inhibiting the cells in their *de novo* protein synthesis would therefore lead to a breakdown of the cellular resistance and increased P-CagA levels in pre-infecting conditions.

Cycloheximide is regularly used in research to inhibit *de novo* protein synthesis by ribosomes in eukaryotic cells. It prohibits elongation during translation of mRNA into proteins (Schneider-Poetsch T et al., 2010). To determine the role of new protein synthesis, cells were pre-treated with cycloheximide before the co- and pre-infection experiments with *H. pylori*.

In infection conditions IL-8 is freshly synthesised by the ribosomes. Therefore, effective ribosome inhibition by cycloheximide was evaluated by measuring IL-8 secretion after infection. Figure VI.13B shows that the infected cells, which were treated with cycloheximide, secreted very low levels of IL-8, similar to uninfected cells. Consequently, translation inhibition was successful.

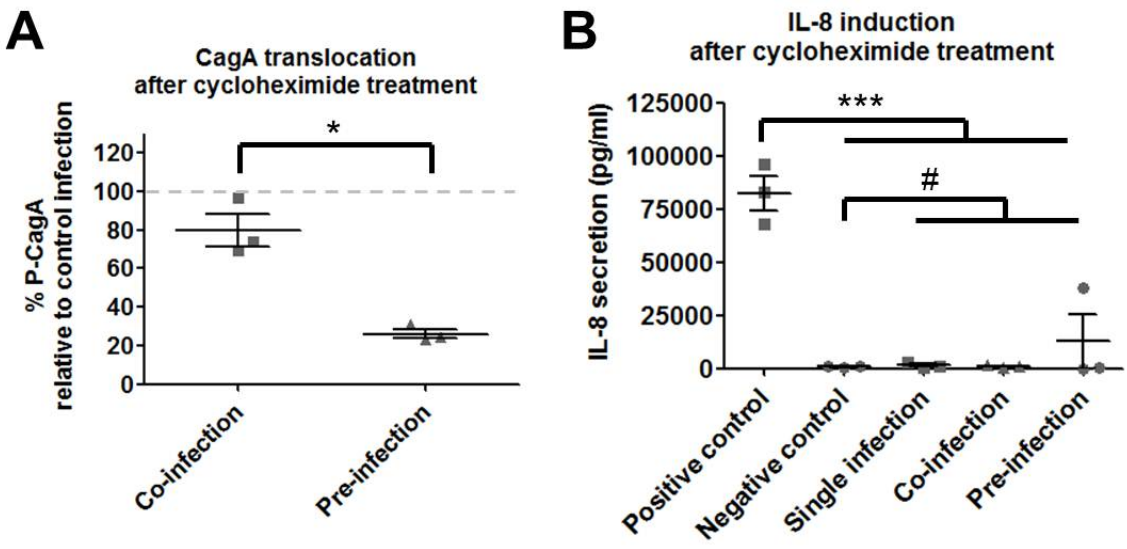

Figure VI.13: Resistance phenomenon after inhibition of protein translation in the host cells.
AGS cells were incubated with cycloheximide before pre-infection with P12ΔT4SSs for 60 minutes. P12 wildtype was used for secondary infection. (A) CagA translocation relative to the control infection. (B) Unprocessed amount of IL-8 secretion after cycloheximide treatment as control for successful inhibition of protein translation. Uninfected AGS cells as negative control; untreated and uninfected AGS cells as positive control. Scatter dot blots (mean and SEM). Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #.

The results of the P-CagA levels are shown in Figure VI.13A. Despite the cycloheximide treatment pre-infection triggered a resistance in the host cells against CagA translocation. Also in co-infection conditions the behaviour of the host cells was consistent to previous observations. The resistance phenomenon seems to be independent of *de novo* protein synthesis, suggesting that another mechanism has to be responsible.

VI.2.5. Relevance of calcium for the cell resistance

Multiple infections trigger a cellular resistance against CagA translocation and IL-8 induction already in co-infecting conditions (see chapter VI.2.3.1.). Signalling pathways have to be very fast to organize this response. One of the fastest ways of communication in a cell is the change in cytoplasmic calcium concentration. Active transporters create a physiological transmembrane calcium gradient by pumping calcium out of the cytoplasm into the extracellular space, or the intracellular calcium stores. As a second messenger, calcium flushes into the cytoplasm via pores, following this gradient and changing intracellular calcium concentration. This calcium influx mediates further signalling cascades.

The first contact of *H. pylori* with the host cell might lead to a rapid change in intracellular calcium concentration, which could mediate a prohibition of further CagA injection.

VI.2.5.1. No specific change in intracellular calcium level during infection

Changes in intracellular calcium levels can be visualized by a calcium sensitive fluorescent dye (e.g. quenching dye of the FLIPR Calcium 6 Assay Kit). The dye accumulates in the cytoplasm and, when binding calcium, increases in its fluorescence. If the resistance phenomenon relies on calcium signalling, co- and pre-infections of the host cells will lead to a change in intracellular calcium levels and therefore a change in fluorescence. This was observed by life cell imaging at a spinning-disk confocal microscope.

Calcium Influx during infection

	Repetition 1	Repetition 2	Repetition 3
Positive control	+	+	+
P12	-	+	+
P12ΔT4SSs	-	+	-
P12ΔT4SSs + P12	-	+	-
P12ΔhopQ	+	+	+
P12ΔhopQ + P12	+	-	+

Figure VI.14: Changes in intracellular calcium level during single and pre-infection.
ST3051 cells were incubated with FLIPR Calcium 6 Assay Kit and imaged during infection with P12ΔT4SSs, P12 wildtype and P12ΔhopQ. P12ΔT4SSs and P12ΔhopQ were also used as pre-infecting strains. Calcium influx, detected by an increased fluorescence, is labeled with (+), stable calcium levels with (-).

The videos acquired during the infections were examined for changes in fluorescence (videos are available upon request), which are displayed in Figure VI.14. The positive control with ionomycin showed reliable results of calcium influx, which validated this setup as a calcium sensitive experiment. Unfortunately, all other samples behaved differently in all three repetition experiments. Sometimes the application of bacteria provoked strong fluorescence signals, on other occasions fluorescence levels did not change at all. No correlation was found between any infection treatment and changes in intracellular calcium concentration.

VI.2.5.2. Forced calcium influx does not influence the cell resistance

Specific changes in intracellular calcium levels during co- or pre-infection treatment were difficult to capture. However, if calcium plays a role in the resistance phenomenon, a forced calcium influx, e.g. by the ionophore ionomycin, will interfere with its mechanisms. Excessive intracellular calcium concentrations might either disable the host cells to organize a resistance against CagA injection and IL-8 induction, because a specific, moderate or low level of calcium is required. The other possibility is that a calcium influx is essential for the establishment of the cellular resistance. Then control samples, incubated with the secondary

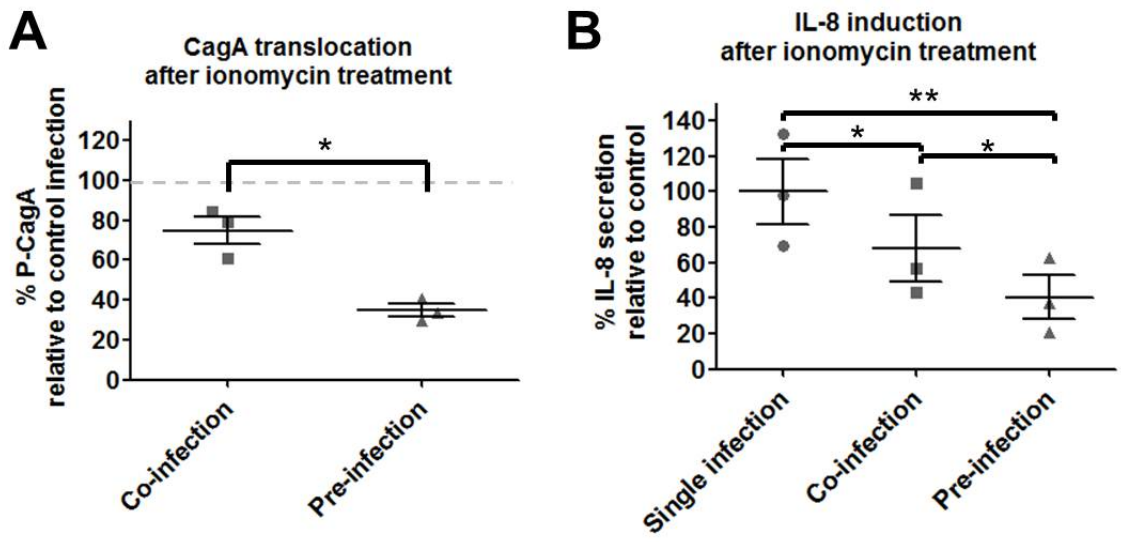

Figure VI.15: Resistance phenomenon after forced calcium influx into the host cells.
AGS cells were incubated with ionomycin before pre-infection with P12ΔT4SSs for 60 minutes. P12 wildtype was used for secondary infection. (A) CagA translocation relative to the control infection. (B) Amount of IL-8 secretion relative to the single infection with P12 wildtype. Scatter dot blots (mean and SEM). Significance: P<0,05, *; P<0,01, **; P<0,001, ***; P>0,05, #.

infecting strain alone, will show the same reduction in P-CagA and IL-8 levels after calcium influx as the co- or pre-infected cells. Either way, the amount of P-CagA and IL-8 should equalize between single infection, co- and pre-infection. No significant difference should be detectable.

In Figure VI.15 P-CagA and IL-8 levels of cells treated with the calcium ionophore ionomycin shortly before the infections are displayed. Despite the forced calcium influx, the host cells were able to built up their resistance in co- and pre-infection conditions. P-CagA as well as IL-8 levels were reduced down to 40%. These values showed no difference to the previous results, where intracellular calcium has not been altered. Interference with the resistance phenomenon by changes in intracellular calcium levels could not be seen. These results, therefore, supported the previous observation that the second messenger calcium seems to be not essential for the mechanism of cellular resistance.

VII Discussion

VII.1. Adaption of Stain-Free technology on infection assays

Semi-quantitative analysis of Western blots always includes the process of normalizing the values against the estimated protein load of the blot. After the revelation, that the broadly used housekeeping proteins interacted with the investigated treatments and that, against former assumptions, their signals did not reflect the amount of total protein in a sample, the Stain-Free technology was suggested as an alternative (Li R et al., 2013) (Gürtler A et al., 2013).

Although Stain-Free technology provides meanwhile the most reliable, most robust and most sensitive quantification procedure, additional difficulties disturb the quantification process in the field of infection biology. In this dissertation two challenges were identified: I) interference of bacterial proteins with the normalization process, and II) insufficient protein load of immune cells in the size range needed.

The first challenge arose, when infected cell samples presented with altered protein patterns compared to uninfected cell lysates. The bacterial proteins were clearly distinguishable from the eukaryotic cell proteins and influenced the normalization values. Finally, the results of our infection experiments were highly variable and unreliable. To address this interference, the Stain-Free read-out was adapted. Most bacterial proteins, which were smaller than 100 kDa, were successfully eliminated from the detection frame by using only the upper part of the blot, which contains proteins bigger than 100 kDa. This way bacterial proteins could not interfere anymore during comparison of infected and uninfected cell lysates. This Upper Lane Normalization (ULN) was successfully validated against the known application of Stain-Free technology and housekeeping protein normalization, presenting with less variability and more reliability.

The second challenge arose from the application of ULN on immune cells. Because their cellular proteins seemed to be smaller than 75 kDa, their Stain-Free signal bigger than100 kDa was too weak to be measured reliably. The solution was to identify one strong band of cellular proteins and measure this band like a housekeeping protein (single band normalization, SBN). Thus, semi-quantitative analysis became more stable.

These two challenges might appear in infection assays with other bacteria species as well. Even though the establishment of the Stain-Free technology was a huge improvement, semi-quantitative analysis of Western blots still needs continuous supervision and re-evaluation for

new interfering factors. The two adjustments elaborated in this dissertation provide methods to minimize interference of bacterial proteins in the normalization process and to make semi-quantitative analysis of Western blots more reliable and efficient. ULN and SBN can be applied on other infection assays, where it is needed to remove bacterial or even viral and parasitic proteins from the normalization process. It might not be suitable for every semi-quantitative analysis of Western blots, but may be an inspiration to solve future challenges in the normalization process.

VII.2. HopQ protein triggers the cellular response to infection

The outer membrane protein HopQ is known to be an adhesin, which binds to the human CEACAM family in order to support the *cag* T4SS during CagA injection and IL-8 induction (Belogolova E et al., 2013) (Königer V et al., 2016). It was also identified as one of the crucial outer membrane proteins needed by the pre-infecting strain to induce cellular resistance (Jiménez-Soto LF et al., 2013). However, the study of Jiménez-Soto *et al* on the resistance phenomenon did not consider the known fact, that *H. pylori* strains can express two different alleles of *hopQ, hopQ* allele I and *hopQ* allele II. Therefore, this dissertation puts an emphasis on the genetic analysis of the *hopQ* genes of all *H. pylori* stains used in the experiments.

Pre-infection assays with P12ΔT4SSs, X47 and Tx30a as pre-infecting strains and P12, P145, P217, 26695 and G27 as secondary infecting strains showed clear differences in the strength of cellular resistance, depending on the strain combination. Especially the choice of the pre-infecting strain, Type I *H. pylori* (P12ΔT4SSs) or Type II *H. pylori* (X47, Tx30a), had the biggest effect on the amount of P-CagA, which indicates the importance of factors defining Type I and Type II *H. pylori*, like *hopQ*, for the resistance mechanism. An analysis of the *hopQ* sequences of each strain used in the pre-infection assay came to the result, that all Type I *H. pylori* strains possessed a *hopQ* allele I, including P12ΔT4SSs, and the two Type II strains possessed a *hopQ* allele II.

These robust findings are in line with the resistance phenomenon described by Jiménez-Soto *et al* and verify this phenomenon with several additional *H. pylori* strains (Jiménez-Soto LF et al., 2013). They further support the preliminary result, that *hopQ* plays a role in the resistance phenomenon and even extend the work by revealing the possible importance of the *hopQ* allele. Type I *H. pylori* with *hopQ* allele I trigger stronger cellular resistance than Type II *H. pylori* with *hopQ* allele II.

These results present a powerful insight into the induction of the resistance phenomenon leading the focus to the characteristics of the pre-infecting strain. Future work on the resistance phenomenon will have to examine the pre-infecting strain thoroughly, starting with the identification of the *hopQ* allele.

VII.3. Pre-infected cells resist against binding of *cag* type IV secretion system

With its syringe-like structure the *cag* type IV secretion system (*cag* T4SS) binds to the host cells and interacts in two ways: It injects its effector protein CagA and induces IL-8 secretion via the NFκB pathway (Odenbreit S et al., 2000) (Yamaoka Y et al., 2004). These two interactions are linked in the way, that a fully functional *cag* T4SS with successful CagA translocation is needed for IL-8 induction (Fischer W et al., 2001). However, CagA itself is not responsible for IL-8 induction (Sokolova O et al., 2013). This is why every investigation of CagA translocation by *H. pylori* must include an investigation of IL-8 secretion by the host cells.

Previous results already demonstrated, how the secondary infecting strain of *H. pylori* is strongly impaired in its capability to inject CagA into its host cells (see chapter VI.2.1.1.). Although this assay was highly sensitive and therefore resulted in high standard deviations, pre-infection repeatedly led to strongly reduced IL-8 induction by the secondary infecting strain. Perfectly corresponding with the findings of translocated CagA, the cells also resisted against IL-8 induction. This means, that the cellular resistance triggered by pre-infection might not only prevent against the injection manoeuvre by the *cag* T4SS, but even its binding.

A slight difference to the P-CagA results was, that the strain P145 induced significantly less IL-8 than the other secondary infecting strains. Thus, the secondary infecting strains, except P145, seem to apply a mechanism distinct from the *cag* T4SS to induce IL-8 secretion in their host cells. This additional mechanism would match with the presence of the outer membrane protein OipA, which enables *H. pylori* to induce IL-8 secretion *cag* T4SS-independently (Yamaoka Y et al., 2000) (Yamaoka Y et al., 2004). Unfortunately, the expression of OipA was not investigated in this dissertation, but should be included in further investigation of IL-8 induction by *H. pylori*.

Together, the findings of P-CagA and the complementary examination of IL-8 secretion are consistent with the conclusion of Fischer *et al.,* that an effective IL-8 induction by the *cag* T4SS only exists conjoined with a successful CagA translocation (Fischer W et al., 2001).

Moreover, they broaden our understanding of the resistance phenomenon by revealing that pre-infection compromises the whole functionality of the *cag* T4SS. These compelling findings lead to the intriguing question whether the resistance phenomenon protects the host cell from several effector proteins of *H. pylori* or if it specifically affects the binding and functionality of the *cag* T4SS. These questions should be subject of future research.

VII.4. Ability of different cell lines to resist CagA translocation

Beside gastric epithelial cells, *H. pylori* also interacts with immune cells. By injecting CagA into their cytoplasm, it paralyses the immune cells and inhibits the regular immune response (Busch B et al., 2015). So far, the resistance phenomenon was only observed in epithelial cell lines representing the gastric mucosa. In order to explore if immune cells are also capable to resist CagA translocation and IL-8 induction by pre-infection treatment, a monocytic leukaemia cell line THP1 and primary leukocytes from voluntary blood donors were tested.

The remarkable results revealed two aspects. First, THP1 cells as well as primary leukocytes resisted CagA translocation and IL-8 induction in pre-infecting conditions. The mechanism behind the cellular resistance seems to be similar in epithelial cells and immune cells, but the effects are even stronger. The second aspect was, that IL-8 secretion of THP1 cells and primary leukocytes was very unusual. IL-8 induction of P12 wildtype was as high as of its T4SSs mutant, contrary to the results with AGS cells. Thus, THP1 cells and primary leukocytes seem to secrete IL-8 independently from the functionality of the *cag* T4SS. This finding supports the exploration, that besides the *cag* T4SS there are other mechanisms for IL-8 induction by *H. pylori*, like OipA (Yamaoka Y et al., 2004).

Overall, the results presented here broaden the number of host cell lines developing a cellular resistance after pre-infection. Additionally, they question our understanding of IL-8 induction by *H. pylori.* It is already known that *H. pylori* possesses different ways to induce IL-8 secretion in its host cells, but these findings provide first evidence that there is more to the IL-8 induction mechanisms by *H. pylori* than known so far. Although OipA was not tested in P12 and P12ΔT4SSs, these data support the theory of additional factors, which are independent from *cag* T4SS and OipA and might be more relevant in immune cells like THP1 cells or primary leukocytes. Further studies should therefore pay attention to which cell line they use and which effector proteins the *H. pylori* strains express.

Furthermore, the crucial data on primary leukocytes proves that it is not only a phenomenon of the laboratory, but may well exist in the physiological interaction of the human being with *H. pylori*. This extraordinary finding is encouraging and stresses the importance of further research on the resistance mechanism. Future work should try to validate the phenomenon in animal models and live tissue samples, which may eventually lead to a better understanding of multiple strain infections in the human stomach.

VII.5. Relevance of cellular processes for resistance

In search of the cellular mechanisms, which build the resistance against CagA translocation and IL-8 secretion, this dissertation explored first the dynamics of the resistance formation. Based on these dynamics two possible elements of the cellular mechanism were identified and examined for their role in the formation of the resistance phenomenon.

VII.5.1. Cellular response in two phases

Previous work documented reduced P-CagA and IL-8 levels in co- and pre-infecting conditions (Jiménez-Soto LF et al., 2013). It stated that co-infection as well as pre-infection triggered the cellular resistance, although the effect of pre-infection was significantly stronger. However, the earlier study only observed pre-infection conditions of 1 hour. In this dissertation different pre-infection periods were tested in order to explore the time line of the resistance formation and define the delay between primary and secondary infection, until the resistance phenomenon first arises.

A pre-infection period of up to 15 minutes showed a stable resistance against CagA injection as strong as the preliminary effect of co-infection. However, after 1 hour of pre-infection treatment the resistance was considerably stronger. Analysis of the secreted IL-8 corresponded with these observations. It seems that the resistance phenomenon has two phases: the first phase is a fast, but weak cellular response followed by a second mechanism, which is active after 60 minutes and intensifies the cellular resistance against CagA translocation.

These discoveries extend those of Jiménez-Soto *et al*, providing exciting insight into the dynamics of the cellular resistance (Jiménez-Soto LF et al., 2013). The data forms the surprising picture of a biphasic response of the host cells to multiple infections. This offers the vital opportunity to make assumptions on the possible candidates involved in the resistance mechanism. Future research should investigate the early-onset and the delayed

mechanism separately. In the early effect membrane processes or membrane lipid composition may play a role, whereas for the later resistance processes the protein household of the cells might be of importance.

VII.5.2. Relevance of the cell cycle for the cell resistance

In this work, as well as in the study of Jiménez-Soto *et al* (Jiménez-Soto LF et al., 2013), the host cells used in experiments on the resistance phenomenon were synchronized in their cell cycle. Since it was assumed that CagA injection by *H. pylori* is dependent on β1 integrin expression, cell cycle synchronization is a method of increasing β1 integrin availability and therefore CagA translocation (Jiménez-Soto LF et al., 2009). The bacteria interact with host cells in one cell cycle status exclusively, which might bias all observations on the resistance phenomenon. Therefore, the co-infection and all pre-infection treatments were validated on unsynchronized host cells.

As expected, multiple infections had the same effect on unsynchronized as on synchronized host cells. Moreover, the same dynamics could be observed: An early-onset weak resistance in the 0-15 minutes pre-infection treatments, resulting in a significantly stronger resistance after 1 hour of pre-infection. Consequently, the resistance phenomenon seems to be independent of cell cycle synchronization.

These solid results first verify that the unique observations in this dissertation and of Jiménez-Soto *et al* are no artefact of the synchronization method and strongly reconfirm the resistance phenomenon (Jiménez-Soto LF et al., 2013). Furthermore, these exceptional findings prove the independence of the resistance phenomenon from cyclic changes in the cell's metabolism, providing the evidence needed to rule out any factor in the cell cycle to participate in the formation of the cellular resistance. Additionally, this independence of the cell-cycle supports the recent observation by Zhao et al. that β1 integrin as a cell cycle-dependent factor might not play a large role in CagA translocation after all (Zhao Q et al., 2018).

In future, cell cycle-dependent factors can be disregarded in the search for the cellular mechanisms of the resistance phenomenon. Additionally, the method of serum-starvation to synchronize the cell cycle can safely be applied to studies on the resistance phenomenon to enhance CagA translocation.

VII.5.3. Protein synthesis is not required for cell resistance

The resistance of eukaryotic cells against CagA injection and IL-8 induction was shown to be biphasic, a weaker response during the first 15 minutes and a second, stronger resistance after about 60 minutes (see chapter VI.2.3.1.). The delay of 60 minutes for the intensification of the cellular resistance indicated a time-intensive process, like *de novo* protein synthesis. After contact with *H. pylori* the host cells might start the synthesis of proteins leading to the resistance, which fully unfold their effect after about 1 hour. In order to explore the relevance of *de novo* protein synthesis, eukaryotic mRNA translation was disrupted during infection treatments by cycloheximide.

Despite effective inhibition of protein synthesis, the cellular resistance in pre-infection conditions against CagA injection presented unchanged. Co- and pre-infection treatments resulted in reduced P-CagA, as it did without the cycloheximide treatment. Therefore, the synthesis of (an) unknown protein(s) is not responsible for the intensification of the resistance phenomenon after 60 minutes.

Although the cellular mechanism behind the resistance phenomenon remains unclear, this solid finding helps to narrow the possibilities down. With *de novo* protein synthesis excluded, an involvement of protein recycling processes or protein modification systems, however, cannot be ruled out. These approaches are a worthwhile starting point for additional explorations of the cellular mechanisms underlying the resistance phenomenon.

VII.5.4. Role of calcium-signalling during the cell response

This dissertation showed that host cells resist against CagA translocation and IL-8 induction already in co-infecting condition (see chapter VI.2.3.1.). Therefore, signalling pathways have to be very fast to organize this response. One of the fastest ways of communication in a cell is the change in cytoplasmic calcium concentration. As a second messenger, calcium flushes into the cytoplasm via pores, and mediates further signalling cascades (Berridge MJ et al., 2003). Recent studies already discovered *H. pylori*`s influence on calcium signalling in T-lymphocytes and macrophages (El-Zaatari M et al., 2010) (Kern B et al., 2015). In order to explore the role of calcium signalling for the resistance phenomenon, intracellular calcium levels were evaluated by a calcium sensitive fluorescence assay during infection. Additionally, the resistance phenomenon was investigated under the influence of a calcium ionophore, which forced strong calcium influx into the host cells.

Surprisingly, in the fluorescence assay no explicit correlation could be found between the co- or pre-infection treatment of the host cells and the detected calcium changes. Calcium influx into the host cells happened independently from the treatment. This could have been the result of unknown interference factors, but the cellular resistance was also unaltered by the ionophore treatment. Although the intracellular department of the host cells was flooded with calcium by the ionophore, the cells still resisted against CagA injection and IL-8 induction in co- and pre-infecting conditions. P-CagA and IL-8 were reduced to the same extend as observed without the manipulation of calcium level. Consequently, the data suggest that the resistance phenomenon has to be unrelated to changes in intracellular calcium level.

Even though these preliminary results offer relevant evidence that the cellular resistance uses another signalling pathway than calcium, one has to acknowledge the limitations of these experiments. This assay was very sensitive to interferences and a correlation could be hidden by false positive incidences. Furthermore, *H. pylori* only interacted with a small percentage of cells in a sporadic way. Because this assay observed cell clusters instead of single cells, changes in a single cell might have been hidden by the cluster.

These unprecedented observations are a first approach to a better understanding of the early phase of the resistance mechanism. As previous findings show that calcium signalling plays a role in inflammatory processes caused by *H. pylori* (Beceiro S et al., 2017), it is advised to further explore the signalling pathway of the resistance phenomenon. The role of calcium remains uncertain and additional research to definitely rule calcium out is strongly needed. For this, it is recommended to focus on the observation of single bacteria/cell interactions. Other possible targets of research on the early cellular response can be changes in membrane processes or membrane lipid composition.

VIII Conclusions

H. pylori infections of the human stomach is common around the world (Eusebi LH et al., 2014). Still, only 15% of the infected people develop gastric diseases (Atherton JC 2006) and eradication of *H. pylori* seems to also have negative effects on human´s health (Labenz J et al., 1997). Research starts to question whether *H. pylori* simply is a human pathogen, or whether it belongs to our flora causing diseases only in context of a disturbed balance. One major lead for this theory are the findings of Jiménez-Soto et al. (Jiménez-Soto LF et al., 2013): Host cells pre-infected with *H. pylori* build up a resistance against secondary infecting *H. pylori* strains, leading to a significantly reduced translocation of the effector protein CagA by the *cag* T4SS.

This dissertation presents various insights into the resistance phenomenon originally described by Jimenez-Soto et al. The phenomenon is biphasic with a fast but weak response and a slow, but strong resistance later. Its strength depends on the HopQ allele of the pre-infecting strain and hardly on the secondary infecting strain. Additionally, the host cells resist not only against CagA translocation, but compromise the *cag* T4SS even in its function to induce IL-8. It seems that the *cag* T4SS cannot adequately bind to the host cell, leading to less cytokine release and thus less inflammation. Continuing studies are necessary to understand the phenomenon better and maybe purposefully influence it in the future.

Apart from that, the presented work confirms the resistance mechanism with various *H. pylori* strains and cell lines of epithelial as well as lymphatic origin. These findings give a strong indication that this phenomenon exists under physiological conditions of the human stomach. Future research needs to validate the cellular resistance in live tissue samples and animal models.

In total, these unprecedented discoveries paint a picture of a strong cellular interference with *H. pylori*'s *cag* T4SS provoked by *H. pylori* itself. It seems to protect its host cells from its own virulence factors, leading to an impairment of the *cag* T4SS and accordingly less inflammation. This interference depends strongly on the strain combination, which means that the wrong combination of strains may result in severe inflammation and disturbance of the cellular mechanisms. Therefore, these results together with the knowledge that multiple strain infections of the human gastric mucosa are frequent and alter the severity of disease (Romo-González C et al., 2009) (Kim YS et al., 2009) (Secka O et al., 2011), strengthen the theory of a gastric microbiota. By understanding this microbiota and its interfering factors better, we might one day be able to influence it in patients and treat gastric pathologies by restoring the balance of this microbiota instead of applying intensive antibiotic eradication.

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X Abbreviations

°C	Degree Celcius
ANOVA	Analysis of variance
BabA	Blood group antigen-binding adhesin
BSA	Bovine Serum Albumin
CagA	Cytotoxin-associated antigen A
*cag*PAI	Cytotoxin-associated antigen pathogenicity island
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
cfu/ml	Colony-forming unite per mililiter
DNA	Desoxyribunucleid acid
EDTA	Ethylenediamine-tetraaceticacid
ELISA	Immunosorbent Assay
EPIYA	Glu-Pro-Ile-Tyr-Ala motifs
FBS	Fetal bovine serum
H. pylori	*Helicobacter pylori*
HopQ	*Helicobacter pylori* outer membrane protein Q
IL-8	Interleukin 8
kb	Kilobase
kDa	Kilodalton
mA	Milli-Amper
MALT lymphoma	Mucosa-associated lymphoid tissue lymphoma
mM	Milimolar
MOI	Multiplicity of infection
nm	Nanometer
OD550	Optic density at 550 nm wavelength
OipA	Outer inflammatory protein A
OMP	Outer membrane proteins
P-CagA	Phosphorylated cytotoxin-associated antigen A
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear neutrophil
PMSF	Phenylmethanesulfonylflouride
PVDF	Polyvinylidenfluorid
rpm	Revolutions per minute
SabA	Sialic acid binding adhesin
SBN	Single band normalization
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean

T4SS	Type IV secretion system
TE	Trypsin/Ethylenediamine-tetraaceticacid
TEMED	Tetramethylethylenediamide
TPN	Total protein normalization
ULN	Upper lane normalization
V	Volt
WHO	World Health Organisation
HKP	Housekeeping protein normalization

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