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Exploring the binding mechanisms of *Helicobacter pylori* to colon epithelial cells and induced downstream signaling

Anna Ralser

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Vorsitz: apl. Prof. Dr. Bernhard Haslinger

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1. Prof. Dr. Markus Gerhard
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Abstract

Helicobacter pylori causes one of the most prevalent infections worldwide, with more than 50% of the world population being affected. *H. pylori* colonizes the human stomach mucosa and this infection is the leading cause for gastric cancer development. Chronic infection has been associated not only with gastric, but also with a variety of extragastric diseases. Emerging studies report an increased risk for colorectal cancer development in *H. pylori* positive individuals. However, the exact mechanism by which *H. pylori* may exert its cancer promoting effects in the distant colon has not been elucidated yet. The attachment of *H. pylori* via outer membrane proteins (OMPs) to host cell receptors, like CEACAMs, is fundamental for *H. pylori*-induced pathogenesis. This interaction enables the translocation of effector molecules, eliciting a chronic inflammatory response to the infection and subsequent activation of downstream signaling. The aim of this thesis is to characterize the binding of *H. pylori* to colon epithelial cells and to identify virulence factors and OMPs involved, as well as signaling pathways induced upon infection. Therefore, colon cancer cells were infected with *H. pylori* and various isogenic mutant strains and binding and downstream signaling was analyzed. Furthermore, *H. pylori*-induced responses in primary intestinal epithelial cells derived from an *in vivo* model allowing CEACAM-HopQ interaction were characterized. *H. pylori* infection was found to alter the expression of CEACAM receptors on colon cancer cells, and successful bacterial binding to colon cells as well as primary epithelial cells was observed. Furthermore, translocation of the pathogenic virulence factor CagA as well as activation of NF-κB signaling and IL-8 secretion upon *H. pylori* infection was detected in colon cells. Those findings identify molecular and pathogenic mechanisms involved in *H. pylori* attachment to colon cancer cells. The successful binding and induction of downstream signaling support a possible direct effect of *H. pylori* in colorectal carcinogenesis.

Zusammenfassung

Mehr als die Hälfte der Weltbevölkerung ist von einer *Helicobacter pylori (H. pylori)* Infektion betroffen. Das Bakterium kolonisiert die Magenschleimhaut und ist die Hauptursache für die Entwicklung von Magenkrebs. Chronische Infektion ist mit einer Vielzahl von nicht nur gastrischen, sondern auch extragastrischen Krankheiten assoziiert, wobei *H. pylori* positive Patienten in den letzten Jahren gehäuft in Zusammenhang mit einem erhöhten Risiko für die Entstehung von Darmkrebs gebracht wurden. Die zugrundeliegenden Mechanismen dieser Assoziation sind jedoch nicht geklärt. Das Andocken von Bakterien über sogenannte äußere Membranproteine an Rezeptoren des Wirts, wie z.B. CEACAMs, sind für die Pathogenese von *H. pylori* von äußerster Bedeutung, indem sie die Translokation von Effektormolekülen, das Auslösen einer chronischer Immunantwort und die Induktion von Signalwegen ermöglichen. In dieser Arbeit wurde die Bindung von *H. pylori* an Kolonzellen, dabei involvierte Virluenzfaktoren und äußere Membranproteine des Bakteriums sowie induzierte Signalkaskaden untersucht. Dafür wurden Kolonkrebslinien mit *H. pylori* und verschiedenen isogenen, mutierten Stämmen infiziert und die Bindung sowie dadurch getriggerte Signalwege analysiert. Außerdem wurden *H. pylori* induzierte Effekte in primären epithelialen Zellen aus dem Darm eines Mausmodells untersucht, welches die CEACAM-HopQ Interaktion simuliert, die für *H. pylori* induzierte Pathogenese im Magen ausschlaggebend ist. Es konnte gezeigt werden, dass *H. pylori* die Expression von CEACAM Rezeptoren in Kolonzellen verändert und durchaus fähig ist, an Kolonzellen sowie primäre epitheliale intestinale Zellen zu binden. Außerdem wurde die Translokation vom pathogenen Virulenzfaktor CagA sowie die Aktivierung vom pro-inflammatorischen NF-κB Signalweg und die Ausschüttung von IL-8 in *H. pylori* infizierten Kolonzellen gezeigt. Diese Ergebnisse decken molekulare und pathogene Mechanismen auf, die der Anheftung von *H. pylori* an Kolonkrebszellen zugrunde liegen. Das erfolgreiche Andocken an Kolonzellen und die daraus resultierenden Effekte lassen einen direkten Effekt von *H. pylori* auf die kolorektale Karzinogenese vermuten.

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1. Introduction

1.1. Helicobacter pylori

H. pylori colonizes the gastric mucosa of about half of the world's population and has been discovered to cause chronic gastritis and peptic ulcers by Barry Marshall and Robin Warren in 1984 [1].

H. pylori is a gram-negative, spiral shaped bacterium [1] with 2 to 6 unipolar, sheathed flagella that allow the organism to move rapidly in viscous solutions, as the mucous layer overlying the gastric mucosa [2]. The bacterium is microaerophilic, requiring at least 2% oxygen [3]. It also requires a highly humid and pH neutral environment, although it can survive short exposure to more acidic conditions, like the gastric pH [4, 5].

A key feature of *H. pylori* is its genetic heterogeneity, with the consequence that every *H. pylori* infected individual carries a distinct strain [6]. These variations are believed to be caused by adaptations of the bacterium to the gastric conditions and immune responses of the host [7] and concern genes encoding virulence, outer membrane proteins and lipopolysaccharide (LPS) biosynthetic enzymes [8-11].

H. pylori infection is the main risk factor for gastric cancer development [12]. Chronic infection can lead, in about 10 to 15 % of infected subjects, to gastric or peptic ulcers, gastric adenocarcinomas and mucosa-associated lymphoid tissue-lymphomas [1]. In addition, *H. pylori* infection has systemic consequences that lead to extragastric diseases.

Disease severity depends on a variety of factors, such as characteristics of the colonizing strain, host immune response as well as environmental factors [13].

1.1.1. Epidemiology

Up to 40 % of the Western population is infected with *H. pylori*, while more than 75% is infected in developing countries (Figure 1). While in the Western world the infection rate of children is decreasing and mostly adults and elderly people are infected, infection prevalence in developing countries is still high among children and adolescents [14, 15]. This prevalence of *H. pylori*

infection depends on the socioeconomic status, especially during childhood [16]. Overall, the infection is commonly acquired during childhood from close family members and persists a lifelong if not treated [1, 17-21].

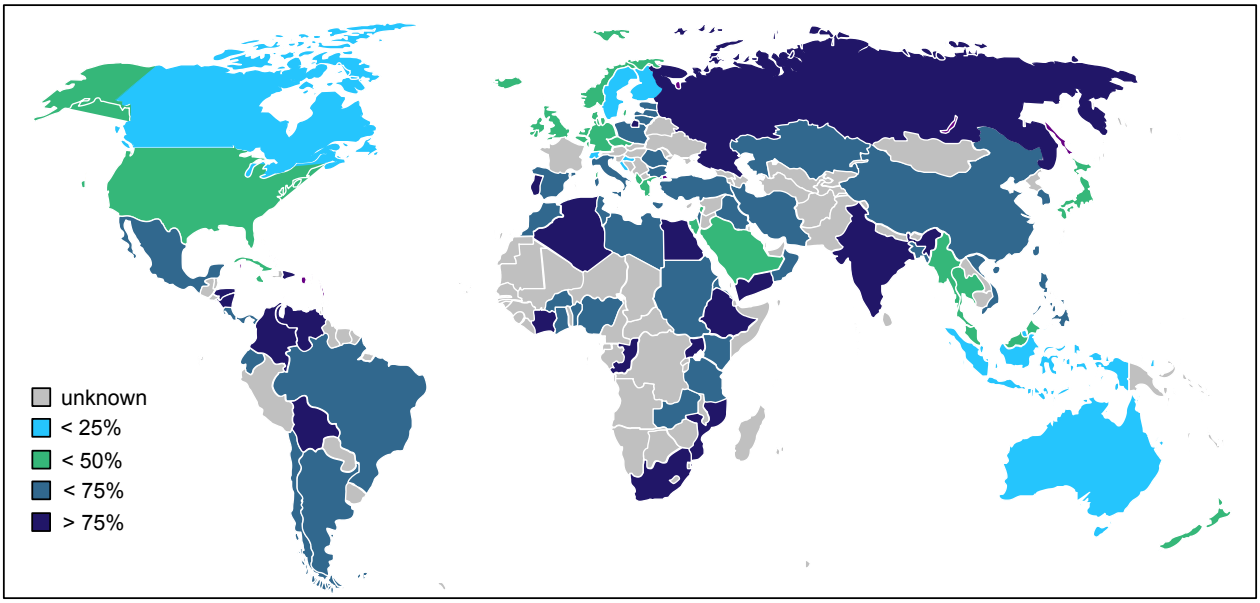

Figure 1: *H. pylori* infection prevalence worldwide.

Percentages of *H. pylori* infected individuals across countries are shown. An apparent connection between developmental status and infection rate is visible [22].

1.1.2. Pathogenesis of Infection

1.1.2.1. Adhesins and epithelial receptors

The outer membrane of *H. pylori* consists of five protein families: adhesins, porins, iron transporters, flagellum-associated proteins and proteins of unknown function [17, 23-26]. It contains phospholipids and LPS, the former consisting of very rare cholesterol glucosides and the latter of lipid A, core oligosaccharide and an O side chain, which mimics Lewis blood group antigens [17, 27-31].

The ability of *H. pylori* to persist in the challenging environment of the stomach is owed to various factors, including the ability to buffer the acidic pH by synthesizing urease, the crossing of the gastric mucus due to its special morphology and finally the binding to epithelial receptors via adhesins [32]. In this study, the best described adhesins BabA and SabA as well as the OMP

HopQ will be further adressed, as they are playing an important role in the attachment of *H. pylori* to epithelial cells. Other known adhesins are summarized in Table 1.

Table 1: *H. pylori* adhesins and their epithelial receptors and their tissue specific effects upon binding.

Adhesin	Epithelial receptor	Pathogenic mechanism after binding	Ref.
BabA	MUC5AC carrying Lewis B antigen	• Proinflammatory response and IL-8 secretion • Enhancing translocation of CagA via T4SS • DNA and mucosal damage	[33] [34, 35]
SabA	Sialyl Lewis X and Lewis A, Lewis X	• Oxidative burst in granulocytes • Hemagglutination of erythrocytes	[36] [37]
HopQ	CEACAM1, 3, 5 & 6	• Translocation of CagA via T4SS • IL-8 secretion • Changes in cell morphology	[38, 39]
AlpA and AlpB	Laminin	• IL-8 secretion • Proinflammatory response	[40, 41]
OipA	unknown	• IL-8 secretion (contribution of OipA is questionable, due to linked presence with CagA)	[42, 43]
HopZ	unknown	• unclear function	[44]
Hsp60	receptor-like sulfatide (sulfoglycolipid)	• induces IL-6, IL-8, TNF-α and GRO production • activates NF-κB signaling	[45]
Neutrophil activating protein A (NAP A)	glycosphingolipids expressed on the neutrophil surface	• induces release of IL-8, macrophage inflammatory protein (MIP)-1α, and MIP-1β by neutrophils	[46]

The best described adhesins, blood-group-antigen-binding adhesin (BabA) and sialic acid-binding adhesin (SabA), are part of the largest outer membrane protein (OMP) family, the Hop family, and mediate epithelial adherence via the Lewis antigens [44].

The first identified adhesin BabA mediates binding to highly glycosylated mucus protein Mucin 5AC (MUC5AC) carrying the fucosylated Lewis^b blood group antigens in the gastric mucosa [10, 47]. The BabA-mediated adhesion of *H. pylori* to the gastric epithelium leads to a proinflammatory response and has therefore a role in the virulence of *H. pylori* [48]. Furthermore, the binding of *H. pylori* to MUC5AC contributes to double-strand breaks of DNA, stronger IL-8 secretion in the mucosa and its injury [33-35].

By upregulating sialyl-dimeric Lewis X expression, babA/Lewis B binding enables SabA mediated adherence [44]. Additionally, the adhesin can bind to sialylated structures on granulocytes and erythrocytes and induce an oxidative burst and hemagglutination [36, 37].

Another member of the Hop family is HopQ, which is binding to members of the carcinoembryonic antigen-related cell adhesion molecules (CEACAM) receptor family expressed on epithelial cells [38].

HopQ binds the N-terminus of CEACAM1, 5 and 6 through a protein-protein interaction, in contrast to the sugar-dependent binding modes exhibited by previously identified pathogens. This interaction was also found to be species specific, with HopQ only binding CEACAM1 in humans and rats, but not murine, bovine or canine CEACAM1 [38]. Additionally, it was found that HopQ-CEACAM interaction is essential for the translocation of the virulence factor cytotoxin-associated gene A product (CagA). The deletion of HopQ resulted in reduced bacterial adhesion and decreased IL-8 release by epithelial cells in response to the infection as well as in abrogation of the cellular elongation phenotype or hummingbird phenotype, suggesting that HopQ likely plays an important role in *H. pylori* pathogenesis [38, 39].

1.1.2.2. Virulence Factors

H. pylori is known to lead to chronic inflammation in the gastric mucosa. The inflammatory response and the disease severity is influenced by several bacterial virulence factors leading to activation of host-signaling pathways regulating epithelial cell homeostasis and reprogramming innate immune cells [44]. The virulence factors described below are important for the induction of signaling pathways and disease outcome.

The *cag* pathogenicity island (*cag* PAI) is *H. pylori*'s best-characterized virulence trait. *The cag PAI* is a 40 kilobase DNA insertion element derived from a bacteriophage, consisting of about 32 genes encoding a type 4 secretion system (T4SS) [49-52]. About 60-70% of Western strains and nearly 100% of Asian strains are *cag* PAI positive (*cag*$^{+}$) [49-52]. This is of high importance due to its association with disease severity, as *cag*$^{+}$ strains harbor a much higher risk for the development of severe gastritis, atrophy, dysplasia, and gastric adenocarcinoma [53, 54].

The T4SS enables the translocation of bacterial factors into host cells, namely CagA and peptidoglycan [55, 56]. CagE is a structural component of the T4SS and its abrogation renders the secretion system nonfunctional, which means that effector molecules are no longer delivered to the host cells.

The protein CagA contains tyrosine phosphorylation motifs (EPIYA sites), which, after translocation, are phosphorylated by host cell SRC and ABL kinases [57]. Subsequently, phosphorylated CagA activates eukaryotic phosphatase SHP-2 and extracellular signal-regulated kinase 1 and 2 (Erk1/2), leading to changes in cell morphology, like cell elongation [58], and activation of Nuclear factor-κB (NF-κB) with subsequent IL-8 release [59-61]. Furthermore, it has been shown that CagA is involved in immune tolerance via DCs, by activating signal transducer and activator of transcription 3 (STAT3) signaling via increased IL-10 expression [62].

Peptidoglycan, once translocated and recognized by Nucleotide-binding oligomerization domain-containing protein 1 (NOD1), activates NF-κB, p38 and Erk signaling in the host and leads to the release of MIP-2, β-defensins, IL-8 and type I interferon, which affects Th1 cell differentiation and therefore host immune recognition [63-65].

The second most important virulence factor of *H. pylori* is the pore-forming toxin Vacuolating Cytotoxin A (VacA). It induces epithelial cell apoptosis, autophagy and inhibits T cell activation by forming anion-selective channels and entering the host cell by endocytosis [44, 66-68]. The toxin activates NF-κB, which leads to proinflammatory IL-8 secretion [69], and inhibits the autophagy process, contributing to gastric inflammation [70]. Furthermore, VacA seems to be crucial to induce tolerogenic dendritic cells (DC), promoting regulatory T (Treg) cells [71].

Another important virulence factor is γ-glutamyltranspeptidase (gGT), which regulates inflammatory effects via distinct mechanisms. It is involved in cycle arrest of T-cells by disruption

of the Ras signaling pathway [72] and induces Treg cells via the tolerization of DCs [71]. The induction of a regulatory phenotype is promoted by the enzymatic activity of gGT, converting glutamine to glutamate, which leads to activation of gluatamate receptors on DCs and inhibition of IL-6 expression [73]. In addition to the induction of a tolerogenic phenotype, the enzymatic property of gGT interferes with T-cell function and proliferation via deprivation of glutamine. This leads to the reduction of effector cytokines and metabolic reprogramming of T-cells [74]. Furthermore, it acts proinflammatory, by activating NF-κB and subsequent IL-8 upregulation by producing H_2O_2 [75].

Besides those virulence factors and their well-studied roles in *H. pylori* induced pathogenesis, other virulence factors include duodenal ulcer-promoting gene (dupA), induced by contact with epithelium gene (iceA) and high temperature requirement A (HtrA), which contribute to the inflammatory response elicited by the bacterium.

1.1.2.3. *H. pylori* induced signaling pathways

Several signaling pathways play a role in the pathogenesis of *H. pylori* infection. Here, the focus will lay on NF-κB and STAT3 signaling, as those pathways are known to play a role in gastric carcinogenesis [76-79].

H. pylori infection can activate the NF-κB pathway by two mechanisms: CagA-dependent and CagA-independent (Figure 2). Once CagA is translocated via the T4SS in the cytoplasm of host cells, it activates IKK by interacting with TRAF6 and TAK1 [79]. Cag-independent activation is mediated via LPS recognition by TLRs [80-83]. This activation may be also influenced by peptidoglycan- mediated NOD1 activation, which depends on the *cag*PAI but not on CagA [84]. Once activated, NF-κB leads to proinflammatory cytokine release (TNFα, IL-1β and IL-6) [85-88], regulates chemokine response (IL-8, MCP-1), blockade of apoptosis (cIAPs, c-FLIP, A20, and BclX), angiogenesis (VEGF, IL-8) and invasion (MMP-2, MMP-9) and thereby contributes to carcinogenesis [77].

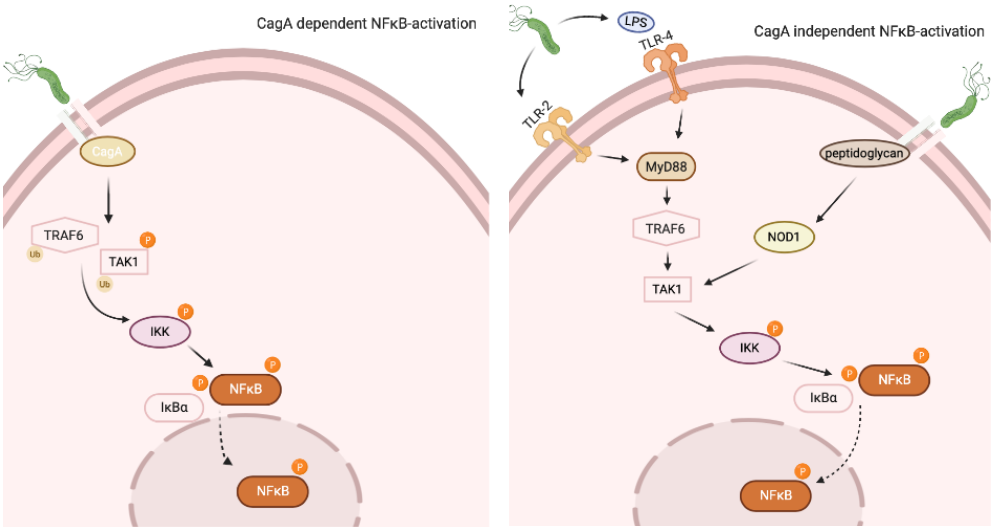

Figure 2: *H. pylori* - induced NF-κB activation.

CagA dependent activation: translocated CagA activates IKK by interacting with TRAF6 and TAK1. CagA independent activation: *H. pylori* LPS is recognized by TLRs leading to activation of NF-κB. Alternatively, T4SS delivered peptidoglycan activates signaling via stimulation of NOD1 [81].

The JAK/STAT3 signaling pathway has been shown to be involved in gastric inflammation and progression to gastric cancer, with CagA further potentiating this process [76]. Several mechanisms and cytokines are involved in STAT3 signaling activation in the context of *H. pylori* infection. One route of activation is enabled by binding of the cytokines IL-6 and IL-11 to the gp130 subunit of the IL-6 family receptor, which can be further driven by CagA mediated induction of IL-11. Additionally, CagA can directly activate STAT3 via recruitment of SHP-2 [79]. After phosphorylation by JAK, STAT3 transcribes genes involved in angiogenesis, like VEGF, cell cycle progression (e.g. cyclinD1) and cell survival, as Bcl/xL and survinin [89] (Figure 3). Additionally, STAT3 activation in immune cells is supposedly an important factor for the outcome of *H. pylori* infection, e.g. STAT3 dependent induction of Th17 responses [90]. Furthermore, as described previously, STAT3 signaling can be activated upon CagA translocation via IL-10, leading to the induction of tolerogenic DCs.

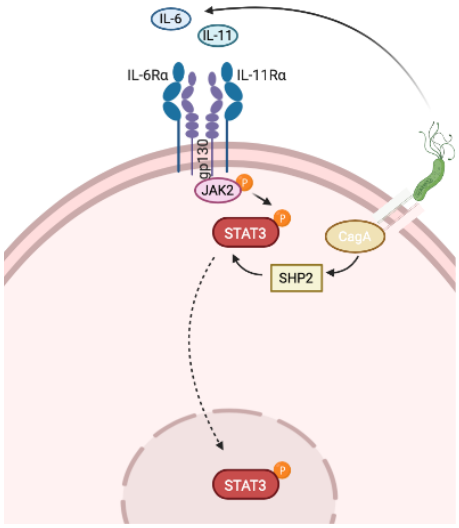

Figure 3: Activation of STAT3 signaling in response to *H. pylori* infection.

Interaction of IL-6 with gp130 leads to STAT3 activation, which is further driven by IL-11. Translocation of CagA activates STAT3 signaling either directly via SHP2, or via induction of IL-11. Subsequently, phosphorylated STAT3 translocates to the nucleus and induces the transcription of genes typically involved in carcinogenic processes [91].

1.1.2.4. Immune Response elicited by *H. pylori*

The innate immune response is activated as a first line of defense against *H. pylori,* by recognition of the bacterial heat shock protein Hsp60 [92], as well as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on epithelial cells [1, 93]. Thereby, toll like receptor (TLR) 2 (recognizing peptidoglycan) and TLR5 (recognizing flagellae) engagement induce NF-κB activation and subsequent chemokine upregulation, whereas TLR4 (LPS) is only a weak inducer of this pathway [94-97]. Another key activator of the innate immune response is intracellular peptidoglycan (after cagPAI mediated translocation) recognized by NOD1 [84].

However, *H. pylori* infection is primarily characterized by an adaptive immune response, consisting of the recruitment of Th1 and Th17 cells and their cytokines leading to gastritis and chronic pathology [98-101]. Nevertheless, the strong immune response towards *H. pylori* almost never results in clearance of the infection, but rather in a downregulation of the inflammation and overtaking of the host's response by the invading organism [17]. As described previously,

virulence factors VacA and gGT, by compromising T-cell effector functions, are the main bacterial components involved in this immune evasion of *H. pylori*.

Additionally, regulatory T cells are specifically induced upon infection and seem to downregulate the immune response towards *H. pylori* infection via CD25+ cells and IL-10 production, as they control inflammation and enable the persistence of the organism in the gastric mucosa [17, 102]. This tolerogenic milieu is formed upon the interaction of virulence factors VacA, CagA and gGT with DCs.

1.1.3. *H. pylori* associated diseases

H. pylori infection is associated with an increased risk for developing mainly gastric but also extragastric pathologies, and therefore plays an important role in diagnostic and preventive clinical measures [17].

1.1.3.1. Gastric diseases

1.1.3.1.1. Acute and chronic gastritis

The colonization of the gastric mucosa with *H. pylori* results in infiltration with neutrophils and mononuclear cells of the corpus and the antrum.

The acute phase of infection is often accompanied by transient dyspeptic symptoms such as fullness, nausea and vomiting, shows histologically an inflammation of the proximal and distal stomach mucosa and may be caused by hypochlorhydria [103].

Once the colonization becomes persistent, the distribution of inflammation and the disease development are strongly dependent on the acid production. In case of normal or high acid secretion, the inflammation is localized in the antrum (antrum-predominant gastritis), as it is the part with lowest acid secretion. This type of colonization appears less harmful histologically, showing limited chronic inactive inflammation and low superficial bacterial counts. This antral-dominant inflammation triggers acid secretion though IL-1ß, one of the most potent inducers of acid production [17]. The clinical consequence of such hyperacidity can be the development of peptic ulcer disease in the duodenum.

In contrast, "low acid producers", e.g. patients with IL-1ß polymorphisms, show an even distribution of bacteria in antrum and corpus, a so called corpus-predominant pangastritis [104]. Their lower acid production is fostered due to loss of parietal cells in the course of atrophic gastritis or by their loss of function due to acid-suppressive drugs, in particular, proton pump inhibitors (PPI) [104]. Furthermore, the inflammation itself further increases hypochlorhydria, as cytokines as IL-1β strongly suppress parietal cell function, which predisposes those individuals to atrophic gastritis, intestinal metaplasia and gastric cancer [17].

1.1.3.1.2. Peptic ulcer disease

Peptic ulcers are mucosal lesions penetrating the muscularis mucosa layer, forming a cavity surrounded by acute and chronic inflammation and measuring at least 0.5 cm [17, 105]. Gastric ulcers are mostly located along the lesser curvature in the transition zone from corpus to antrum [106] and duodenal ulcers in the duodenal bulb, the area with the highest gastric acid concentration [17]. They are strongly related to *H. pylori* infection and as a consequence their incidence decreased with the decrease of *H. pylori* prevalence, having a current incidence in Europe of 0.10 - 0.19 % per year [107].

Symptoms of a duodenal ulcer are waking at night with burning, upper abdominal pain, that improves with eating and general symptoms like belching, vomiting, weight loss and poor appetite [107]. The same applies to gastric ulcer, except that pain worsens with eating [108].

Ulcer complications include bleeding, perforation and stricture formation, whereas bleeding is the most common one, occurring in 15% to 20% of the cases [17]. If caused by *H. pylori* infection, the risk of renewed bleeding can be reduced with eradication therapy [109]. Even in case of perforation and stricture formation in *H. pylori* positive patients, eradication therapy has positive effects on outcome and should be performed after initial therapy [17].

1.1.3.1.3. Atrophic gastritis, intestinal metaplasia and gastric cancer

Atrophic gastritis is characterized by destruction of gastric glands, loss of parietal cells, and replacement of the normal gastric mucosal architecture by fibrosis and, in case of intestinal metaplasia, by intestinal-like epithelium [17]. These processes are dependent on the distribution

of chronic active inflammation, occur in about 50 % of all *H. pylori* infections [110], and increase the risk of gastric cancer development [111]. This risk is associated with host and bacterial factors, which influence the inflammatory response and is increased in case of cagA-positivity [112, 113], smoking and high salt diet [114-117].

Epidemiologically, gastric cancer is the fifth most common cancer in the world [118] and 89% of non-cardia gastric cancers are associated with chronic *H. pylori* infection [119]. A meta-analysis of 1526 Japanese individuals with duodenal ulcers, gastric ulcers, hyperplasia and nonulcer dyspepsia revealed that gastric cancer develops in around 3% of infected patients, compared to none in uninfected patients [12]. In patients without premalignant lesions, eradication of *H. pylori* results in a far lower risk of cancer development, which supports the fact, that the bacterium is involved in early stages of gastric carcinogenesis [120].

Furthermore, gastric mucosa-associated lymphoid tissue (MALT) is a common response to chronic *H. pylori* infection, but only less than 1% progress to a MALT lymphoma by developing a monoclonal population of B cells [17, 121].

1.1.3.2. Extragastric diseases

Even though *H. pylori* infection is strictly colonizing the gastric mucosa, it has systemic effects in other organs. Some of these extragastric effects are attributed to T-cell responses elicited by the bacterium in distant organs.

1.1.3.2.1. Esophageal diseases

H. pylori infection might have a protective effect on erosive esophagitis, Barett's esophagus (BE) and esophageal adenocarcinoma (EA), whereas the negative association with gastroesophageal reflux disease (GERD) symptoms is controversial [1]. This protection arises from the hypothesis that *H. pylori* infection results in destruction of acid-producing parietal cells and is therefore acid-suppressive. This is mostly attributed to CagA and VacA bearing strains [122].

1.1.3.2.2. Inflammatory Bowel Disease

Several studies support a strong negative association between *H. pylori* colonization and inflammatory bowel disease (IBD). This effect may be explained by a high ratio of immunoregulatory to immunostimulatory sequences of *H. pylori* DNA [123] and IL-10 production in mesenteric lymph nodes (MLNs) in response to the infection [124]. Furthermore, especially in asymptomatic carriers, Treg response, directed by TLR2 signaling suppressing dendritic cells (DC) [125], will predominate and suppress Th1, Th17 and Th2 and lead to anti-inflammation and immune suppression (B14). TLR2/NLRP3/caspase-1/IL-18 signaling contributes as well to *H. pylori* specific immunomodulation and IBD protection [1].

1.1.3.2.3. Allergic disease manifestations

A protective effect of *H. pylori* infection has been addressed by several studies on asthma, atopic dermatitis and other allergies with respiratory manifestations, particularly in children and early onset allergies [126-132].

Regarding allergic asthma, meta-analyses report lower *H. pylori* infections in asthmatics than in controls with an odds ratio of 0.81 to 0.84 [133, 134], and even animal models confirmed this protective effect of *H. pylori* infection [135].

The persistence factors and immunomodulators VacA and gGT [71] promote chronic infection as they lead to tolerization of DCs and support Treg differentiation. As a consequence, DC/Treg-derived IL-10 suppresses Th2 and Th17 responses in the lung, and thus prevents allergic reactions [1].

1.1.3.2.4. Colorectal Cancer

An increasing amount of literature has been supporting a link between *H. pylori* infection and colorectal cancer (CRC) [136-141]. This positive association has been reported with an odds ratio between 1.15 and 10.6 [138, 140, 142-145]. However, possible underlying mechanisms of this relationship have not been identified yet.

1.2. Colorectal Cancer

1.2.1. Epidemiology

Being the third most common cancer and the second most frequent cause of cancer related deaths, almost 2 million individuals worldwide develop colorectal cancer every year [146]. With a lifetime incidence of 5 %, this cancer entity is more common in developed countries [147-149].

1.2.2. Pathways involved in Colorectal Carcinogenesis

Three carcinogenic pathways have been reported to be involved in the development of CRC: the adenoma-carcinoma "Correa" sequence, the serrated pathway and the inflammatory pathway (Figure 4) [150].

The majority of sporadic CRCs arise from the adenoma-carcinoma sequence, with more than 85% of cases affected. This pathway is characterized by stepwise mutations of tumor-suppressor genes (*APC, p53*) and oncogenes (*KRAS*) [151], regulating growth and leading from adenomas, as precursors, to cancer [150].

In contrast stands the serrated pathway, where cancer arises from serrated adenomas, after mutation of the oncogene *BRAF* and subsequent CpG island methylated phenotype (CIMP) fostered progression [152, 153]. This pathway accounts for about 10-15% of CRC cases.

Finally, in the inflammatory pathway, chronic inflammation drives the progression of normal cells from low to high-grade dysplasia and finally to cancer [154]. Patients with IBD, mostly ulcerative colitis, are affected [155], however only around 2% of CRC cases can be explained by this pathway [150].

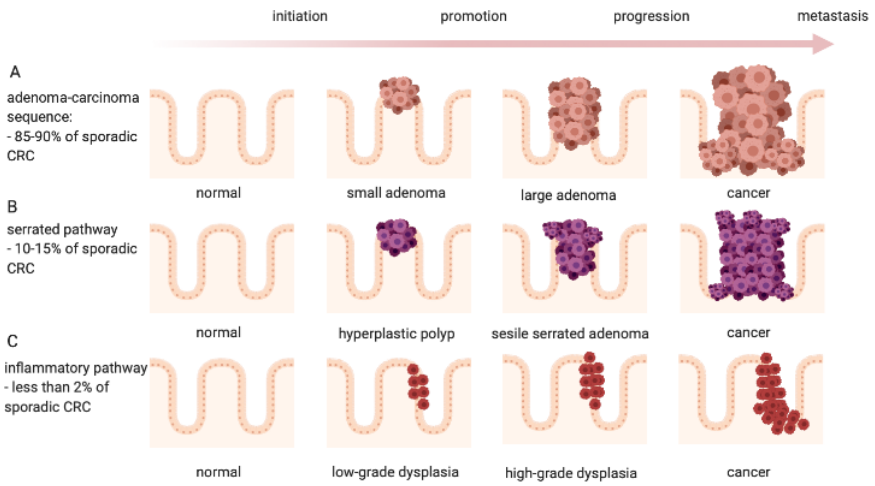

Figure 4: Carcinogenic pathways and their incidence in the development of CRC.

A. The adenoma-carcinoma sequence is accounting for 85%-90% of sporadic CRC cases and is a stepwise progression model involving mutations of tumor-suppressors and oncogenes. B. The serrated pathway accounts for 10-15% of the cases and those cancers arise from serrated adenomas. C. Chronic inflammation, mostly ulcerative colitis, drives the progression from normal cells to dysplasia and finally to cancer in around 2% of the cases [150].

1.2.3. Genetics and risk factors

Around 60-65% of CRC cases arise sporadically due to genetic and epigenetic aberrations, which are highly influenced by modifiable risk factors. The second major proportion constitutes of hereditary cases, with 25% of them having a family history without any known genetic cause and around 5% belonging to hereditary cancer syndromes, like familial adenomatous polyposis (FAP) or hereditary nonpolyposis colorectal cancer (HNPCC) [156].

However, despite this hereditary component, environmental factors highly contribute and determine CRC carcinogenesis.

The most common risk factors connected to CRC can be divided into general factors, such as increasing age, male sex, previous colonic polyps and previous CRC, and environmental factors, such as diet (rich in meat, fat; poor in fiber, folate and calcium), sedentary lifestyle, obesity, diabetes mellitus, smoking and high alcohol intake [157, 158]. Among these known risk factors, a rising amount of literature supports the contribution of *H. pylori* infection to the development and aggressiveness of CRC.

2. Objectives

The aims of this thesis were to explore the molecular mechanisms involved in *H. pylori* induced colorectal carcinogenesis. Therefore, the binding of *H. pylori* to colon epithelial cells as well as primary intestinal cells from CEACAM transgenic mice were assessed and virulence factors and OMPs involved in this adherence were determined. Furthermore, signaling pathways induced upon infection were studied in order to shed light on carcinogenic processes elicited in colon epithelial cells upon *H. pylori* infection.

3. Material and Methods

3.1. Material

Table 2: Instruments used within this study.

INSTRUMENT	COMPANY
Agarose Gel chamber	Bio-Rad Laboratories
Thermal cycler C1000 Touch	Bio-Rad Laboratories
Biophotometer, 6131 Spectrophotometer	Eppendorf
C100 Touch thermal cycler	Bio-Rad Laboratories
CFX384 Touch Real-Time PCR Detection System	Bio-Rad Laboratories
CytoFlex S	Beckman Coulter
Fine Balance (max: 120g, d~0.0001g)	ScalTec
Forma Series II Water Jacket CO_2 incubator, bacteria incubator	Thermo scientific
Heating plate/magnetic stirrer	ARE VELP Scientifica
Haemocytometer, Neubauer improved (0.100 mm, 0.0025 mm^2)	Marienfeld superior Germany
Hera cells 240 incubator, cell culture incubator	Thermo electorn corporation
Hera Safe Bio-Flow cell culture	Hera Instruments
Heraeus Biofuge Primo centrifuge	Thermo Electron corporation
Heraeus Megafuge 40R centrifuge	Thermo Fisher Scientific
Ice machine CoolNat	Ziegra Eismaschinen
MaxWell RSC48	Promega
Mini Gel Tank	LifeTechnologie
Mini shaking oven, hybridisation open; OV3	Biometra
Molecular Imager Gel Dox XR+ (Eagle Eye)	Bio-Rad Laboratories
Nanodrop 1000	Thermo Fisher Scientific
NIKON eclipse TS-100 microscope	NIKON
PowerPac (WB)	Bio-Rad Laboratories
Rocker-shaker MR-12	BioSan

Sunrise plate reader	Tecan
Thermomixer compact	Eppendorf
Trans-Blot Semi-Dry Transfer Cell	Bio-Rad Laboratories
Ultrasonic water bath, SONOREX DIGITEC	Bandelin
Water bath	GFL
WB detection machine	INTAS Science Imaging
XcitaBlue Conversion Screen	Bio-Rad Laboratories

Table 3: Consumables used within this study.

CONSUMABLES	COMPANY
10 cm petri dish, not treated	Falcon
12-well plate	Falcon
15 mL falcon	Greiner Bio-one GmbH
24-well plate	Falcon
50 mL falcon	Greiner Bio-one GmbH
75 cm^2 flask	Labsolute – Th. Geyer GmbH & Co. KG
96-well plate (V bottom)	Kuhnle
ClarityTM Western ECL substrate	Bio-Rad Laboratories
Cotton Buds ROTILABO wood, 5 mm	Carl Roth GmbH & Co. KG
Cell Strainer, 70µm	Corning
Framestar 384 well skirted PCR plate	4titutde
Gel-Blotting Paper 580x600mm, 195g/m^2 (100Bg.)	A. Hartenstein
Inoculation loop, 10 µL	VWR
MaxWell RSC simplyRNA tissue kit	Promega
NUNC, maxi sorb, flat bottom plates	Thermo Fisher
PCR tube stripes with lid (8 tubes)	Kisker Biotech GmbH & Co. KG
Pierce ECL Western Blotting substrate	Thermo Scientific
Plastic pipettes (10 mL, sterile)	Greiner Bio-one GmbH
Plastic pipettes (25 mL, sterile)	Greiner Bio-one GmbH

Plastic pipettes (5 mL, sterile)	Greiner Bio-one GmbH
Restore PLUS Western Blot Stripping buffer	Thermo Scientific
Microscope slides	Roth
40 % Acrylamide/Bis Solution 37.5:1	Bio-Rad Laboratories
2.0 mL microtubes, CapLock	TreffLab – Nolato Treff AG
1.5 mL microtubes, CapLock	TreffLab – Nolato Treff AG
0.5 mL microtubes, CapLock	TreffLab – Nolato Treff AG
Amersham Protran 0.45 NC nitrocellulose Western blotting membranes (300mm x 4m)	GE Healthcare Life Science

Table 4: Buffers used within this study.

BUFFER	INGREDIENTS	STORAGE
10% APS	in 100 mL dH_2O: • 10g ammonium persulfate	-20°C
1M DTT	in 100 mL dH_2O: • 15,43 g	-20°C
0,5 M EDTA	in 100mL dH_2O, pH 8: • 18,61 g	-4°C
10x TBS 1x TBS-T	in 1L dH_2O, pH: 7.5 • 50 mM Tris ultra-pure (60.5 g) • 150 mM NaCl (87.6 g) diluted with dH_2O + 0.1% Tween	RT
1 M Tris pH 8.8	in 1L dH_2O: • 181,71 g	RT
0.5 M Tris pH 6.5	in 1L dH_2O: • 60.57 g	RT
1 M Tris pH 7.4	in 1L dH_2O: • 121.14 g	RT
RIPA buffer	in 250 mL dH_2O: • 50 mM Tris ultra-pure; pH 7.4 • 1% NP-40 (Igepal = NP-40 substitute) • 150 mM NaCl • 0.25 % DOC	RT

	• 1 mM EGTA Ready to use, in 10 mL: • 1 tablet of protease + phosphatase inhibitor	
10x SDS running buffer	in 1L dH_2O: • 25 mM Tris ultra-pure (30.3 g) • 200 mM Glycine (150.1 g) • 0.1 % SDS (10g)	RT
Semi Dry buffer	in 2L dH_2O: • 48 mM Tris ultra-pure (11.64 g) • 39 mM Glycine (5.86 g) • 0.037 % SDS • 20 % MeOH	-4°C
Separating gel buffer	in 0.5L dH_2O: • 1 M Tris pH 8.8 • 0.4 % SDS	RT
Stacking gel buffer	in 0.5L dH_2O: • 0.5 M Tris pH 6.5 • 0.4 % SDS	RT
SDS lysis buffer stock 1x SDS lysis buffer	in 50mL dH_2O: • 250 mM Tris pH 6.8 • 6 % SDS • 10 % Glycerol • 0.01% bromophenol blue Add 500µl 1 M DTT to 9.5 mL stock	RT

Table 5: Reagents used within this study.

REAGENT	COMPANY
Acetic Acid (0,02M)	Roth
Albumin (BSA) Fraction V (pH 7.0)	AppliChem
Ammonium Persulfate (APS)	Sigma
Bromophenol Blue Sodium Salt	Sigma
CFSE (10mM)	Sigma
Dimethyl Sulfoxide (DMSO) for cell culture	AppliChem

DL-Dithiothreitol (DTT)	Sigma
dNTP mix (10 mM in NFW)	Promega
D-sorbitol	Sigma
Ethanol absolute, molecular biology grade	AppliChem
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Glycerol	Roth
Glycine	Roth
GoTaq® qPCR Master Mix	Promega
HRP-conjugated anti-mouse secondary antibody	Promega
HRP-conjugated anti-rabbit secondary antibody	Promega
IL-8 Human Elisa Kit	Thermo Scientific
KCl	Roth
KH_2PO_4	Roth
Methanol	Merck
M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant	Promega
M-MLV RT 5x buffer	Promega
Na_2HPO_4	Roth
NaCl	Roth
NaOH	Roth
Powdered milk, blotting grade	Roth
Precision Plus Protein Dual Color Standards	Bio-Rad Laboratories
Random Primers 150ng/µl	Promega
Sodium Dodecyl Sulfate (SDS), pellets	Roth
Staurosporine, from Streptomyces sp.	Sigma
Sucrose	Millipore
Temed (N, N, N', N'-Tetramethylethylenediamine)	Sigma
Thiazolyl Blue Tetrazolium Bromide, approx. 98 % TLC (MTT)	Sigma
TPCA-1	Sigma

TRIS ultra-pure	AppliChem
Trypan Blue Stain (0.4%)	Gibco
TWEEN® 20 molecular biology grade	AppliChem
ß-Mercaptoethanol (cell culture grade)	Sigma

Table 6: Media used within this study.

MEDIA	COMPANY
0.05 %Trypsin-EDTA (1x)	Gibco
Collagen I, Bovine 5mg/mL	Gibco
Glutamax (100x)	Gibco
HEPES (1M)	Gibco
Dulbecco`s Modified Eagle Medium (DMEM)	Gibco
Fetal Bovine Serum (FCS), heat inactivated	Sigma
Penicillin (5000 units/mL)/Streptomycin (5000 µg/mL) (P/S)	Gibco
Phosphate Buffered Saline (PBS) pH 7.4	Gibco

Table 7: Software used within this study.

SOFTWARE	COMPANY
Affinity Designer	Serif
Affinity Photo	Serif
BioXRad	Bio-Rad Laboratories
FlowJo	FlowJo
GraphPad Prism	Graphpad Software
LabImage 1D	Kapelan Bio-Imaging
Magellan7 software	Tecan

3.2. Methods

3.2.1. Cell Culture

Cells (summarized in table 1) were thawed from long-term storage (in liquid nitrogen) and held in culture using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FCS) and 1% Penicillin/Streptomycin (P/S). Cells were maintained at 37° C in a 5% CO_2 atmosphere and split every three to four days with 3ml 0.05% Trypsin-EDTA after washing with phosphate buffered saline (PBS). After incubation, trypsinization was stopped by adding DMEM+10% FCS+1% P/S (culture medium) and the collected cell suspension was centrifuged at 1000 rpm for 5 min. The pellet was resuspended in 1 ml culture medium and depending on the cell number split from 1:5 to 1:20.

Table 8: Cell lines used for cell culture based analyses.

CELL LINE	REFERENCE	CHARACTERISTICS
HT-29	ATCC® HTB-38™	Organism: human Tissue: colon Cell Type: epithelial Morphology: adherent
LS174T	ATCC® CL-188™	
HCT116	ATCC® CCL-247™	
DLD-1	ATCC® CCL-221™	
MKN-45	[159]	Organism: human Tissue: stomach Cell Type: epithelial Morphology: adherent

3.2.2. Infection of cells with *H. pylori*

On day -2, adherent cells were trypsinized as described in 1. and counted using the Neubauer hemacytometer cell counting chamber. Therefore, 10 µl of the resuspended pellet was added to 90 µl trypan blue solution and the mean of the counted cells of 4 quarters was adjusted to the desired cell number (100.000 - 250.000) and seeded into 24-well plates in 500 µl culture medium.

On day -1, the culture medium of the seeded cells was changed into antibiotic-free medium: DMEM+10% FCS.

On the day of infection, cells were counted again using the Neubauer chamber and, after changing the medium into DMEM+10% FCS + 10% brain heart infusion (BHI) containing 20% FCS, infected at a multiplicity of infection (MOI) of 20. After 3, 6 and 24 hours, supernatants were collected, centrifuged at 13.000 rpm for 5 min. and frozen at -20° for later analysis. After washing the adherent cells with 500 µl PBS, cells were lysed using 100 µl SDS lysis buffer, ultrasound sonicated for 10 min., heated to 95° for 10 min and then frozen at -20° for later analysis.

3.2.3. Preparation of bacteria for infection

Bacterial strains (listed in table 2) were put in culture on Wilkins-Chalgren (WC) Dent agar plates in a microaerophilic atmosphere (5% O2, 10% CO2) on day -5. On day -2, bacteria were split onto new plates using a swab.

On the day of infection, bacteria were collected in 1 ml BHI+20 % FCS with an inoculation loop and the optical density (OD) was measured at 600 nm. Cells were infected at an MOI of 20, against the background that *H. pylori* OD 1 corresponds to $2x10^8$ bacteria.

Table 9: *H. pylori* strains used for infection experiments.

STRAIN	MUTANT	ANTIBIOTIC SELCTION
P12	WT	WC Dent
P12	CagA	WC Dent Kanamycin
P12	HopQ	WC Dent Chloramphenicol
G27	WT	WC Dent
G27	CagA	WC Dent Kanamycin
G27	CagE	WC Dent Kanamycin
G27	HopQ	WC Dent Chloramphenicol
G27	HopQ complemented	WC Dent Chloramphenicol

G27	BabA	WC Dent Kanamycin
G27	SabA	WC Dent Chloramphenicol
G27	BabA + SabA	WC Dent Kanamycin + Chloramphenicol
PMSS1	WT	WC Dent

3.2.4. C57BL/6 human ceacam1+/+ × mouse ceacam1–/– mice

C57BL/6 human *ceacam1*$^{+/+}$ × mouse *ceacam1*$^{-/-}$ mice were kindly provided by Bernhard B. Singer (University Hospital Essen, Essen, Germany) and generated by crossing a human *ceacam1* transgenic line with murine *ceacam1* knock-out mice and backcrossing to C57BL/6 [160, 161].

The human *ceacam1* transgenic line was created using bacterial artificial chromosome (BAC) injection and inserting the BAC clone randomly into chromosome 11, whereas mouse *ceacam1* is known to be on chromosome 7 [162-164].

Mice were housed under specific pathogen free conditions in microisolator cages and fed standard mouse chow.

3.2.5. Primary epithelial cell isolation and culture

Small intestine and colon were harvested from 3-8 weeks old mice. After removing faeces and washing with PBS+10% FCS, villi were removed by applying pressure on the tissue between two glass object slides. After two incubations in 0,5M EDTA and several washing steps with PBS, the crypt fraction was harvested by applying pressure on the tissue with an object slide and filtering the cell suspension through a 70µm cell strainer. Cells were cultured in DMEM F12 + 10%FCS + 1% Glutamax + 1% HEPES + 1%P/S and cultured in coated (with 15µg/cm2 bovine collagen I in 20 mM acetic acid) 12-well plates. Infection of primary epithelial cells were performed as described in section 2.

3.2.6. Flow cytometry based binding assay

On the day of the assay, bacteria, grown and split as described in 3., were collected in 1 ml BHI+20% FCS with an inoculation loop and the measured OD was adjusted to OD 1. 1 µl CFSE was added and incubated at 37° C shaking at 150 rpm for 30 min. In the meantime, cells were trypsinized as described in 1. and seeded into 96-well V-bottom plates at a density of 100.000/well in a volume of 200 µl DMEM+10% FCS. After incubation with CFSE, bacteria were centrifuged at 3.500 rpm and washed twice with 1 ml PBS. After resuspending the washed pellet in 1 ml BHI+20% FCS, the OD was measured again and cells were infected at an MOI of 20 and incubated for 30 min. at 37° C, shaking at 150 rpm. After incubation, cells were centrifuged at 3.000 rpm for 5 min. and washed with 200 µl PBS for four times and then fixed with 0.5% PFA for 5 min. After one more washing step with PBS, cells were acquired with the flow cytometer. The data analysis was conducted with FlowJo software. The gating strategy is shown in figure 5.

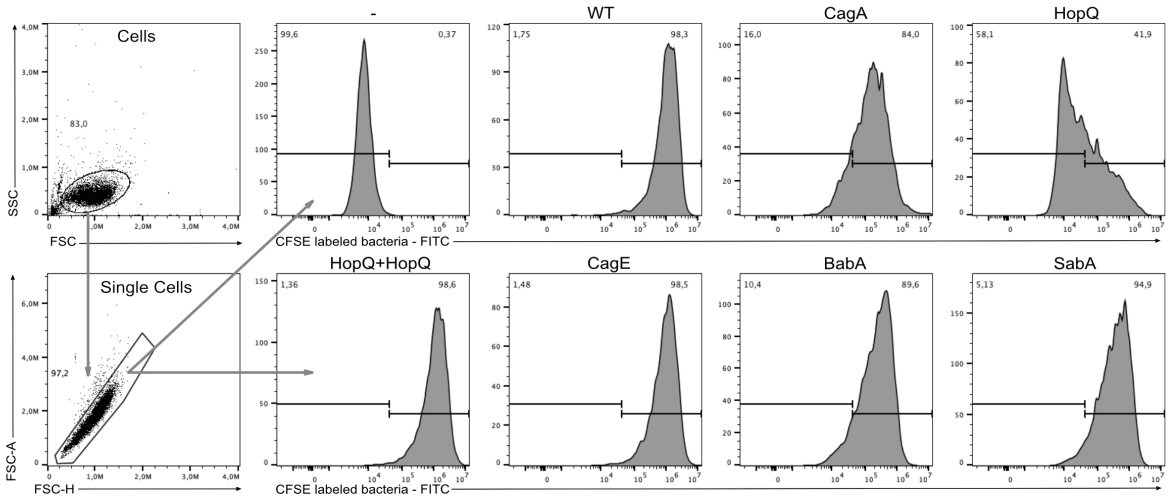

Figure 5: Gating strategy used for binding assay.

3.2.7. Western blot

Cell lysates from infection experiments (described in 2.) were loaded on SDS-PAGE gels, separated proteins were transferred onto a 0.45 µm nitrocellulose membrane and membranes blocked with 5% powdered milk blotting grade in TBS-T before applying primary antibodies diluted in 5% BSA in TBS-T overnight at 4° C. CEACAM expression was detected using

CEACAM1 or CEACAM5 antibody (kindly provided by Prof. B. Singer, University Hospital Essen, Essen, Germany), CagA phosphorylation was detected using a rabbit anti-CagA antiserum (kindly provided by Dr. R. Vogelmann, University Hospital Mannheim II, Mannheim, Germany), ß-Actin, GAPDH, pp65, p65, pSTAT3 and STAT3 were detected with respective antibodies (Table 3). After washing with TBS-T, horseradish peroxidase-conjugated secondary antibodies (Promega, USA) against mouse or rabbit (diluted 1:3000) were applied for 1 hour at room temperature in blocking buffer (5% milk in TBS-T) and after washing, protein bands were detected with Pierce ECL Western Blotting substrate on an Intas imager. Bands were quantified using LabImage 1D. In case of detection of phosphorylated protein, the membrane was stripped with Stripping Buffer and after blocking, primary antibody was applied and proceeded as described above.

Table 10: Antibodies used for protein detection.

TARGET	CLONE	DILUTION	2ND	COMPANY
ß-actin	AC-15	1:5000	rabbit	Sigma
CagA		1:3000	rabbit	provided by Dr. R. Vogelmann, Mannheim
pCagA		1:500	rabbit	provided by Dr. R. Vogelmann, Mannheim
CEACAM1&5		2.5µg/ml	mouse	provided by Prof. B. Singer, Essen
GAPDH	14C10	1:1000	rabbit	Cell Signaling Technologies
p65	D14E12	1:1000	rabbit	Cell Signaling Technologies
pp65	Ser536	1:1000	rabbit	Cell Signaling Technologies
STAT3	124H6	1:1000	rabbit	Cell Signaling Technologies
pSTAT3	D3A7	1:1000	rabbit	Cell Signaling Technologies

3.2.8. ELISA

The secretion of Interleukin-8 (IL-8) was detected using an IL-8 ELISA kit, according to manufacturer's instructions. Briefly, on day 1 NUNC, maxi sorb, flat bottom plates were coated with coating antibody in coating buffer and incubated overnight at 4°C. On day 2 of the assay, after washing and blocking with Assay Diluent, diluted cell supernatants and standard were added and incubated overnight at 4°C. On day 3, detection antibody was added to washed cells

and incubated for 1 hour at room temperature (RT). After washing, Avidin-HRP was incubated for 30 min. at RT, washed again, substrate solution incubated for 15 minutes at RT and finally stop solution added. The signal was measured at 450 nm minus 570 nm wavelength subtraction using Tecan Plate Reader.

3.2.9. RNA isolation, cDNA synthesis and qPCR

RNA was isolated with the MaxWell RSC48 simply RNA tissue kit, including DNAse treatment. RNA concentrations were measured on Nanodrop and adjusted to 1µg/µl. cDNA was synthesized in two steps, first by incubating RNA with random primers at 70° for 5 minutes, followed by applying the following master mix (Reverse Transcriptase negative master mix for controls):

Table 11: cDNA master mix.

REAGENT	VOLUME	VOLUME RT NEGATIVE CONTROL
Nuclease free water	3,75 µl	4,75 µl
dNTPs	1,25 µl	1,25 µl
M-MLV Reverse Transcriptase	1 µl	-
M-MLV 5x RT buffer	5 µl	5 µl

Afterwards, the following conditions were applied:

Table 12: cDNA cycling conditions.

TEMPERATURE	TIME
22°	10:00
50°	50:00
70°	15:00
12°	∞

For qPCR, cDNA and primers were diluted 1:10 in NFW and the following master mix and cycling conditions were applied (Table 13, 14 and 15).

Table 13: qPCR master mix.

REAGENT	VOLUME
Diluted cDNA	4 µl
GoTaq qPCR Master Mix	5 µl
Diluted forward primer	0,5 µl
Diluted forward primer	0,5 µl

Table 14: qPCR cycling conditions.

TEMPERATURE	TIME
95°	5:00
95°	0:10
60°	0:30
Go to step 2	39x
65°	0:05
95°	0.5°/cycle
12°	∞

Table 15: qPCR primers used within this study.

GENE	FORWARD SEQUENCE	REVERSE SEQUENCE
GAPDH	GAA GGT GAA GGT CGG AGT	GAA GAT GGT GAT GGG ATT TC
CEACAM1	GCA ACA GGA CCA CAG TCA AG	CCA GGG CTA CTG CTA TCA G
CEACAM3	CAG CTC TGC CTT CTC GAT G	CTC ATA GAT GGA AGC TGC TG
CEACAM5	AGG CCA ATA ACT CAG CCA GT [165]	GGC TTG GGC AGC TCC GC
CEACAM6 [166]	CGT CGG CAT CAC GAT TGG	TGG GAT TGG AGG AGC TAG AAG
CEACAM3	CAG CTC TGC CTT CTC GAT G	CTC ATA GAT GGA AGC TGC TG

3.2.10. Statistics

GraphPad Prism was used for graphical presentation of results and to determine statistical significances with two-way ANOVA with Tukey's multiple comparisons test. Results were considered significant with a p value under 0.05 and shown as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

Figures were constructed with Affinity Photo and Affinity Designer.

4. Results

4.1. Colon epithelial cells express different levels of CEACAM receptors

The presence of *H. pylori* in the colon has been suggested by several studies [167-169]. However, whether the bacterium can interact with colon cells and induce any downstream signaling remains unknown.

To investigate whether *H. pylori* can bind to colon epithelial cells, the expression of CEACAM receptors on different colon cancer cells was analyzed, since binding of *H. pylori* could be mediated by an interaction between the outer membrane protein HopQ and CEACAMs expressed on epithelial cells.

Gene expression analysis revealed that CEACAM1, 5 and 6 are expressed on HT-29 and LS174T cells, CEACAM5 additionally on DLD-1 cells, whereas none of the tested CEACAMs were found to be expressed on HCT-116 cells. CEACAM3 levels were detected at very low levels in HT-29 cells (Figure 6).

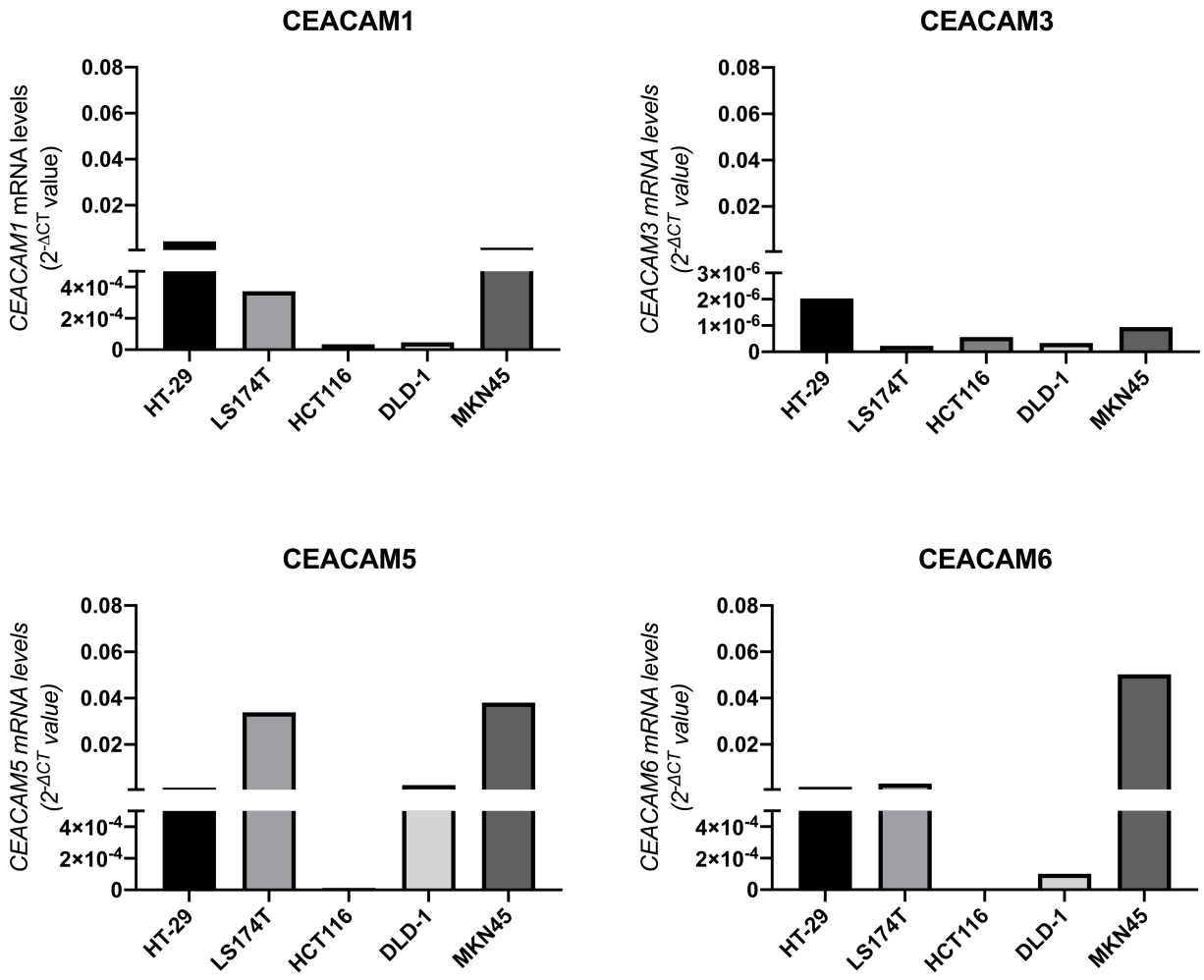

Figure 6: Screening of CEACAMs expressed on human colon cells.

Gene expression levels of *CEACAM1, 3, 5* and *6* were determined via qPCR. Lysed cells were tested as duplicates and normalized to *GAPDH* ($2^{-\Delta CT}$-Value). The gastric cell line MKN45 served as a control.

Next, the protein levels of CEACAM1 and CEACAM5 were confirmed by western blot using an antibody detecting both CEACAMs. HT-29 and LS174T cells were found to express high protein levels, whereas HCT116 cells did not show any CEACAM1 or CEACAM5 expression. DLD-1 only showed a weak signal (Figure 7), which is in concordance with the gene expression analysis (Figure 6).

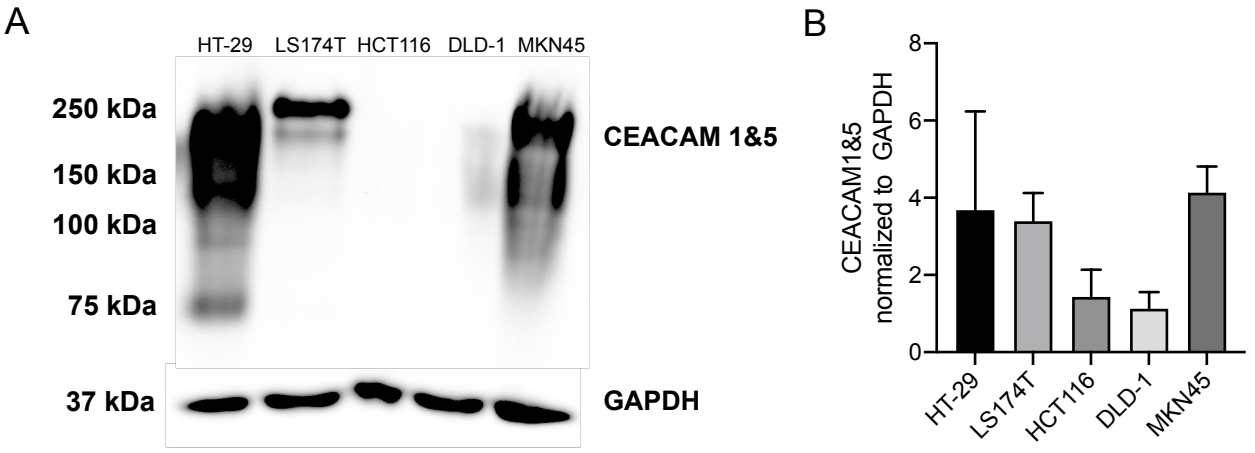

Figure 7: CEACAM1 and 5 expression on human colon cell lines.

(A) Protein levels of CEACAM1 and 5 in lysed cells were analyzed by Western blot. GAPDH was used as loading control. One representative experiment of three independent experiments is shown. (B) Quantifications of CEACAM 1 and 5 expression, normalized to GAPDH, are shown as mean ± SD.

In a next step, whether *H. pylori* infection altered the expression of CEACAM was addressed. HT-29 (Figure 8A) and LS174T cells (Figure 8B) showed a homogenous expression of glycosylated CEACAM (upper smear), which remains stable over time and is not influenced by infection with *H. pylori* knockout strains for the virulence factor CagA and OMP HopQ. *H. pylori* infection seemed to enhance CEACAM expression on HCT116 cells after 3 hours of infection, while it remained at similar levels over time and strain used for infection (Figure 8C). DLD-1 cells expressed CEACAM only after infection but the protein seemed not to be glycosylated (lower band) (Figure 8D).

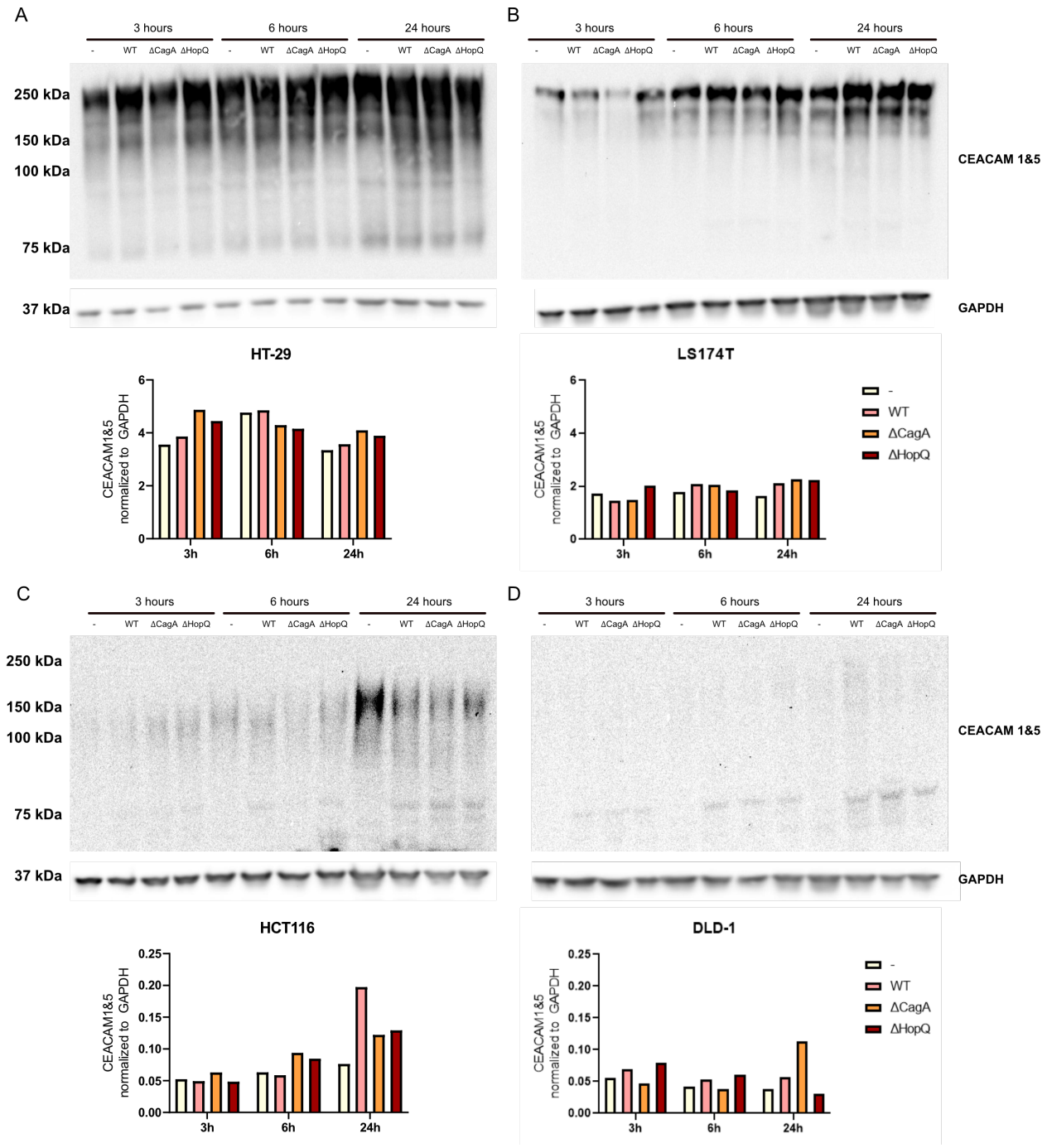

Figure 8: *H. pylori* infection increases CEACAM1 and 5 expression in human colon cell lines.

Colon cell lines were infected with *H. pylori* strain P12 and isogenic mutant strains ΔCagA, ΔHopQ) at an MOI of 20 for 3, 6 and 24 hours. Expression levels of CEACAM1 and 5 in (A) HT-29, (B) LS174T, (C) HCT116 and (D) DLD-1 cell lysates were analyzed by Western blot. GAPDH was used as loading control. Quantifications of CEACAM 1 and 5 expression levels were normalized to GAPDH.

In addition to its expression in colon cell lines, CEACAM expression in primary epithelial cells isolated from the intestine of C57BL/6 human ceacam1$^{+/+}$ × mouse ceacam1$^{-/-}$ mice (CEACAM transgenic mice) was assessed. C57BL/6 wildtype mice were used as a control, as they do not express human CEACAMs. Human CEACAM1 and 5were expressed in the intestine of CEACAM transgenic mice. Infection with *H. pylori* WT increased their expression, whereas CagA and HopQ mutant strains only had a mild effect on the expression (Figure 9).

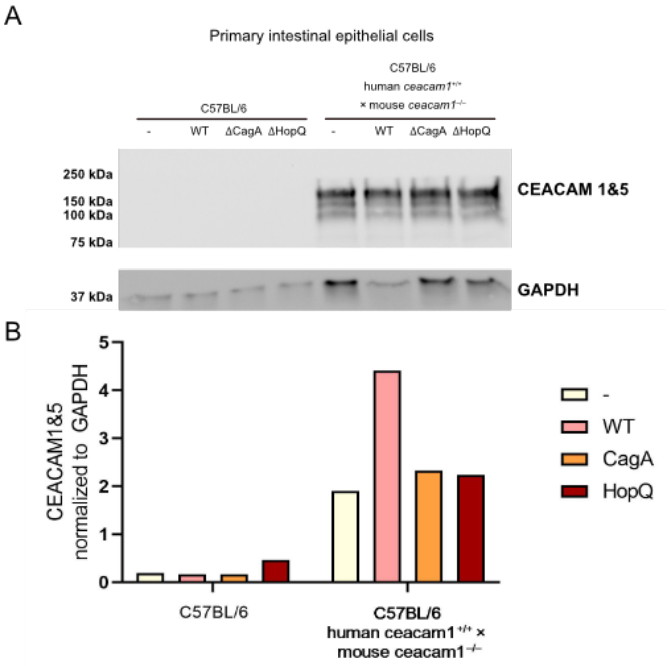

Figure 9: CEACAM1 and CEACAM5 expression is increased upon *H. pylori* infection in intestinal primary epithelial cells from CEACAM transgenic mice.

(A) Primary epithelial cells isolated from intestine were infected with *H. pylori* strain G27 and isogenic mutant strains, ΔCagA, ΔHopQ) at an MOI of 20 for 6 hours. Expression levels of CEACAM1 and CEACAM5 of lysed cells were analyzed by Western blot. GAPDH was used as loading control. (B) Quantifications of CEACAM 1 and 5 expression were normalized to GAPDH.

4.2. *H. pylori* binds to colon epithelial cells

To assess the binding ability of *H. pylori* to CEACAM expressing colon cells, a flow cytometry based binding assay was performed. *H. pylori* was indeed able to bind to colon epithelial cells.

Reduced binding was observed for strains deficient for outer membrane proteins HopQ and SabA and surprisingly also for the CagA mutant strain (Figure 10).

In detail, H. pylori CagA and HopQ knockout strains showed impaired binding to cell lines with high expression of CEACAMs, namely HT-29 and LS174T cells, whereas the binding ability was reconstituted when HopQ was complemented (Figure 10A and 10B). In contrast, *H. pylori* strains devoid in the virulence factor CagA, as well as outer membrane protein SabA displayed impaired binding to cell lines HCT116 and DLD-1, expressing low or no CEACAMs, when infected with (Figure 10C and 10D), indicating that in the absence of CEACAMs, binding might be mediated via SabA-Lewis antigens interaction. The gastric cell line MKN45 was used as a control and confirmed that in the stomach, HopQ deletion leads to a lower binding ability of *H. pylori* (Figure 10E).

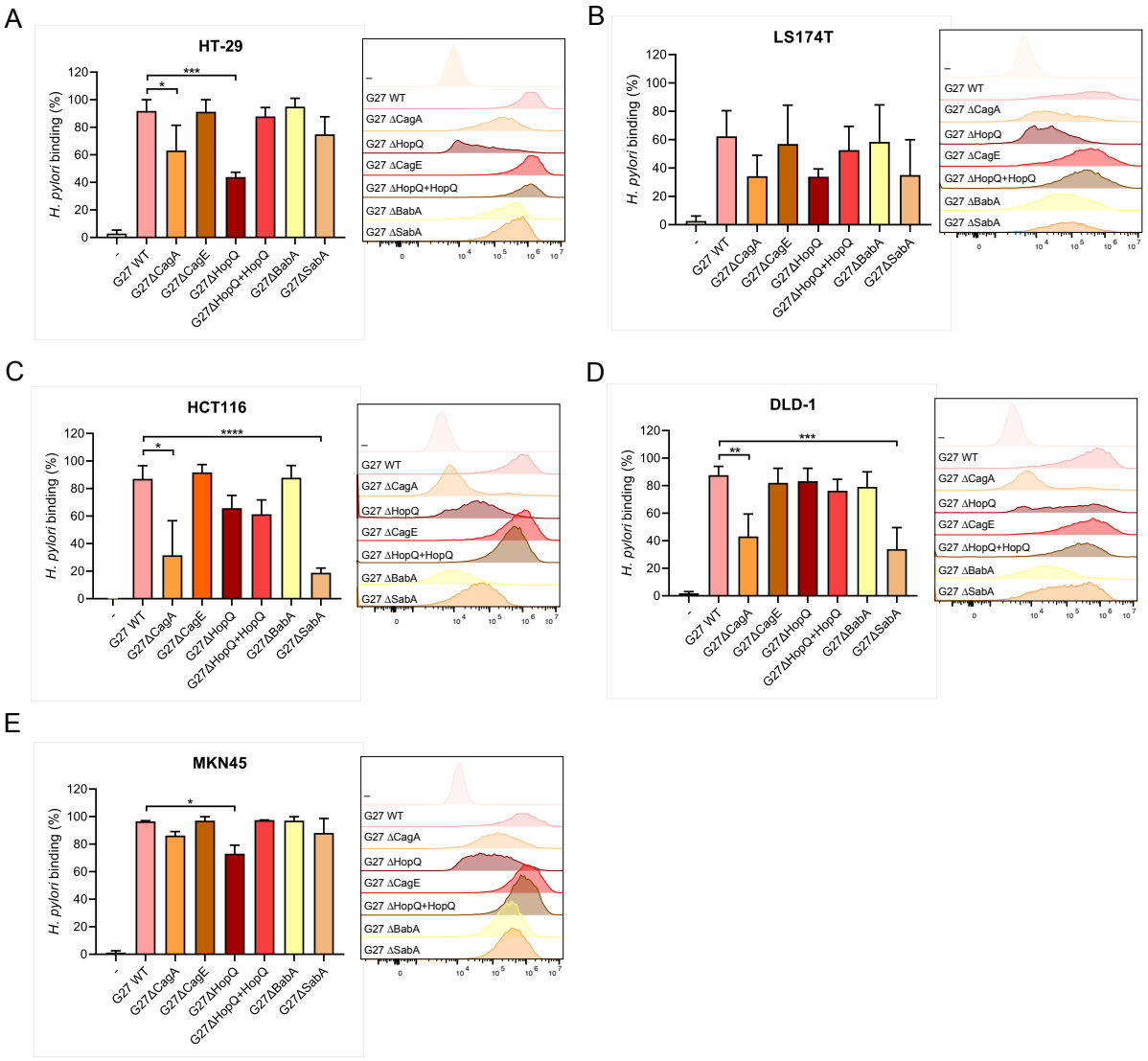

Figure 10: Binding ability of *H. pylori* to colon cells.

The cell lines (A) HT-29, (B) LS174T, (C) HCT116, (D) DLD-1 and (E) MKN45 were infected with CSFE labeled *H. pylori* strain G27 and isogenic mutant strains, ΔCagA, ΔCagE, ΔHopQ, ΔHopQ+HopQ, ΔBabA and ΔSabA for 30 minutes at an MOI of 20 and the binding ability was assessed by flow cytometry. Representative stacked histograms of binding are shown. Graphs show percentage (%) of *H. pylori* binding to cells detected by flow cytometry. Results are shown as mean ± SD, n= 3. Two-way ANOVA with multiple comparisons, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

Furthermore, the binding ability of *H. pylori* to primary epithelial cells isolated from the intestine of CEACAM transgenic and C57BL/6 mice was assessed. The assays revealed that *H. pylori* was able to bind to intestinal epithelial cells, however, neither significant differences between

CEACAM transgenic and C57BL/6 mice, nor between different mutants of *H. pylori* were detected (Figure 11).

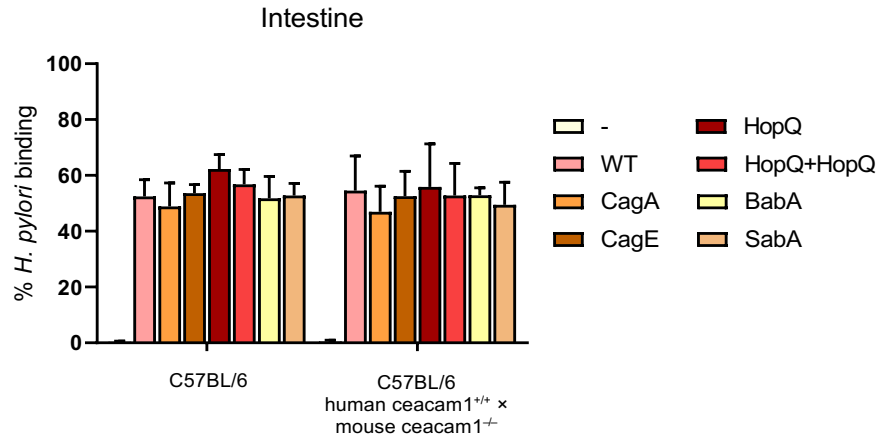

Figure 11: Binding ability of *H. pylori* to murine primary intestinal cells.

Primary epithelial cells were isolated from the small intestine of C57BL/6 human ceacam1+/+ × mouse ceacam1–/– mice and Control C57BL/6 mice and infected with CSFE labeled *H. pylori* strain G27 and the isogenic mutant strains ΔCagA, ΔCagE, ΔHopQ, ΔHopQ+HopQ, ΔBabA and ΔSabA) for 30 minutes at an MOI of 20. The binding ability was assessed by flow cytometry. Graphs show % of *H. pylori* binding to cells detected by flow cytometry. Results are shown as mean ± SD, experiments, n= 3.

Together, these results confirm that colon cells express different CEACAM receptors, and that *H. pylori* infection alters this expression. Furthermore, the binding assays revealedthat *H. pylori* can bind to colon cells as well as primary epithelial intestinal cells, however HopQ-CEACAM interaction seems not to be the only mode of binding.

4.3. H. pylori CagA is translocated into colon epithelial cells

In the stomach, a crucial step after colonization and involved in subsequent pathogenesis of *H. pylori* infection is the translocation of virulence factors and activation of signaling pathways.

An important *H. pylori* virulence factor related to gastric pathogenesis is the oncogenic protein CagA, which is translocated into gastric epithelial cells through a T4SS. Similar to what occurs in gastric cells, *H. pylori* was able to detect a successful translocation of CagA into colon epithelial

cells. CagA translocation (detected by phosphorylated CagA) remained stable over time and seemed to be independent of HopQ, in contrast to gastric cell lines, where translocation of CagA is HopQ-dependent (Figure 12).

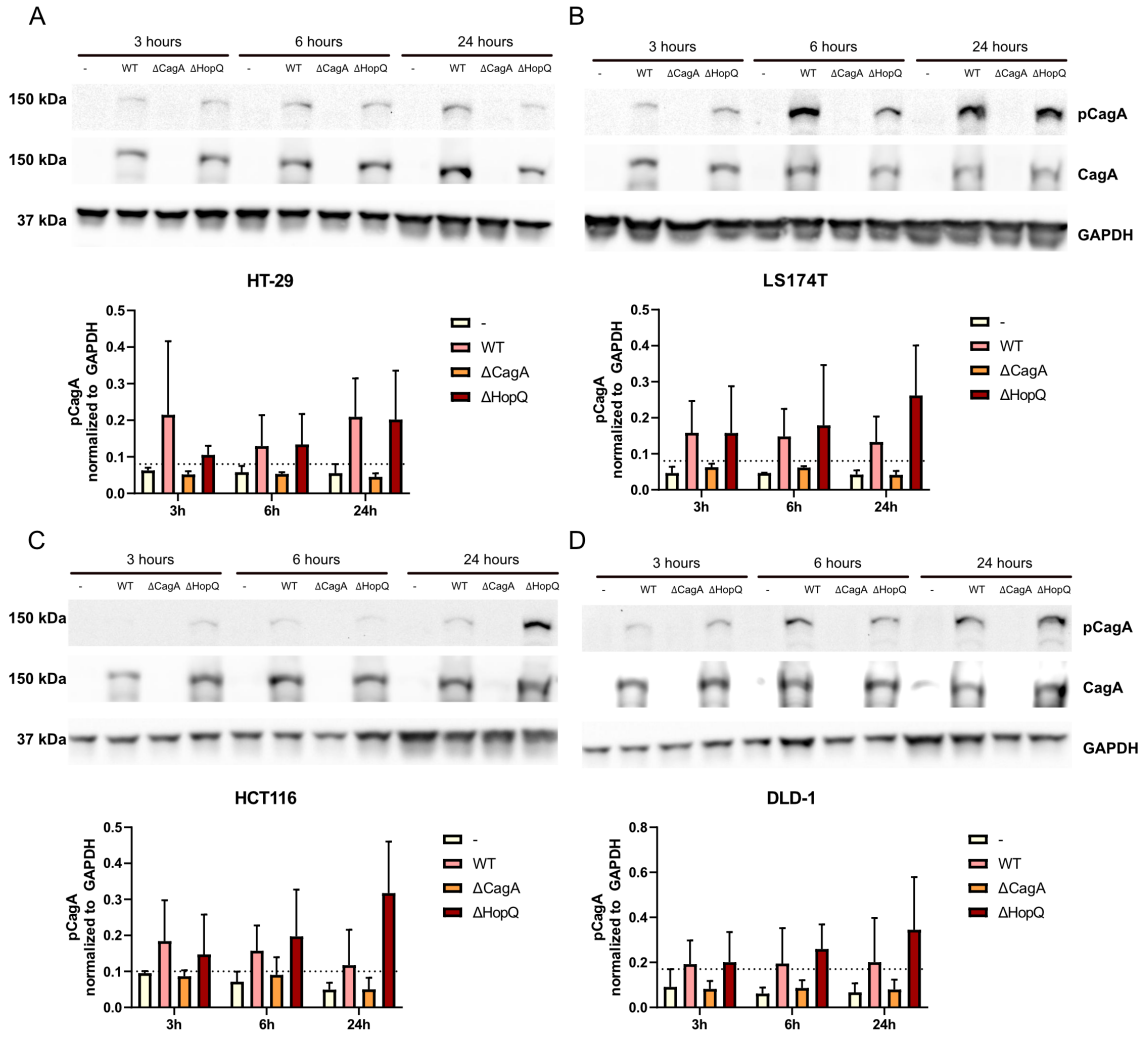

Figure 12: *H. pylori* CagA is translocated into colon epithelial cells

Colon cell lines (A) HT-29, (B) LS174T, (C) HCT116 and (D) DLD-1 were infected with *H. pylori* strain P12 and the isogenic mutant strains ΔCagA, ΔHopQ at an MOI of 20 for 3, 6 and 24 hours. Expression levels and phosphorylation of CagA in cell lysates were analyzed by Western blot. GAPDH was used as loading control. One representative experiment of three independent experiments is shown. Quantification of pCagA, normalized to GAPDH, is shown as mean ± SD.

To confirm that CagA can be translocated to colon cells independently of the presence of HopQ, an additional strain was included as control. This strain is deficient of CagE, a structural part of the T4SS. The results show that CagA is not translocated in the absence of a functional T4SS (lack of CagE), as expected, while in the absence of HopQ, CagA translocation is still detected (Figure 13).

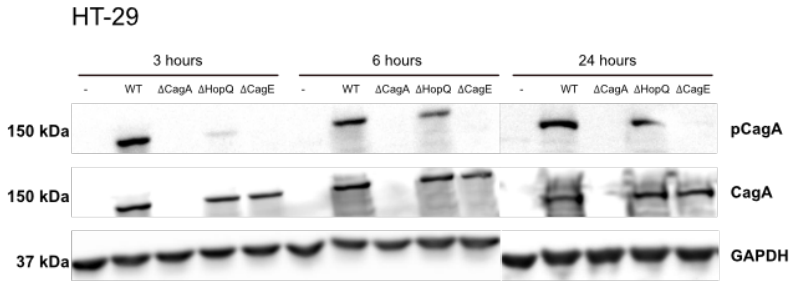

Figure 13: Translocation of the virulence factor CagA into colon cells depends on the T4SS.

HT-29 cells were infected with *H. pylori* strain G27 (WT, ΔCagA, ΔHopQ, ΔCagE) at an MOI of 20 for 3, 6 and 24 hours. Expression levels and phosphorylation of CagA in lysed cells were analyzed by Western blot. GAPDH was used as loading control.

Next, the ability of *H. pylori* to translocate CagA into primary cells from CEACAM transgenic mice and C57BL/6 mice was assessed. CagA was not translocated into primary cells of the intestine, as no phosphorylated CagA was observed in infected cells (Figure 14), despite the successful binding of H. pylori to these cells (Figure 11).

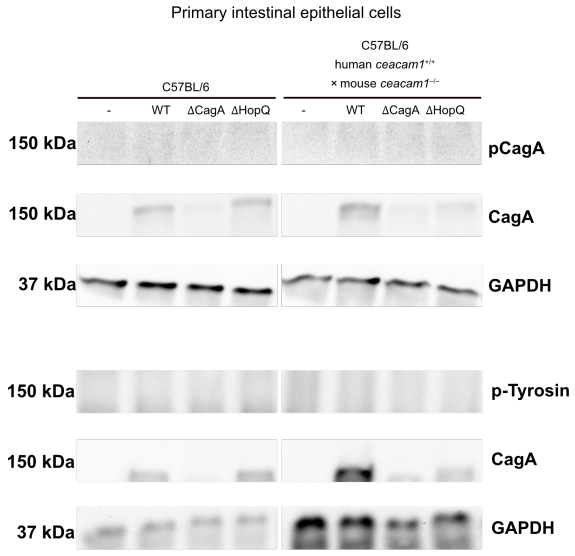

Figure 14: CagA does not translocate into murine primary intestinal cells.

Primary cells were infected with *H. pylori* strain G27 (WT, ΔCagA, ΔHopQ,) at an MOI of 20 for 6 hours. Expression levels and phosphorylation of CagA (pCagA and pTyrosin) in lysed cells were analyzed by Western blot. GAPDH was used as loading control.

To summarize, *H. pylori* is able to translocate its virulence factor CagA into colon cancer cells. This depends on a functional T4SS but is independent of HopQ. However, *H. pylori* fails to translocate CagA into primary epithelial cells isolated of the intestine from CEACAM transgenic mice and C57BL/6 mice.

4.4. *H. pylori* activates NF-κB signaling in colon cells

Once attached to the gastric epithelium and able to translocate virulence factors, the next step of *H. pylori's* pathogenesis cascade is the activation of proinflammatory signaling pathways. The NF-κB signaling pathway is known to be the first pathway activated in the stomach upon *H. pylori* infection. Therefore, we investigated whether *H. pylori* infection leads to activation of this signaling pathway in colon epithelial cells and whether OMPs and virulence factors are involved. Infected colon cells showed a higher activation of the NF-kB pathway, as detected by phosphorylation of p65, compared to non-infected cells. This activation was not observed when cells were infected with HopQ deficient strains, while it was increased after infection with CagA mutant strains. However, these effects varied over time and were different for the cells tested. (Figure 15).

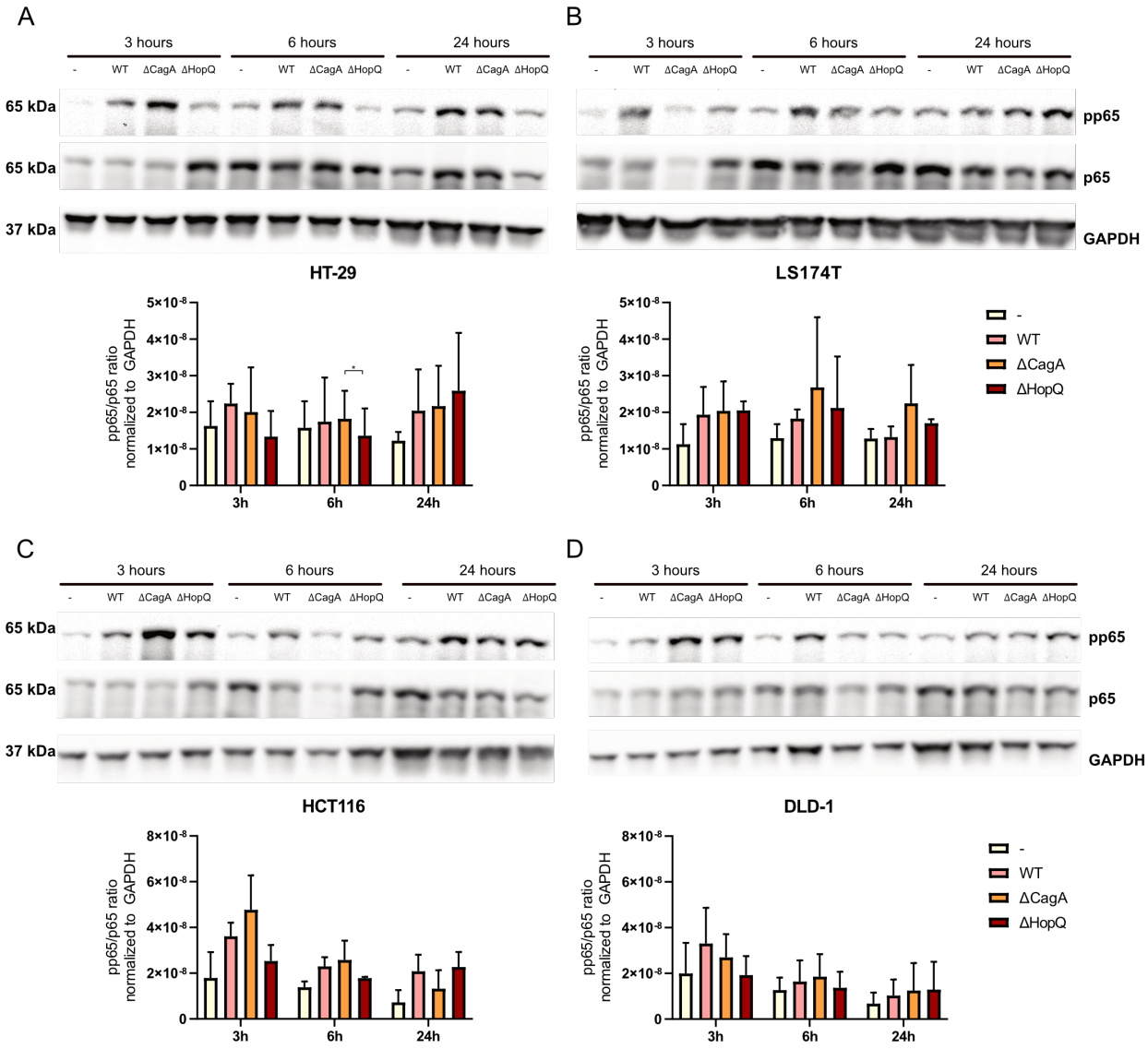

Figure 15: NF-κB signaling is activated upon *H. pylori* infection.

(A) HT-29, (B) LS174T, (C) HCT116 and (D) DLD-1 cells were infected with *H. pylori* strain P12 and the isogenic mutant strains ΔCagA, ΔHopQ at an MOI of 20 for 3, 6 and 24 hours. Expression levels and phosphorylation of p65, a marker of canonical NF-κB signaling, in lysed cells were analyzed by Western blot. GAPDH was used as loading control. One representative experiment of three independent experiments and quantification of pp65, normalized to p65 and GAPDH, are shown. Results are shown as mean ± SD. Significant differences were determined by 2way ANOVA with multiple comparisons, * = $p<0.05$.

Secretion of IL-8 is known to be a marker for NF-κB activation and induction of inflammation. The secretion of this inflammatory chemokine was evaluated via ELISA. An elevated secretion of IL-8 in *H. pylori* infected colon cells was observed, which was dependent on the presence of the outer membrane protein HopQ. This effect was most prominently detected in HT-29 and LS174T cells (Figure 16).

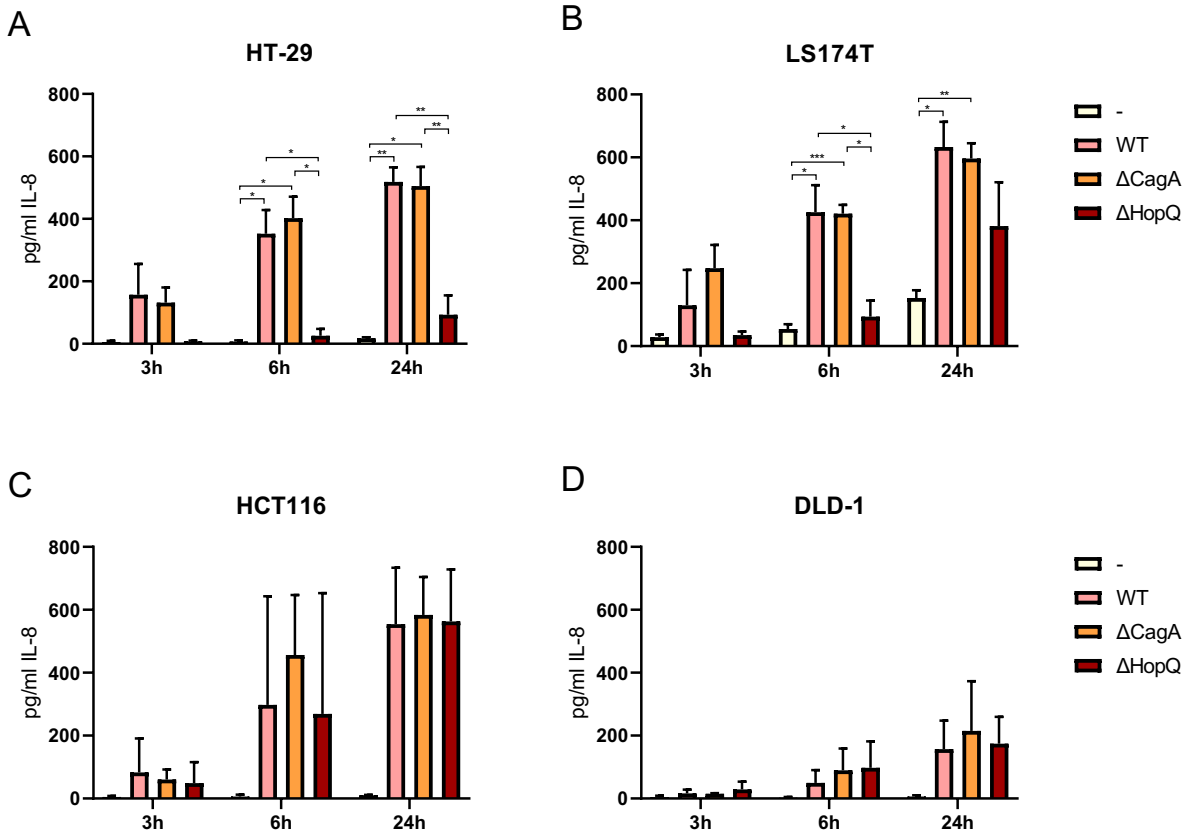

Figure 16: *H. pylori* infection induces secretion of IL-8 in colon cells.

(A) HT-29, (B) LS174T, (C) HCT116 and (D) DLD-1 cells were infected with *H. pylori* strain P12 and the isogenic mutant strains ΔCagA, ΔHopQ at an MOI of 20 for 3, 6 and 24 hours and IL-8 was determined via ELISA in collected supernatants. n= 3. Results are shown as mean ± SD. Statistics determined by 2way ANOVA with multiple comparisons, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

However, activation of NF-κB signaling was not detected in intestinal primary epithelial cells of CEACAM transgenic mice and Control C57BL/6 mice, neither under basal conditions nor upon *H. pylori* infection (Figure 17).

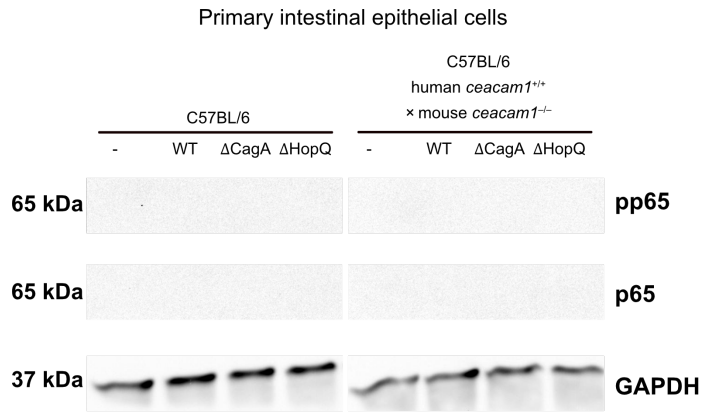

Figure 17: NF-κB signaling is not detectable in murine primary intestinal cells.

Primary epithelial cells from intestine infected with *H. pylori* strain G27 and the isogenic mutant strains ΔCagA, ΔHopQ at an MOI of 20 for 6 hours. (A) Expression levels of pp65 and p65 in lysed cells were analyzed by Western blot. GAPDH was used as loading control.

Together, *H. pylori* infection results in activation of NF-κB signaling and subsequent IL-8 secretion in colon cancer cells. This depends on the presence of the OMP HopQ.

4.5. *H. pylori* alters STAT3 signaling in colon cells

STAT3 is known to play an important role in colorectal carcinogenesis and is known to be activated upon *H. pylori* infection in the stomach. A tendency towards increased activation of STAT3 upon infection was found, which seemed to be dependent on the presence of the virulence factor CagA and the OMP HopQ. However, these results were not significant and showed variances among the tested colon cells and the different time points (Figure 18).

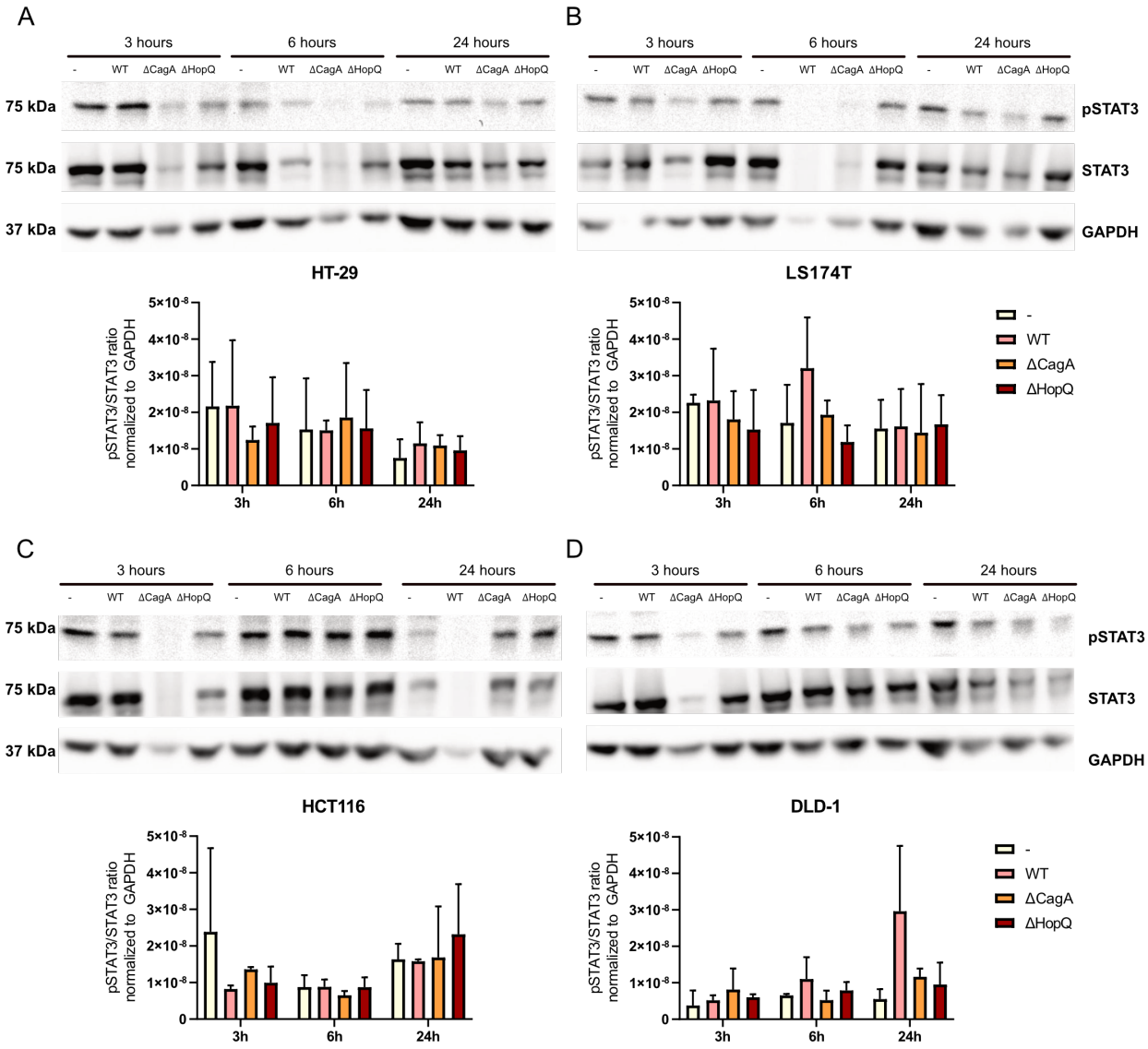

Figure 18: STAT3 signaling in colon cell lines upon *H. pylori* infection.

(A) HT-29, (B) LS174T, (C) HCT116 and (D) DLD-1 cells were infected with *H. pylori* strain P12 and the isogenic mutant strains ΔCagA, ΔHopQ at an MOI of 20 for 3, 6 and 24 hours. Expression levels and phosphorylation of STAT3 of lysed cells were analyzed by Western blot, GAPDH was used as loading control. One representative experiment of three independent experiments is shown. Quantification of pSTAT3, normalized to STAT3 and GAPDH, is shown in the right panels. Results are shown as mean ± SD.

STAT3 signaling was slightly activated in *H. pylori* infected CEACAM transgenic mice derived intestinal cells. This activation depended on the virulence factor CagA and the OMP HopQ.

However, *H. pylori* infection had no effect on STAT3 signaling in primary cells derived from wild type C57BL/6 mice (Figure 19).

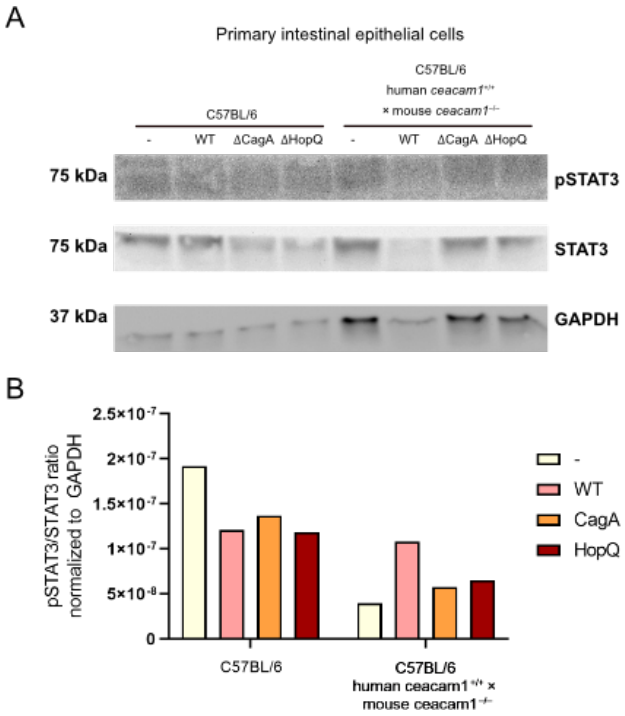

Figure 19: *H. pylori* infection affects STAT3 activation in murine primary intestinal cells.

Primary epithelial cells were isolated from intestine and infected with *H. pylori* strain G27 and the isogenic mutant strains ΔCagA, ΔHopQ at an MOI of 20 for 6 hours. (A) Expression levels of pSTAT3 and STAT3 in lysed cells were analyzed by Western blot. GAPDH was used as loading control. (B) Quantifications of pSTAT3, normalized to STAT3 and GAPDH, are shown.

Altogether, these results indicate that there is no significant and consistent effect of *H. pylori* infection on the activation of STAT3 signaling in colon epithelial cells.

5. Discussion

H. pylori infection has been discussed as a possible risk factor for CRC development by a number of studies. However, the molecular mechanisms involved in this association have not been reported yet. One possible mechanism could be a direct interaction of H. pylori with colonic epithelial cells. The aim of this study was to explore the binding capacity of *H. pylori* to colon cancer cells as well as primary colon epithelial cells, and the role of CEACAM receptors in this context. Furthermore, *H. pylori* induced pathogenesis *in vitro*, including the translocation of effector molecules (CagA), inflammatory response to infection (IL-8 secretion) and subsequent downstream signaling (NF-κB and STAT3) was assessed.

H. pylori HopQ interacts with several CEA antigen family members, including CEACAM1, CEACAM3, CEACAM5, and CEACAM6 on the epithelial surface. This interaction has shown to be a prerequisite for successful translocation of CagA in gastric cells [38, 39]. In colon cancer cells HT-29 and LS174T, CEACAM1, CEACAM5 and CEACAM6 were stably expressed, in contrast to CEACAM3. In HCT-116 and DLD-1 cells, however, CEACAM expression was observed only upon induction by *H. pylori* infection of the cells. The expression patterns under naïve conditions can be attributed to the known tissue-specific expression of CEACAMs: CEACAM1 is known to be widely expressed across epithelial cells, CEACAM5 is mainly expressed on epithelial cells and CEACAM6 on both epithelial cells and neutrophils. CEACAM3 expression, however, is restricted to neutrophils, which explains the observed lack of CEACAM3 expression in colon cancer cells [170, 171]. In healthy individuals, opportunistic bacteria are kept from adhering to intestinal cells by low expression of CEACAMs [172]. However, an inflammatory environment can induce their upregulation, which has been shown for inflammation induced by *Escherichia coli* in Crohn's Colitis [173]. Similarly, *H. pylori* infection of colon cancer cells resulted in upregulation of CEACAM receptors. Besides *H. pylori* and *E. coli*, this notion of pathogens upregulating their host receptors has been described for *Haemophilus influencae*, which was shown to induce the expression of its receptor, ICAM-1, on human respiratory epithelial cells [174]. In addition to inflammation, epithelial disorders and malignancies can lead to differential expression of CEACAMs: CEACAM1, 5 and 6 e.g., are not detected in normal stomach but are upregulated in

gastritis and gastric cancer [38, 39]. As a result, those CEACAMs are widely used as biomarkers in gastrointestinal cancers [170, 175].

In order to substantiate the *in vitro* findings, primary epithelial cells were isolated from C57BL/6 human ceacam1$^{+/+}$ × mouse ceacam1$^{-/-}$ mice. These mice have been shown to exhibit inducible human CEACAM1 expression on CD8+ T-cells [160] and might therefore be a suitable model to study *H. pylori's* interaction with human CEACAMs in mice. A stable baseline expression of human CEACAM1 and CEACAM5 in the intestine of transgenic CEACAM mice was observed. Even in the in-vivo model, levels were further increased upon infection with *H. pylori*, substantiating the upregulation of CEACAM expression in response to *H. pylori* infection in the colon. The OMP HopQ and the virulence factor CagA seem to play a role in this inducible CEACAM expression, as infection with respective knockout strains led to similar expression levels as observed in non-infected primary cells. In this context, CagA might have a role in regulating CEACAMs via downstream mechanisms that are induced upon its translocation. For example, a study in colon biopsies of IBD patients showed an increase of CEACAM expression upon stimulation with a proinflammatory cytokine cocktail including TNFα, IL-1β and IFNγ [176]. Human studies have shown that CagA positive strains induce significantly more pro-inflammatory cytokines compared to CagA negative strains [88]. Therefore, it can be speculated that CagA is involved in the regulation of CEACAM expression via proinflammatory downstream mechanisms induced upon translocation. Furthermore, the reduced CEACAM expression in the absence of HopQ might be related to a ligand (HopQ)-mediated induction of the receptor (CEACAM). This phenomenon has been observed in gastric cell lines, where, similarly to the results observed here, the infection with *H. pylori* WT strains induced the expression of CEACAMs, whereas strains devoid of HopQ did not result in an increase of expression [177]. However, further research is needed to determine the exact role and mechanism of HopQ and CagA mediated upregulation of CEACAM expression, characterizing cytokines as well as signaling pathways involved in this process.

These findings indicate that *H. pylori* might employ human CEACAMs as receptors to bind to colon epithelial cells via its OMP HopQ, and that this HopQ-CEACAM interaction is important for subsequent pathogenesis. Furthermore, the upregulation of CEACAM receptors upon

infection might indicate that *H. pylori* is able to establish a niche in the colon by promoting sufficient receptor abundance for attachment to epithelial cells.

In order to investigate whether *H. pylori* is able to attach to colon cells and to determine the importance of different adhesins and virulence factors in this process, binding assays in colon cell lines as well as primary epithelial cells were performed.

H. pylori successfully attached to colon cancer cells as well as primary epithelial cells isolated from intestine of CEACAM transgenic mice. The adhesion of *H. pylori* to stomach epithelial cells is mediated by distinct surface proteins engaging with host receptors, such as BabA and SabA interacting with Lewis antigens [44] or HopQ interacting with CEACAM receptors [38, 39]. *H. pylori* binding to colon cells was reduced in HopQ- and SabA-mutant strains. Furthermore, a decreased binding in the absence of the virulence factor CagA was found, which might relate to the previously described reduced CEACAM expression upon CagA knockout, therefore reducing receptor availability for HopQ and resulting in less binding. In contrast, BabA did not seem to play an important role in *H. pylori* binding to colon cells. The gastric cancer cell line MKN45 served as a control and confirmed that in the stomach, *H. pylori* binding is strongly mediated by HopQ-CEACAM interaction, whereas the lack of other OMPs BabA and SabA did not result in decreased binding, which however can be attributed to the high basal CEACAM expression of this cell line. Indeed, it has been shown that also in the stomach, binding in gastric cancer cells with low basal CEACAM expression was less affected by HopQ knockout [177]. Taken together, these data support a connection between basal CEACAM expression and dependence of binding on HopQ, and confirm the importance of the interaction of bacterial HopQ with host CEACAMs for binding to epithelial cells. However, in case of low basal CEACAM expression in colon cells, the bacterium was binding in a SabA dependent manner, which suggest that *H. pylori* adheres to colon cells via distinct mechanisms and adapts its mechanism of adherence to the presence of cell surface receptors.

Importantly, the successful binding ability of the bacterium to colon epithelia suggests a possible direct interaction of *H. pylori* with human colon cells and shows that the prerequisite of adherence to exert its cancer promoting effects is given.

After successful attachment of *H. pylori* to the stomach mucosa, a crucial step for subsequent pathogenesis is the translocation of virulence factors [178, 179]. Among these, the effector protein CagA, which is transferred via the T4SS, induces inflammatory responses and triggers pathogenic signaling cascades and is therefore pivotal for *H. pylori*-induced pathogenesis. The functionality of the T4SS is affected by OMPs. For instance, BabA, enhances its activity and HopQ was identified as an important cofactor for its function [180, 181].

H. pylori was able to translocate the virulence factor CagA into colon cancer cells. This translocation was mediated through the T4SS and independent of HopQ. All tested cell lines showed a consistent phosphorylation of CagA, regardless of their basal or *H. pylori* induced CEACAM expression. This finding is controversial, as in AGS stomach cells, HopQ-CEACAM interaction is a prerequisite for translocation [38, 39]. CagA translocation requires the host cell $\alpha_5\beta_1$ Integrin as a receptor for the T4SS and for subsequent phosphorylation and functionality of the effector protein [182]. The required adherence to the colonic epithelial cells might, however, also be mediated by other outer membrane proteins of the Hop family, such as BabA or SabA. In line with the previously described ability of *H. pylori* to adapt the mechanism of adherence on the presence of cell surface receptors to colon cells, those findings could suggest a less prominent role of HopQ-CEACAM interaction in *H. pylori*'s attachment to colon epithelia as compared to the stomach.

CagA translocation was not detected in primary epithelial cells isolated from murine intestine, although CEACAM expression and *H. pylori* binding in those cells were successfully detected.

One possible explanation could be the polarization of these epithelial cells. In contrast to non-polarized cell lines, where host cell receptors are easily accessible, in polarized cells integrins required for T4SS pilus formation are located at the basolateral membrane. In order to engage with those receptors and to successfully translocate its effector proteins, *H. pylori* has to overcome tight and adherence junctions. Studying E-Cadherin positive gastric cell lines, it has been shown that for *H. pylori* this is achieved by the bacterial serine protease HtrA [183]. In line with that, it has been shown that *Campylobacter jejuni* was using the same protease to overcome tight junctions in order to invade gut epithelial cells [184, 185]. However, none of these studies have assessed the ability of bacteria to overcome cell-cell junctions in primary epithelial cells.

Together, these results show the ability of *H. pylori* to inject its effector molecules into human colon cancer cells, however, it is not clear, whether *H. pylori* is able to do so in the in vivo situation. Although the mouse model used within this study does express human CEACAMs, other host specific factors, which are important for CagA translocation, may not be present, and therefore this host might lack other necessary conditions for this step in *H. pylori* pathogenesis. Further studies are required to determine the ability of *H. pylori* to attach to the gut epithelia and to assess whether the bacterium is able to exert its hallmark functions via the T4SS there. Especially, the knowledge whether CagA can be delivered into the colonic cell might be of high clinical relevance, considering the association of CagA seropositivity with an increased risk for CRC [186].
A crucial step in *H. pylori* induced pathogenesis is the modulation of the host immune response and the initiation of downstream signaling. In the gastric mucosa, successful binding of *H. pylori* to epithelial cells and subsequent colonization is followed by the activation of the NF-**κ**B pathway. This first host epithelial response is characterized by the activation of the innate immune response via pro-inflammatory cytokines and chemokines such as IL–6 and IL-8 and. Even though CagA translocation, mediated by the T4SS, is known to be important for perturbations in host immune responses and activation of signaling pathways, the activation of NF-**κ**B does not fully depend on CagA translocation. Instead, the OMP HopQ has been reported to be essential for T4SS dependent NF-**κ**B activation in gastric cells [181]. Another example for such a T4SS dependent and CagA independent activation of proinflammatory signaling is the delivery of peptidoglycan into the host cell [84].
NF-**κ**B activation and subsequent IL-8 release were detected in colon cells upon *H. pylori* infection and could confirm a HopQ dependent but CagA independent activation of NF-**κ**B. The lack of induction of IL-8 secretion in cells infected with HopQ deficient strains further substantiates the importance of HopQ in this signaling cascade. However, activation of NF-**κ**B signaling was not detected in murine intestinal primary cells, which could be attributed to the previously mentioned limitations of this model to study *H. pylori* pathogenesis. Additionally, although the NF-**κ**B pathway can be activated in a CagA independent manner, it has been shown that CagA is important for NF-**κ**B-mediated pro-inflammatory responses in the stomach [59] and

therefore the fact, that CagA was not translocated in those cells upon *H. pylori* infection, might contribute to this failure to activate NF-κB.

A possible mechanism of activation of the NF-κB-IL-8 signaling cascade could be via Toll-like receptors (TLRs) expressed on colon epithelial cells, which must recognize and react to the bacterium. Future investigations on the role of *H. pylori* LPS and T4SS-mediated transfer of intracellular peptidoglycan are required in order to shed light on the mechanisms involved in the activation of the innate immune response in colon epithelial cells upon *H. pylori* infection.

Cytokines associated with chronic inflammation activate JAK2-STAT3 signaling, which leads to the overexpression of anti-apoptotic and pro-metastatic genes involved in gastric and colon carcinogenesis [187]. IL-6 and IL-11 are among those cytokines and they are released upon chronic *H. pylori* infection [188]. Furthermore, translocated CagA has been shown to engage with gp130 and determine the signal switch between SHP2/ERK and JAK/STAT3 signaling. In fact, tyrosine phosphorylated CagA is rather activating SHP2/ERK signaling, whereas unphosphorylated CagA leads to signaling via the STAT3 axis [189].

The activation of STAT3 signaling upon *H. pylori* infection in colon cells and primary epithelial cells was inconsistent. This could be attributed to the known limitation of *in vitro* experiments, which lack the environment and signals of cells other than epithelium, that might be crucial in activation of this pathway. More detailed investigations are required to address the importance of CagA phosphorylation status as well as the mechanisms involved in a possible activation of STAT3 signaling in colon epithelial cells upon *H. pylori* infection. Mouse models of colon tumor development might be required to properly address the stages of activation of this signaling pathway in colon epithelia, the cytokines involved as well as the role and importance in malignant transformation of epithelial cells.

5.1. Proposed model of *H. pylori* induced colorectal carcinogenesis

Diverging hypothesis exist on the occurrence of *H. pylori's* relationship with increased CRC development. On the one hand, the hypothesis of a direct effect of *H. pylori* on colonic epithelia, which would require the presence of the bacterium or at least its toxins in the colon, is supported.

On the other hand, an indirect effect via distant modulations of immune responses, signaling pathways and microbiota could be the underlying mechanism.

This study revealed that *H. pylori* is indeed able to bind to colon epithelial cells, with HopQ-mediated CEACAM binding being one possible mechanism of adherence. Subsequently, CagA is translocated via the T4SS into the colon epithelial cell. Translocation is, however, not dependent on the presence of HopQ. The activation of the NF-κB signaling pathway and induction of IL-8 secretion lays the foundation of a proinflammatory environment created by *H. pylori* within the colon (Figure 20).

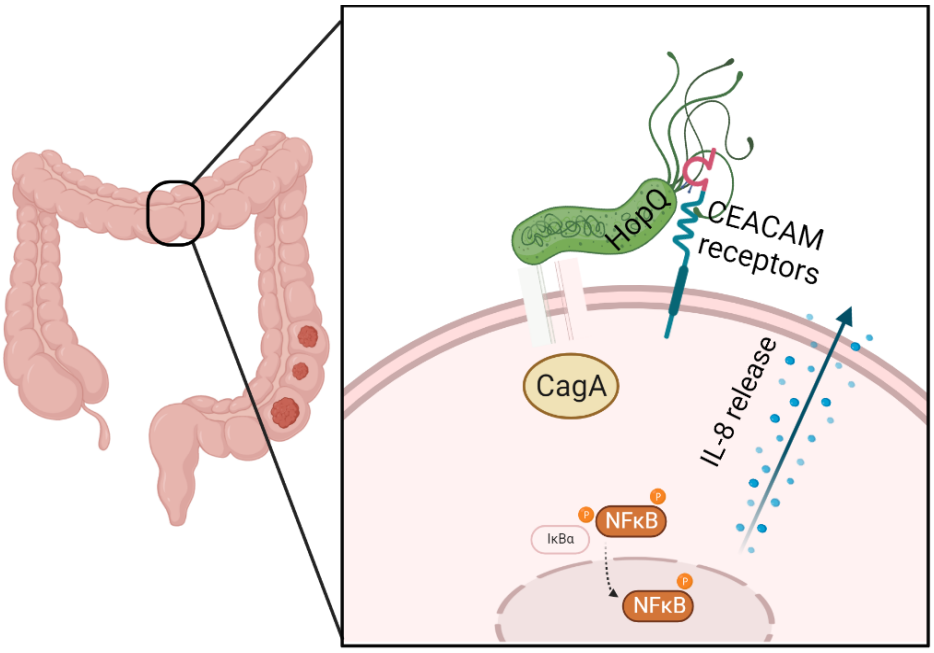

Figure 20: Simplified proposed model of mechanisms involved in *H. pylori* induced carcinogenesis

H. pylori attaches to the colon epithelium via HopQ-CEACAM interaction, translocates CagA, activates NF-κB signaling and induces IL-8 secretion.

5.2. Outlook and therapeutic potential

The mechanisms identified within this study support the assumption that *H. pylori* might exert direct effects on the colon epithelium. However, a prerequisite for the described *H. pylori* colonic pathogenesis would be the presence of the living bacterium within the colon. Future studies are needed to prove the presence of the living bacterium within the human colon and its ability to exert its pathogenic functions.

While the basic ability of the bacterium to create a proinflammatory environment has been shown, however, *in vivo* studies are essential to determine the exact immune cell populations involved and downstream mechanisms affected by these changes. Along this line, it would be highly interesting to assess the activation of NF-**κ**B as well as STAT3 signaling in human colon tissue as well as mouse models and to address how these signaling pathways are activated upon *H. pylori* infection.
Finally, the assessment of the direct effect of *H. pylori* on tumorigenesis by means of infecting CRC models is of utmost importance.

In this study, a basis for future research investigating the underlying mechanisms of *H. pylori* induced colorectal carcinogenesis was established. Furthermore, important proteins and factors of the bacterium possibly involved in cancer promoting effects, which might be potential targets for risk assessments and future therapies, were highlighted.

6. Abbreviations

BabA	blood-group-antigen-binding adhesin
BAC	bacterial artificial chromosome
BE	Barett's esophagus
BHI	brain heart infusion
cag PAI	*cag* pathogenicity island
cag^{+}	*cag* PAI positive
CagA	cytotoxin-associated gene A product
CEACAM transgenic	C57BL/6 human ceacam1$^{+/+}$ × mouse ceacam1$^{-/-}$ mice
CEACAM	carcinoembryonic antigen-related cell adhesion molecules
CIMP	CpG island methylated phenotype
CRC	colorectal cancer
DC	dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
dupA	duodenal ulcer-promoting gene
EDTA	ethylenediaminetetraacetic acid
Erk1/2	extracellular signal-regulated kinase 1 and 2
FAP	familial adenomatous polyposis
FCS	fetal Bovine Serum
GERD	gastroesophageal reflux disease
gGT	γ-glutamyltranspeptidase
H. pylori	*Helicobacter pylori*
HNPCC	hereditary nonpolyposis colorectal cancer
HtrA	high temperature requirement A
IBD	inflammatory bowel disease
iceA	epithelium gene
IL	Interleukin
MALT	mucosa-associated lymphoid tissue

MLN	mesenteric lymph node
MOI	multiplicity of infection
MUC5AC	mucin 5AC
NFW	nuclease-free water
NF-κB	nuclear factor-κB
NOD1	nucleotide-binding oligomerization domain-containing protein 1
OD	optical density
OMP	outer membrane protein
P/S	Penicillin/Streptomycin
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PPIs	proton pump inhibitors
PRRs	pattern recognition receptors
RT	room temperature
SabA	sialic acid-binding adhesin
SHP-2	eukaryotic phosphatase
STAT3	signal transducer and activator of transcription 3
T4SS	type 4 secretion system
TLRs	toll-like receptors
VacA	vacuolating cytotoxin
WC	Wilkins-Chalgren
WT	Wildtype

7. List of Figures

8. List of Tables

9. Acknowledgements

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