Charakterisierung der *Helicobacter pylori*-assoziierten Magenadenokarzinogenese am Tiermodell des Mongolischen Gerbils: Rolle der *cag*-Pathogenitätsinsel bei der Induktion präkanzeröser Prozesse

DISSERTATION

zur Erlangung des Grades eines
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Inhaltsverzeichnis

1. Zusammenfassung

Helicobacter pylori ist ein spiralförmiges, gramnegatives, mikroaerophiles und motiles Bakterium. Der humanpathogene Keim adhäriert an die Magenepithelzellen, wodurch eine Signaltransduktionskaskade gestartet wird, die in der Induktion von Zytokinen, der Infiltration von immunreaktiven Zellen und letztendlich in einer chronischen Gastritis resultiert. Eine über Jahre anhaltende chronische Entzündung stellt *per se* ein erhöhtes karzinogenes Risiko dar. *H. pylori* als Karzinogen der Klasse I wird somit eine bedeutende Rolle bei der Umwandlung der Magenmukosa, entlang der präkanzerösen Stufen Atrophie, Metaplasie, Dysplasie bis hin zum Magenadenokarzinom zugeschrieben. Aufgrund der Fähigkeit virulenter *H. pylori*-Stämme (Typ I) das Effektorprotein CagA in die Wirtszelle zu translozieren, wird vermutlich eine Signaltransduktionskaskade gestartet, die auf die Physiologie und Architektur der Magenmukosa direkt regulatorisch wirkt. Ein Ziel dieser Arbeit war es, den Regulationsmechanismus der *H. pylori*-induzierten Karzinogenese im Magen am Modell des Mongolischen Gerbils zu analysieren. Mit Hilfe einer Zeitverlaufsstudie konnte zum ersten Mal gezeigt werden, dass eine frühzeitig hochgradige Entzündung der Antrum- und Korpusmukosa zu späten physiologischen Veränderungen führt (Hypochlorhydrie und Hypergastrinämie). Diese *H. pylori* Typ I-Stamm-induzierte Entzündung und die damit verbundenen physiologischen Veränderungen sind kausal für die Entwicklung präkanzeröser Transformationen der Magenmukosa verantwortlich.

Anhand der Ergebnisse diverser Studien wurde vermutet, dass dem gastrointestinalen Hormon Gastrin eine bedeutende Schlüsselrolle bei der Entstehung maligner Veränderungen der gastrointestinalen Mukosa zukommt. Aufgrund der im Tiermodell gemessenen Hypergastrinämie und der damit verbundenen Entwicklung präkanzeröser Veränderungen wurde im zweiten Teil dieser Arbeit die Regulation des humanen Gastrinpromotors durch *H. pylori* Typ I-Stämme untersucht. Hierfür wurden in diesem Teilprojekt die stabil mit einem humanen Gastrinpromotor-Luziferase-Reporterkonstrukt transfizierten Magenkarzinomzellen AGS verwendet. Es konnte erstmalig β_5-Integrin als wichtiger Wirtsrezeptor für die *H. pylori*-induzierte Gastrinpromotoraktivierung identifiziert werden. Weitere Untersuchungen bestätigten einen neuartigen β_5-Integrin-ILK-Signalkomplex, der für die *H. pylori*-induzierte Gastrinexpression verantwortlich ist.

Zusätzlich konnte eine Beteiligung des EGF-Rezeptors sowie des Liganden TGF-α bei der Aktivierung der Gastrinexpression nachgewiesen werden.

Das von *H. pylori* induzierte, aus der Epithelzelle der Magenmukosa sekretierte Chemokin IL-8 bewirkt im Tiermodell eine frühe Infiltration von immunreaktiven Zellen, sowie die daraus resultierende hochgradige Entzündung. Aus diesem Grund wurde die Regulation der *H. pylori*-induzierten IL-8-Sekretion mit Hilfe von AGS-Zellen *in vitro* untersucht. Das Ergebnis zeigt, dass eine spezifische C-terminale „coiled-coil"-Region des *H. pylori* CagL-Proteins eine essentielle Rolle bei der Adhärenz des Bakteriums an die Epithelzelle besitzt. Bedeutend war, dass isogene *H. pylori*-Mutanten, bei denen die „coiled-coil"-Region des CagL-Proteins deletiert wurde, keine IL-8-Ausschüttung mehr zeigten.

1. Summary

Helicobacter pylori is a spiral-shaped, gram-negative, microaerophilic and motile bacterium. The human pathogen adheres to the epithelial cells of the stomach and induces a signal cascade that leads to the induction of cytokines, the infiltration of immune cells and eventually chronic gastritis. A persisting chronic inflammation intrinsically increases the risk for developing cancer. Hence, *H. pylori* a class I-carcinogen plays a crucial role in the malignant transformation of the gastric mucosa including all described precancerous steps leading to cancer (atrophy, metaplasia, and dysplasia). The physiology and architecture of the gastric mucosa are probably regulated via signal cascades that are induced by the translocated *H. pylori* type I effector protein CagA. One aim of this study was to analyze the regulatory mechanisms that are involved in *H. pylori*-induced carcinogenesis of the stomach. To examine these regulatory mechanisms, we used the Mongolian gerbil animal model. By applying a time course experiment, we could show for the first time that an early severe inflammation in antral and corpus mucosa later leads to physiological changes (hypochlorhydria, hypergastrinemia). This *H. pylori* type I-strain induced inflammation and the associated physiological changes are responsible for precancerous transformations of the gastric mucosa.

Several studies indicated that the gastrointestinal hormone gastrin plays an essential role in regulating the normal and malignant development of the gastrointestinal mucosa. Based on the observed hypergastrinemia and induced precancerous conditions in Mongolian gerbils, we analyzed the mechanisms involved in *H. pylori* type I-strain-induced gastrin expression. In order to explore the regulation of the human gastrin expression by *H. pylori*, we used an *in vitro* study applying gastric epithelial cell line (AGS) stably transfected with a human gastrin promoter luciferase reporter construct. We have identified β5-integrin as a host-recognition receptor essential for *H. pylori*-induced gastrin promoter activation. Further investigations revealed a novel β5-integrin-ILK signaling complex that is responsible for *H. pylori*-induced gastrin expression. Additionally, we determined the EGF receptor and one of its ligands, TGF-α, were involved in *H. pylori*-induced gastrin expression.

An early infiltration of immune cells and the resulting severe inflammation of the gastric mucosa observed in the animal model infected with *H. pylori* were mainly caused by IL-8, a chemokine secreted by epithelial cells. For this reason, we wanted to examine the *H. pylori*-induced IL-8 secretion by applying an *in vitro* approach. The

results of this study demonstrate that a specific C-terminal coiled-coil region of CagL plays a crucial role in *H. pylori*-adherence to the epithelial cells. However, the most important finding is that isogenic *H. pylori* mutant-strains missing the specific coiled-coil region are completely unable to induce IL-8 expression.

2. Einleitung

2.1 Entdeckung und Charakterisierung von *Helicobacter pylori*

Im Jahre 1982 entdeckten B. Marshall und R. Warren bei der Untersuchung von Magenbiopsien aus Gastritispatienten spiralförmige, gramnegative Bakterien (Warren and Marshall, 1983). Bis zu diesem Zeitpunkt nahm man an, dass aufgrund der hohen Säurekonzentration im Magen eine bakterielle Kolonisation der Magenschleimhaut nicht möglich ist. Nach der Kultivierung dieses Bakteriums wurde es in die Gattung *Campylobacter* eingegliedert und bekam nach seinem Fundort den Namen *Campylobacter pyloridis*. Mit fortschreitender Erforschung und somit immer neueren Erkenntnissen über diesen Keim wurde er schließlich wegen seiner besonderen Charakteristika umbenannt und einer vollkommen neuen Gattung zugeordnet. Im Jahr 1989 wurde der erste Vertreter dieser neuen Gattung *Helicobacter pylori* genannt (Goodwin *et al.*, 1989).

H. pylori (Abb.1) ist ein spiralförmiges, gramnegatives Bakterium, das eine Länge von circa zwei bis vier Mikrometer aufweist (Vandamme *et al.*, 1991). Es besitzt zwei bis sieben unipolar angeordnete Flagellen, die ihm die notwendige Motilität für eine erfolgreiche Besiedelung der Magenschleimhaut ermöglichen. *H. pylori* wird im Labor unter mikroaerophilen Bedingungen (5% O_2; 10% CO_2; 85% N_2) bei 37^0 C auf Blut oder Serum enthaltenden Vollmedienplatten kultiviert (Goodwin and Armstrong, 1990; Goodwin and Worsley, 1993). Durch eine verlängerte Kultivierung wird die kokkoide Form von *H. pylori* induziert, die metabolisch noch aktiv, jedoch nicht mehr *in vitro* kultivierbar ist (Bode *et al.*, 1993; Nilius *et al.*, 1993; Kusters *et al.*, 1997). Die Rolle dieser kokkoiden Form bei der Infektionsübertragung ist jedoch bis heute nicht geklärt (She *et al.*, 2003; Wang *et al.*, 1997).

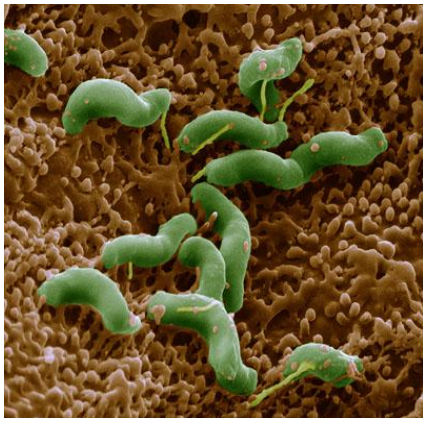

Abb.1: *H. pylori* auf der Magenschleimhaut des Menschen

Rasterelektronenmikroskopische (REM) Aufnahme 9000x [© *eye of science*]

2.2 Epidemiologie der *H. pylori*-Infektion

Mit einer Prävalenz von circa 50% ist die Infektion mit *H. pylori* die zweithäufigste Infektionskrankheit weltweit. Die Infektion mit *H. pylori* kommt in allen Bevölkerungen und Gesellschaftschichten vor, variiert jedoch nach geografischer Region, Alter und sozioökonomischem Status (Malaty *et al.*, 1992; Malaty and Graham, 1994; Malaty *et al.*, 2001). Risikofaktoren für eine Ansteckung sind beispielsweise ein niedriger sozioökonomischer Status, schlechte hygienische Verhältnisse sowie das Wohnen auf zu engem Raum (Webb *et al.*, 1994). Diese Kriterien erklären, warum die Infektionsrate in Entwicklungsländern ~ 90%, in Industrieländern aber nur durchschnittlich 20% - 30% beträgt (Dunn *et al.*, 1997). Die Übertragung von *H. pylori* von Individuum zu Individuum ist bis heute nicht vollständig geklärt. Es wird hauptsächlich von einer oral-oralen oder fäkal-oralen Übertragung vorrangig von der Mutter auf das Kind ausgegangen (Drumm *et al.*, 1990). Diese Annahme ergibt sich durch die Beobachtung, dass in der Regel bei nahen Angehörigen dieselben *H. pylori*-Stämme gefunden wurden und die Infektion meist schon im Kindesalter erworben wurde (Bamford *et al.*, 1993; Konno *et al.*, 2005; Granstrom *et al.*, 1997; Mitchell *et al.*, 1992). Bei der Frage nach dem Hauptreservoir des Keims gehen Forscher davon aus, dass es der menschliche Magen ist, obwohl *H. pylori* auch im Zahnstein und Stuhl nachgewiesen werden konnte (Desai *et al.*, 1991; Namavar *et al.*, 1995).

2.3 Pathogenitätsfaktoren

2.3.1 Urease

Um den menschlichen Magen kolonisieren zu können, müssen die Bakterien für einige Zeit im sauren Milieu des Magenlumens überleben, bevor sie ihre Nische im epithelnahen, fast neutralen Magenschleim erreichen. Hierfür besitzt *H. pylori* das Enzym Urease, das aus den Untereinheiten UreA und UreB aufgebaut ist und den im Magen vorkommenden Harnstoff in Kohlendioxid und Ammoniak aufspaltet (Marshall *et al.*, 1990; Stingl *et al.*, 2002). Bei diesem Prozess wird der Harnstoff durch einen pH-abhängigen Harnstoffkanal, der durch das Membranprotein UreI gebildet wird, aufgenommen und im Zytosol durch die Urease gespalten (Weeks *et al.*, 2000). Dieser Mechanismus dient der Aufrechterhaltung des neutralen pH-Wertes im bakteriellen Zytosol. Der entstandene Ammoniak puffert die einströmenden Protonen der Magensäure ab und gewährleistet somit einen neutralen pH-Wert. Damit wird ein Milieu geschaffen, welches das Membranpotential und die Energiegewinnung aufrechterhält und somit das Überleben des Bakteriums ermöglicht (Rektorschek *et al.*, 1998). Die Regulation des Harnstoffkanals ist gekoppelt an den pH-Wert des Zytosols. Steigt der pH-Wert über ~ 7, wird der Kanal geschlossen und eine Alkalisierung des Zytosols vermieden (Clyne *et al.*, 1995). Um die Pufferung des Periplasmas zu gewährleisten, besitzt *H. pylori* das Enzym α-Karboanhydrase. Dieses Enzym wandelt das durch die Ureaseaktivität entstehende CO_2 zu HCO_3^- um (Marcus *et al.*, 2005). Die Urease ist hauptsächlich ein zytoplasmatisches Enzym, das aber auch an der Oberfläche des Bakteriums vorkommt (Phadnis *et al.*, 1996; Marcus and Scott, 2001). Es wird angenommen, dass die an der Oberfläche lokalisierte Urease eine Ammoniakwolke erzeugt und somit das Bakterium vor der Säure des Magens schützt. In Maus- und Gerbil-Tiermodellen konnte gezeigt werden, dass eine funktionsfähige Urease für die Kolonisierung des Magens essentiell ist. Konstruiert man *H. pylori* UreI-Mutanten, in denen der Harnstoffkanal defekt ist, kommt es nur in säuregeblockten Tieren zur Kolonisierung. In unbehandelten Tieren, die einen normalen Magen pH-Wert von ~ 1 haben, kann keine *H. pylori*-Kolonisierung festgestellt werden (Sachs *et al.*, 2003). Aufgrund dieser Resultate konnte die Urease als wichtiger Kolonisierungsfaktor für die *H. pylori*-Besiedelung des Magens charakterisiert werden (Kavermann *et al.*, 2003). Eine Beteiligung des Enzyms bei Epithelzellschäden durch die Anreicherung des

Zellgiftes Ammoniak (Megraud *et al.*, 1992; Sommi *et al.*, 1996; Suzuki *et al.*, 1992) oder bei der Induktion von Apoptose wird bis heute kontrovers diskutiert (Igarashi *et al.*, 2001).

2.3.2 Flagellen

Um eine lebenslange Persistenz des Keims in einem extremen Habitat wie dem menschlichen Magen zu ermöglichen, ist *H. pylori* auf seine Begeißelung und die damit verbundene Motilität angewiesen. Durch sie erreicht das Bakterium entlang des pH-Gradienten die schützende Bikarbonat-gepufferte Schleimschicht, welche die Magenepithelzellen vor der aggressiven Säure des Magens schützt (Yoshiyama *et al.*, 1999; Schreiber *et al.*, 2004). Um Verluste durch Peristaltik zu verhindern ist es für den Keim essentiell, mit Hilfe seiner hohen Motilität die Magenepithelzellen zu erreichen und an ihnen zu adhärieren. Für die Beweglichkeit des Bakteriums sind zwei bis sieben unipolar angeordnete Flagellen zuständig, die aus den Flagellinproteinen FlaA und FlaB aufgebaut sind (Leying *et al.*, 1992; Suerbaum *et al.*, 1993). Eine lipidhaltige Flagellenhülle schützt die Geißeln vor dem Abbau durch die Magensäure (Geis *et al.*, 1993). Der Schutz dieser Hülle ist jedoch zeitlich begrenzt und setzt daher eine relativ schnelle Kolonisierung des Bakteriums voraus (Schreiber *et al.*, 2005). Im Tiermodell mit gnotobiotisch gehaltenen Ferkeln konnte gezeigt werden, dass neben der Urease (siehe 2.3.1) auch die Flagellen einen essentiellen Kolonisierungsfaktor darstellen (Eaton *et al.*, 1992).

2.3.3 Adhäsine

Untersuchungen zur Verteilung von *H. pylori* auf der Magenmukosa zeigten eine vermehrte Lokalisierung des Keims 0 - 25 Mikrometer über dem Epithel. Überraschend jedoch war die Beobachtung, dass nur ungefähr 30% der Bakterien im Bereich 0 - 5 Mikrometer oberhalb der Mukosa zu finden waren, und nur circa 20% an den Epithelzellen gebunden vorlagen (Hessey *et al.*, 1990; Schreiber *et al.*, 1999). Dabei ist die Bindung von *H. pylori* an das Epithel essentiell, da sie den Verlust des Keims durch Magenperistaltik verhindert. Die Bindung an die Epithelzelle erfolgt meist über eine Gruppe von äußeren Membranproteinen, den Adhäsinen. Die bislang wichtigsten Vertreter dieser Adhäsionsmoleküle sind die Adhäsine BabA, SabA, AlpA und AlpB. BabA (blood group antigen-binding adhesin) bindet fukosylierte Lewis^b-Blutgruppenantigene auf der Oberfläche der Epithelzellen (Boren *et al.*, 1993; Ilver *et al.*, 1998; Aspholm-Hurtig *et al.*, 2004). Aufgrund epidemiologischer Studien konnte

gezeigt werden, dass BabA-positive *H. pylori*-Stämme im Gegensatz zu BabA-negativen Stämmen mit verstärkter mukosaler Entzündung, atrophischer Gastritis und Magenadenokarzinom assoziiert sind (Prinz *et al.*, 2001). SabA (sialic acid-binding adhesin) bindet an sialylierte Glykoproteine, die vorrangig in der entzündeten Magenmukosa und eher selten in der gesunden Magenmukosa vorkommen (Aspholm *et al.*, 2006). AlpA und AlpB sind zwei homologe, 518 Aminosäuren lange „outer membrane“-Proteine (OMP). Diese wurden ebenfalls der Gruppe der Adhäsine zugeordnet, obwohl ihr Rezeptor an den Epithelzellen noch nicht identifiziert werden konnte (Odenbreit *et al.*, 1999).

2.3.4 VacA

Das 88 kDa große Protein VacA (vacuolating cytotoxin A) ist ein von *H. pylori* sekretiertes Zytotoxin, das die Ausbildung großer, zytoplasmatischer Vakuolen in Epithelzellen induziert (Abb.2) (Cover *et al.*, 1992). Alle aus menschlichen Biopsien reisolierten *H. pylori*-Stämme produzieren VacA, obwohl deutliche Variationen in der Sequenz erkennbar sind. Aufgrund dieser Sequenzpolymorphismen ergeben sich verschiedene VacA-Allele, die sich in ihrer Vakuolisierungsaktivität unterscheiden. Es konnte gezeigt werden, dass eine Infektion mit *H. pylori*-Stämmen, die ein vakuolisierungsaktives VacA-Allel (s1m1 oder s1m2) tragen, mit schwereren Krankheitsverläufen einhergeht (Atherton *et al.*, 1995; van Doorn *et al.*, 1998). VacA wird nach der Sekretion in die kovalent verbundenen Domänen p33 (33 kDa) und p55 (55 kDa) aufgespalten (Telford *et al.*, 1994). Diese beiden Untereinheiten weisen unterschiedliche Funktionen auf. Die p55-Untereinheit vermittelt die Bindung des Toxins an die Wirtszelle (Reyrat *et al.*, 1999). Das kleinere p33-Fragment entfaltet mit Hilfe des N-terminalen Teils von p55 die vakuolisierende Aktivität im Zytoplasma der Wirtszelle (de *et al.*, 1998). VacA bildet als reifes Toxin hexamere Ringe aus, von denen sich jeweils zwei aneinanderlagern und hochmolekulare Dodekamere ausbilden (~1 MDa). Diese Dodekamere zerfallen unter sauren (pH-Wert <4) oder alkalischen (pH-Wert >9) Bedingungen wieder in Monomere und erlangen so eine vielfach erhöhte Toxizität (Cover *et al.*, 1997). Diese Monomere inserieren in die Zytoplasmamembran der Wirtszelle und formen dort Membranporen, die für die Aufnahme des Toxins essentiell sind (Szabo *et al.*, 1999; McClain *et al.*, 2003; Vinion-Dubiel *et al.*, 1999). Weitere Eigenschaften des VacA-Toxins sind die Induktion der Apoptose (Galmiche *et al.*, 2000; Kuck *et al.*, 2001) sowie die Inhibition

(I) des intrazellulären Vesikeltransports (Montecucco *et al.*, 1996; Satin *et al.*, 1997), (II) der Antigenpräsentation in B-Zellen (Molinari *et al.*, 1998) und (III) der T-Zell-Aktivierung (Boncristiano *et al.*, 2003; Gebert *et al.*, 2003; Sundrud *et al.*, 2004; Sewald *et al.*, 2008).

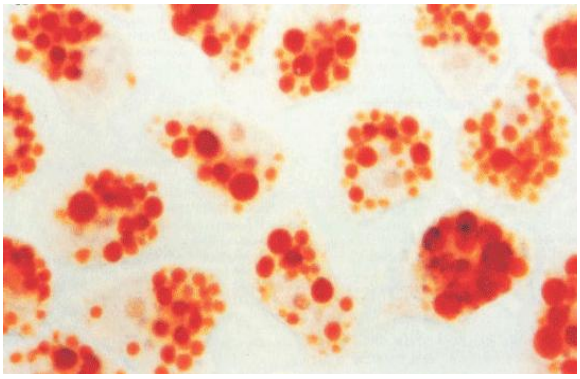

Abb.2: Vakuolisierung von HeLa-Zellen durch VacA Zugabe. Färbung mit Neutralrot.

2.3.5 Die *cag*-Pathogenitätsinsel (*cag*-PAI)

H. pylori wird in sogenannte Typ I- oder Typ II-Stämme unterteilt. Der Unterschied zwischen ihnen liegt darin, dass Typ I-Stämme im Gegensatz zu Typ II-Stämmen eine komplett funktionelle *cag*-PAI sowie ein aktives VacA besitzen (Xiang *et al.*, 1995). Die *cag*-PAI ist eine 40 kb große Genregion mit circa 29 Genen (Abb. 3). Die Gene der *cag*-PAI codieren für ein Typ IV-Sekretionssystem (T4SS) sowie dessen Substrat CagA (Abb.4) (Censini *et al.*, 1996).

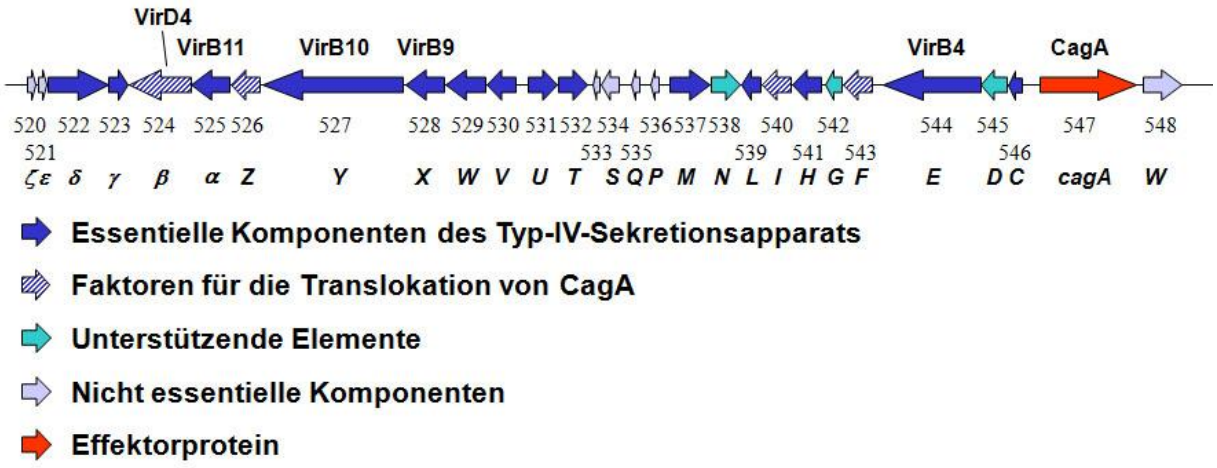

Abb.3: Schematische Darstellung der *cag*-PAI von *H. pylori* P12 (Fischer *et al.*)

Die offenen Leseraster (orfs) sind als Pfeile dargestellt und wurden entsprechend ihrer Sequenzanordnung in *H. pylori* P12 mit durchlaufenden Nummern hp520-548 bezeichnet. Die Buchstaben kennzeichnen die bisher bekannten *cag*-Gene (z.B. X = *cag*X). Orfs mit Homologien zu den *vir*-Genen von *Agrobacterium tumefaciens* wurden oberhalb gekennzeichnet.

CagA wird durch das T4SS in die Wirtszelle injiziert und entfaltet dort vielfältige Funktionen (siehe 2.3.6.2) (Backert *et al.*, 2000; Naumann, 2005; Fischer *et al.*, 2001). Es konnte gezeigt werden, dass eine Infektion mit *H. pylori* Typ I-Stämmen mit der Entwicklung schwererer Krankheitsverläufe wie chronischer und atrophischer Gastritis, Ulkuserkrankungen sowie Magenadenokarzinom assoziiert ist (Peek, Jr. *et al.*, 1995; Yamaoka *et al.*, 1997; Covacci *et al.*, 1993; Graham and Yamaoka, 1998; Walker and Crabtree, 1998; Webb *et al.*, 1999; Blaser *et al.*, 1995; Parsonnet *et al.*, 1997).

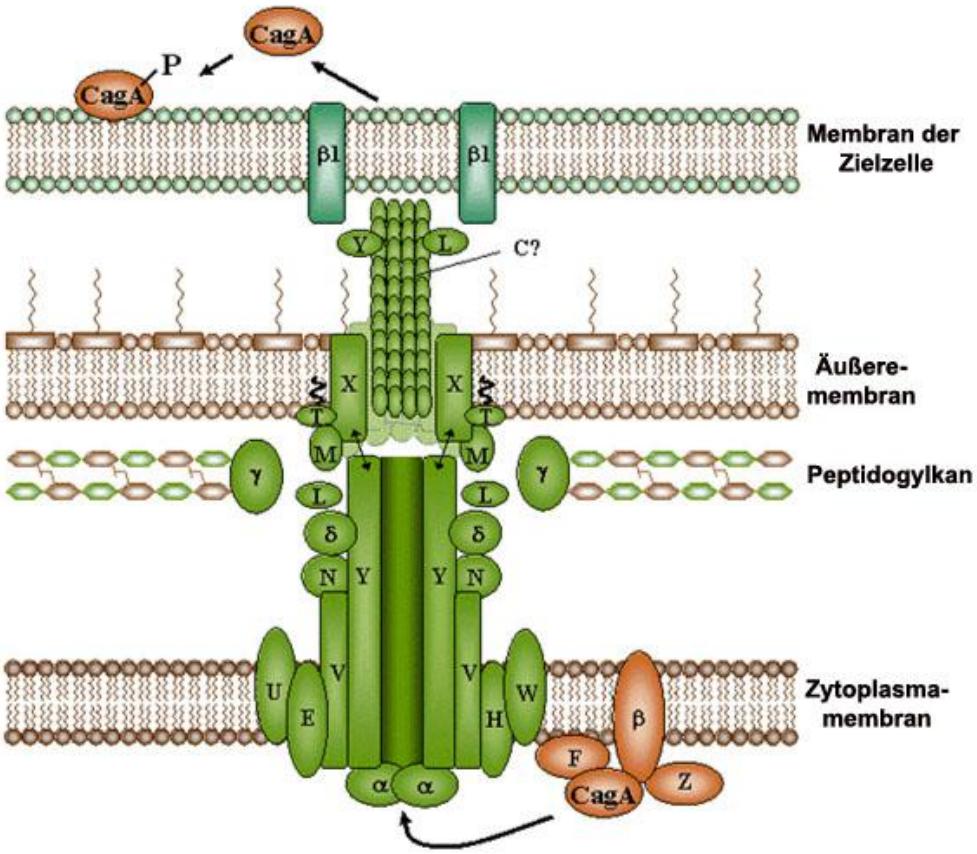

Abb.4: Hypothetisches Modell des Typ-IV-Sekretionsapparates (Fischer *et al.*)

Das T4SS ist ein Multiproteinkomplex, der die innere und äußere Membran von *H. pylori* durchspannt. An diesem Modell wird nach aktuellem Wissensstand vereinfacht die Lokalisierung der Einzelkomponenten und die Translokation von CagA in die Wirtszelle dargestellt.

2.3.6 CagA

2.3.6.1 CagA-Translokation

Lagert sich ein *H. pylori* Typ I-Stamm an eine Epithelzelle des Wirts, so kommt es durch das T4SS zur Injektion von CagA (130-170 kDa). Dieser Prozess wird durch $\alpha_5\beta_1$-Integrine der Wirtszelle und dem CagL-Protein am Pilus des T4SS von *H. pylori* vermittelt (Kwok *et al.*, 2007). CagL ist auf der *cag*-PAI codiert (siehe Abb.3). Es enthält ein sogenanntes RGD (Arg-Gly-Asp)-Motiv, das die Bindung an $\alpha_5\beta_1$-Integrin

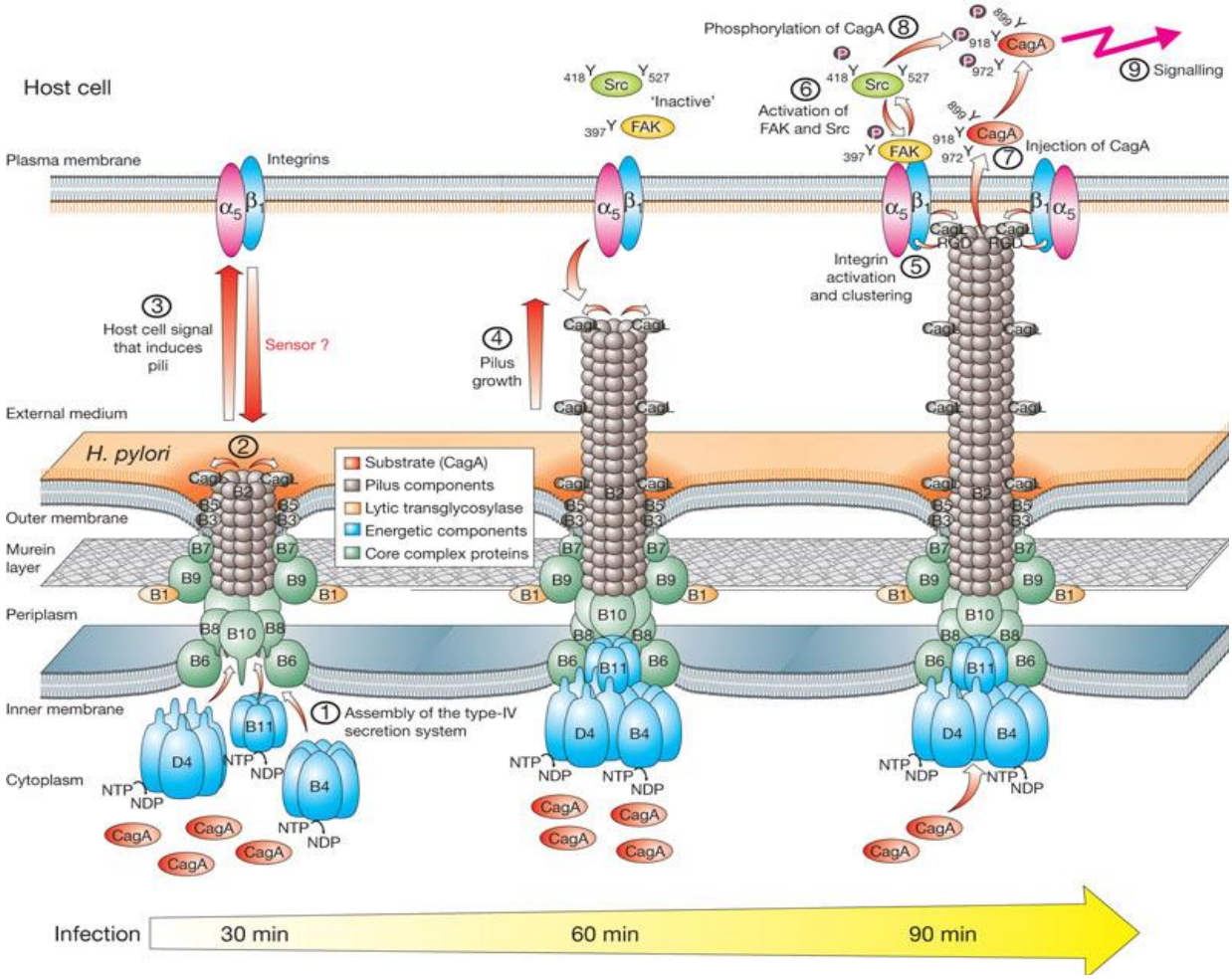

Abb.5: Modell zur Veranschaulichung der *H. pylori* CagA-Translokation (Kwok *et al.* 2007)

(1+2) Ohne Kontakt zur Wirtszelle wird CagL zur Bakterienoberfläche oder spezifisch an die Oberfläche des Pilus transportiert. **(3)** Während der Infektion bekommt *H. pylori* Kontakt mit der Wirtszelle und CagL bindet an den Transmembranrezeptor $\alpha_5\beta_1$-Integrin des Wirts. **(4)** Durch bisher unbekannte Mechanismen verlängert sich der Pilus aufgrund der Interaktion zwischen CagL und $\alpha_5\beta_1$-Integrin. **(5)** Während der Bindung des RGD-Motivs von CagL an den Rezeptor $\alpha_5\beta_1$-Integrin wird dieser aktiviert. **(6)** Das aktivierte $\alpha_5\beta_1$-Integrin löst nun die „downstream"-Signalkaskaden aus, die zu einer Phosphorylierung von FAK und Src führen. Diese Aktivierung induziert unmittelbar danach sogenannte „actin-cytoskeletal rearrangements", „plasma-membrane dynamics", die Zusammenballung von Integrinen sowie die Aktivierung anderer Rezeptoren. **(7)** Diese Veränderungen sind bedeutend für die CagA-Injektion in die Wirtszelle. **(8)** Die aktivierte Src-Kinase phosphoryliert das translozierte CagA an seinen Tyrosinresten. **(9)** Das phosphorylierte CagA stimuliert nun weitere Signalwege (siehe 2.3.6.2).

der Wirtszelle ermöglicht. Die CagL-$\alpha_5\beta_1$-Integrin-Interaktion stimuliert parallel zur CagA Translokation auch FAK (focal adhesion kinase) und Src (Mitra and Schlaepfer, 2006). Die Tyrosinkinase Src phosphoryliert das translozierte CagA-Protein an seinen spezifischen EPIYA (Glu-Pro-Ile-Tyr-Ala)-Phosphorylierungsmotiven (siehe Abb.5) (Backert *et al.*, 2001; Stein *et al.*, 2002). Phosphoryliertes sowie auch unphosphoryliertes CagA induziert diverse Wirtssignalkaskaden, die meist mit malignen Transformationen der Wirtszellen einhergehen (siehe 2.3.6.2).

2.3.6.2 CagA als Effektorprotein

Im Laufe der Zeit konnten diverse Effekte von Tyrosin-phosphoryliertem CagA ($CagA^{P\text{-}Tyr}$) auf die Wirtszelle gezeigt werden. Durch die Phosphorylierung von CagA kommt es zu Dephosphorylierungen von Wirtszellproteinen wie Kortaktin, Ezrin und Vinkulin (Selbach *et al.*, 2003; Selbach *et al.*, 2004; Moese *et al.*, 2007). Die Dephosphorylierung dieser Aktin-bindenden Proteine führt zu charakteristischen Zellausläufern, ein Phänotyp, der als „hummingbird“-Phänotyp bezeichnet wurde. Außerdem kommt es zum Auseinanderweichen der Epithelzellen, dem sogenannten „cell scattering“ (Segal *et al.*, 1999; Backert *et al.*, 2001; Tsutsumi *et al.*, 2003; Churin *et al.*, 2003; Higashi *et al.*, 2004; Suzuki *et al.*, 2005). Zusammenfassend kann festgehalten werden, dass $CagA^{P\text{-}Tyr}$ in viele Signalkaskaden eingreift, die mit der Induktion von sogenannten „cytoskeletal rearrangements“ verbunden sind.

Neben den eben beschriebenen phosphorylierungsabhängigen Funktionen gibt es auch einige zelluläre Funktionen von CagA, die keine Phosphorylierung des Proteins voraussetzen. So konnten Interaktionen des unphosphorylierten CagA-Proteins mit E-Cadherin (Zelladhäsionsprotein), c-Met (Hepatozyten-Wachstumsfaktor-Rezeptor), PLC-γ (Phospholipase), Par1 (Adaptermolekül) und Grb2 (Adaptermolekül) nachgewiesen werden (Mimuro *et al.*, 2002; Churin *et al.*, 2003; Murata-Kamiya *et al.*, 2007; Saadat *et al.*, 2007; Zeaiter *et al.*, 2008). Diese phosphorylierungsunabhängigen Interaktionen von CagA bewirken eine Störung der „tight junctions“, der „adherens junctions“ und der Zellpolarität sowie eine Induktion der Entzündung und Zellteilung. Aufgrund epidemiologischer Studien wurde der Zusammenhang zwischen der CagA-Expression und dem Entstehen eines Magenadenokarzinoms dargestellt. Erst kürzlich konnte gezeigt werden, dass CagA

mit der Bildung gastrointestinaler Tumore in Mäusen assoziiert ist und somit onkogenes Potential besitzt (Ohnishi *et al.*, 2008).

2.3.6.3 T4SS-abhängige, CagA-unabhängige Effekte

Obwohl CagA bis heute das einzige bekannte vom T4SS translozierte Effektorprotein ist, konnten diverse Antworten des Wirts unabhängig von CagA beobachtet werden. Dazu zählen proinflammatorische Reaktionen des Wirts, bei denen die Signalmoleküle NF-κB, AP-1 sowie die Protoonkogene c-Fos und c-Jun eine Rolle spielen (Sharma *et al.*, 1995; Censini *et al.*, 1996; Sharma *et al.*, 1998; Meyer-ter-Vehn *et al.*, 2000). Eine Aktivierung von Rac1 und Cdc42 (Rho-GTPasen) sowie eine Stimulation der Erk („extracellular-signal regulated kinase“) und des EGF (epidermal growth factor)-Rezeptors wurde außerdem beobachtet (Churin *et al.*, 2001; Keates *et al.*, 2005). Diese Resultate sprechen dafür, dass das T4SS möglicherweise noch andere Faktoren neben CagA in die Wirtszelle translozieren kann beziehungsweise Komponenten des Apparats selbst für das Auslösen von Signaltransduktionskaskaden verantwortlich sind. Ein Schritt in diese Richtung wurde von Viala *et al.* (2004) beschrieben, indem Peptidoglykan mit Hilfe des T4SS in die Wirtszelle transloziert werden konnte. Dadurch wurde die proinflammatorische Signalkaskade über den intrazellulären Rezeptor Nod1 und NF-κB und somit die Ausschüttung von IL-8 induziert (Viala *et al.*, 2004).

2.4 Pathogenese der *H. pylori*-Infektion

Nach der Kolonisierung der Magenschleimhaut mit *H. pylori* kommt es zu einer akuten Gastritis, welche durch die Infiltration von Immunzellen in die Magenmukosa gekennzeichnet ist (Genta, 1997; Rossi *et al.*, 2000). Die daraus entstehende chronische Gastritis verläuft jedoch in circa 90% aller Fälle asymptomatisch und kann nur histologisch nachgewiesen werden. Nur in 10% - 20% der Fälle entwickeln sich über einen längeren Zeitraum schwerere gastrointestinale Erkrankungen wie z.B. chronisch atrophische Gastritis, Magen- oder Duodenalulkus, Magenadenokarzinom oder MALT (mucosa associated lymphoid tissue)-Lymphome (siehe Abb.6) (Atherton, 2006). Das Magenadenokarzinom wird histologisch in zwei Typen eingeteilt: Der intestinale und der diffuse Typ. Die Prävalenz einer *H. pylori*-Infektion bei Patienten mit einem Karzinom vom intestinalen Typ liegt mit 89% signifikant höher als beim diffusen Typ (32%) (Parsonnet *et al.*, 1991). Ob sich eine Antrum-prädominante

Gastritis oder eine chronische Korpus-dominante atrophische Gastritis mit einem erhöhten Risiko für die Entstehung eines Magenadenokarzinoms entwickelt, wird zusätzlich von Umwelt- und Wirtsfaktoren beeinflusst. Bei Individuen mit intakter Säuresekretion kolonisiert *H. pylori* vorwiegend die antrale Mukosa, die wenig säureproduzierende Parietalzellen besitzt. Aus dieser Kolonisierung entsteht eine sogenannte Antrum-prädominante Gastritis, mit einer Prädisposition für die Entwicklung von Magen- und Duodenalulkus (Kusters *et al.*, 2006). Dieser Gastritistyp kommt bei circa 15% aller infizierten Individuen vor und ist vermehrt in westlichen Ländern verbreitet.

Ist die Säuresekretion jedoch gestört, kommt es zu einer zunehmenden Besiedelung von *H. pylori* im Korpus. Diese Kolonisierung der Korpusmukosa führt zu einer Korpus-prädominanten Pangastritis (Kuipers *et al.*, 1995). Die daraus resultierende aktive Entzündung im Korpusgewebe führt zu einer verstärkten Hypochlorhydrie, da das proinflammatorische Zytokin IL-1β einen stark suppressiven Effekt auf die säureproduzierenden Parietalzellen ausübt (El-Omar *et al.*, 1997; Ruiz *et al.*, 1996). Somit ist die Korpus-dominante atrophische Gastritis ein Hauptrisikofaktor für die Entwicklung eines Magenadenokarzinoms. Die Prävalenz mit der eine *H. pylori*-infizierte Person aus einer atrophischen Gastritis ein Magenadenokarzinom entwickelt, liegt bei circa einem Prozent.

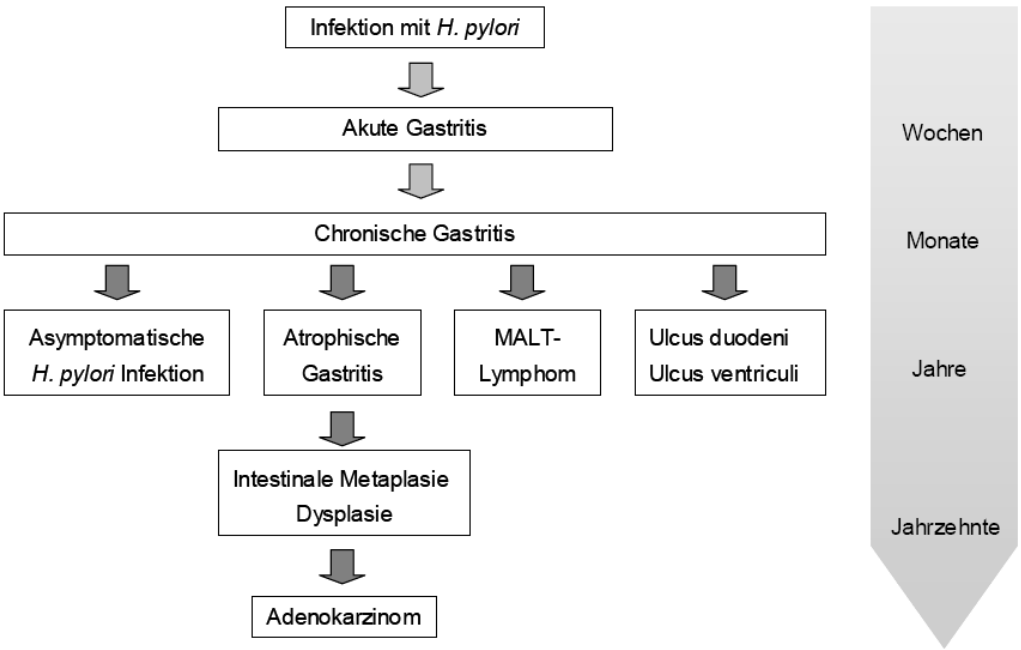

Abb.6: Pathogenese der *H. pylori*-Infektion (nach Telford *et al.* 1997)

Erläuterungen zum Schema siehe Text 2.4.

Die Variabilität in den Krankheitsbildern lässt sich damit erklären, dass es unterschiedlich pathogene *H. pylori*-Stämme gibt (siehe 2.3.5) sowie die Immunantwort des Wirts von Individuum zu Individuum unterschiedlich ausfällt (Shimoyama *et al.*, 1998). Bedeutende Faktoren, die auf die Karzinogenese der Magenmukosa beschleunigend wirken, sind exogene Einflüsse wie beispielsweise salzreiche Lebensmittel (Nitritpökelsalz), die Einnahme entzündungshemmender Schmerzmittel (hauptsächlich Acetylsalicylsäurederivate), Alkohol, Koffein und Nikotin sowie endogene Faktoren wie psychogener Stress, verminderte gastrale Durchblutung und Vitamin C-Mangel (Harrisons Innere Medizin 1995). Des Weiteren spielen bei der malignen Entwicklung der Magenmukosa genetische Wirtsdeterminanten wie z.B. Polymorphismen im IL-1β- oder TNF-α-Gen eine entscheidende Rolle (Garza-Gonzalez *et al.*, 2005; El-Omar *et al.*, 2000).
Epidemiologische Studien zeigen die Assoziation zwischen der Kolonisierung mit *H. pylori* und der Entstehung von Magen- und Duodenalulkus (Ernst and Gold, 2000; Suerbaum and Michetti, 2002) sowie der Entwicklung eines Magenadenokarzinoms auf. Deshalb wurde *H. pylori* 1994 von der WHO (World Health Organisation) als Typ I-Karzinogen eingestuft (Logan, 1994).

2.5 Diagnose und Therapie

2.5.1 Diagnose

Bei der *H. pylori*-Diagnose verwendet man sogenannte invasive und nicht-invasive Methoden.

Invasive Methoden:

Bei einer Magenspiegelung werden Biopsien entnommen, die anschließend durch verschiedene mikrobiologische, biochemische und histologische Nachweismethoden analysiert werden. Der *H. pylori*-Nachweis kann durch Kultivierung des Bakteriums auf komplexen Medien, Urease-Schnelltest, histologische Färbungen oder PCR erfolgen (Makristathis *et al.*, 2004; Malfertheiner, 1994). Bei einem Urease-Schnelltest wird ein Indikatormedium verwendet, das eine Alkalisierung aufgrund der Ureaseaktivität durch einen Farbumschlag anzeigt. Ein großer Vorteil in der Kultivierung der Bakterien liegt darin, dass man sie anschließend einer Antibiotikaresistenzprüfung unterziehen kann, deren Ergebnis eine optimale Eradikation erleichtert.

Nicht-invasive Methoden:

Die am häufigsten verwendete nicht-invasive Nachweismethode ist der sogenannte ^{13}C-Harnstoff-Atemtest. Bei diesem Test wird dem Probanden gelöster ^{13}C-Harnstoff verabreicht und das durch die Urease entstandene $^{13}CO_2$ mittels massen-spektroskopischer Analyse in der Atemluft nachgewiesen (Pathak *et al.*, 2004). Weitere nicht-invasive Methoden zum Nachweis von *H. pylori* sind serologische Untersuchungen aus Blut, Speichel und Urin sowie der Stuhl-Antigen-Test, der spezifische *H. pylori*-Antigene mittels ELISA detektiert (Asfeldt *et al.*, 2004; Kato *et al.*, 2004).

2.5.2 Therapie

Aus ökonomischen Gründen werden Patienten mit einer asymptomatischen Gastritis nicht therapiert. Nach den Richtlinien des Maastricht Konsensus der EHSG sollen Personen mit *H. pylori*-assoziierter symptomatischer Gastritis, Ulkuserkrankungen, MALT-Lymphomen sowie nach der Operation eines Magenadenokarzinoms eradiziert werden (Malfertheiner, 2003; Malfertheiner *et al.*, 2002). Derzeit ist die Tripel-Therapie als Standardtherapie vorgeschlagen. Bei dieser Therapie wird eine Kombination aus zwei Antibiotika (Clarithromycin mit Amoxycillin oder Metronidazol) und einem Protonenpumpeninhibitor (PPI) verabreicht (Lind *et al.*, 1999). Da die Zahl der antibiotikaresistenten *H. pylori*-Stämme immer weiter ansteigt, wird alternativ eine Quadrupeltherapie angeboten, welche sich aus einem PPI, Metronidazol, Tetrazyklin und Wismuthsalz zusammensetzt.

2.6 Problemstellung

Teilarbeit A:

Eine über Jahre hinweg andauernde chronische Entzündung stellt ein erhöhtes Risiko für die Krebsentstehung dar. *H. pylori* als Typ I-Karzinogen könnte somit eine bedeutende Rolle bei der Umwandlung der Magenmukosa, entlang der präkanzerösen Stufen (Atrophie, Metaplasie und Dysplasie) bis hin zum Magenadenokarzinom, zugeschrieben werden. Mit Hilfe des etablierten Tiermodells, der Mongolischen Gerbils (Wüstenrennmäuse, *Meriones unguiculatus*), sollen schrittweise die pathologischen Veränderungen der Magenmukosa unter einer chronischen *H. pylori*-Infektion beleuchtet werden. Es sollen hauptsächlich die Faktoren der Prädisposition für die Entwicklung des Magenadenokarzinoms vom intestinalen Typ untersucht werden. Dabei soll die Frage geklärt werden, was tritt zuerst auf, die Hypochlorhydrie oder die Hypergastrinämie, und was ist die Ursache und was die Folge der damit assoziierten Hyperproliferation, Atrophie und Metaplasie der Korpusmukosa. Ein spezieller Teilaspekt dieses Abschnitts der Arbeit liegt in der Quantifizierung („real-time“ RT-PCR) diverser Mediatoren und Entzündungsfaktoren auf mRNA-Ebene. Da das Genom des Mongolischen Gerbils nicht sequenziert ist, müssen die zu untersuchenden Gene mit Hilfe der sogenannten „cross-species“-Amplifikation isoliert und anschließend sequenziert werden.

Teilarbeit B:

Das Peptidhormon Gastrin, das hauptsächlich die Säureregulation im Magen steuert, beeinflusst zusätzlich diverse wichtige zelluläre Prozesse, die vermutlich eine bedeutende Rolle bei der Pathogenese im Magen besitzen. Zu den zellulären, durch Gastrin regulierten Prozessen zählen beispielsweise die Regulation der Proliferation, Apoptose, Zellinvasion, Zellmigration und Angiogenese. Die Daten von Rieder *et al.* 2005 zeigen, dass es bei einer Infektion mit *H. pylori* nach 32 Wochen zu einer Hypergastrinämie im Gerbilmodell kommt. Deshalb sollen mit Hilfe von *in vitro* Experimenten die durch *H. pylori* ausgelösten Signaltransduktionskaskaden untersucht werden, welche für die Induktion des Gastrinpromotors verantwortlich sind. Hierfür soll eine humane Magenadenokarzinomzelllinie (AGS), die stabil mit einem Gastrin-Luziferase-Reporterkonstrukt (AMO) transfiziert ist, verwendet werden. Das Hauptaugenmerk bei diesem Teilabschnitt liegt auf der Identifizierung

des bislang unbekannten *H. pylori*-Rezeptors der Wirtszelle, der für die Gastrinpromotorinduktion notwendig ist.

Teilarbeit C:

Im dritten Teilprojekt dieser Arbeit sollen die am Tiermodell beobachteten *H. pylori*-induzierten Regulationsmechanismen, im Besonderen die durch Wildtyp-Stämme hervorgerufene erhöhte KC-(IL-8-Homolog)-Expression, *in vitro* an einer humanen Magenadenokarzinomzelllinie (AGS) untersucht werden. Bei diesen Experimenten soll der Fokus auf der Identifizierung des bakteriellen Liganden liegen, der die Ausschüttung von IL-8 induziert.

3. Synopsis

3.1 Mongolische Gerbils als Tiermodell für die *H. pylori*-induzierte Karzinogenese im Magen

Originalarbeit:

Wiedemann T., *et al.* (2009) *Helicobacter pylori cag*-pathogenicity island-dependent early immunological response triggers later precancerous gastric changes in Mongolian gerbils. *PLoS One* 4: e4754.

In den letzten Jahren fanden zahlreiche Tiermodelle Einsatz, um die Pathogenese des Magenadenokarzinoms im Zusammenhang mit der chronischen *H. pylori*-assoziierten Gastritis zu beleuchten. Die am häufigsten dazu verwendeten Nagetiere, die Mäuse, bieten den Vorteil der genetischen Manipulierbarkeit, doch verläuft die *H. pylori*-Infektion nicht dem humanen Entzündungsprozess entsprechend, da die *cag*-PAI in Mäusen nicht stabil ist und somit *H. pylori*-Typ I-Stämme für Infektionsversuche nicht verwendet werden können (Philpott *et al.*, 2002). Aus diesem Grund wurde das bereits etablierte Tiermodell des Mongolischen Gerbils eingesetzt (Rieder *et al.*, 2005). In dieser Teilarbeit soll die *H. pylori*-Infektion über einen Zeitverlauf von 2, 4, 8, 16 und 64 Wochen analysiert werden, um die Reihenfolge der nach 32 Wochen beschriebenen veränderten Parameter in einen größeren Kontext setzen zu können. Es sollen schrittweise die pathophysiologischen Veränderungen der Magenmukosa unter einer chronischen *H. pylori*-Infektion beleuchtet werden. Für diese Untersuchung wurden neben einer nicht infizierten Gerbil-Kontrollgruppe eine weitere Gruppe mit dem Gerbil-adaptierten Typ I-Wildtypstamm B128 (Wt) und eine dritte Gruppe mit einer entsprechenden isogenen Δ*cag*Y-Mutante oral infiziert. Die Δ*cag*Y-Mutante besitzt ein defektes T4SS, wodurch die Translokation des Effektorproteins CagA in die Wirtszelle nicht mehr möglich ist. Dadurch wurde der Effekt der *cag*-PAI auf die zeitliche Veränderung der Magenmukosa betrachtet.

3.1.1 *cag*-PAI-abhängige Kolonisierungsdichte

Durch Kultivierung von Gewebehomogenisaten wurden die *H. pylori* B128 und *H. pylori* B128Δ*cag*Y Kolonisierungsdichten (Cfu) für die jeweiligen Zeitpunkte bestimmt. Nach zwei Wochen Infektion konnte sowohl für die Wt-infizierte als auch für die Δ*cag*Y-infizierte Gruppe eine vergleichbare Kolonisierungsdichte zwischen den

jeweiligen Antrum- (10^5 Cfu/g Magen) und Korpusproben (10^3 Cfu/g Magen) ermittelt werden. Bei der Analyse der späteren Zeitpunkte konnten zwei *cag*-PAI-abhängige Vorgänge beobachtet werden. Zum einen sank die Kolonisierungsdichte in der antralen Mukosa der B128-infizierten Gerbils nach 32 Wochen signifikant, wohingegen die Kolonisierungsdichte dieser Tiere in der Korpusmukosa kontinuierlich von vier bis 32 Wochen anstieg (Fig. 1). In B128Δ*cag*Y-infizierten Tieren konnten keine Schwankungen in der Kolonisierungsdichte festgestellt werden.

3.1.2. *cag*-PAI-abhängige pathohistologische Veränderungen

Durch histologische Untersuchungen von Gewebeschnitten aus Tieren, die mit dem Wt-Stamm oder der Mutante infiziert waren, konnten weitere *cag*-PAI-abhängige Prozesse nachgewiesen werden. Nur Tiere, die mit dem Wt-Stamm infiziert worden waren, wiesen eine frühe, hochgradig aktive und chronische Gastritis in der antralen Mukosa acht Wochen nach Infektion auf. In der Korpusmukosa zeigten diese Tiere eine über das gesamte Zeitverlaufsexperiment ansteigende aktive und chronische Gastritis, was in Tieren, die mit der Mutante infiziert worden waren, nicht der Fall war (Fig. 2A-C). Ein weiterer für die Pathogenese wichtiger Punkt lag in der *cag*-PAI-abhängigen kompletten Parietalzellatrophie der Korpusmukosa acht Wochen nach Infektion (Fig. 4B,C+Tab.1). Betrachtet man die Gesamtarchitektur der Magenmukosa von Wt-infizierten im Vergleich zu B128Δ*cag*Y-infizierten Tieren, so fällt das Hauptaugenmerk auf die in der Korpusmukosa von Wt-infizierten Tieren stattfindende starke Dedifferenzierung des Gewebes in Richtung des intestinalen Typs. Diese Veränderungen konnten im Gewebe von B128Δ*cag*Y-infizierten Tieren nicht beobachtet werden (Fig. 3A+Tab. 1). Somit konnte die sogenannte „Correa-Kaskade“ (chronische Gastritis → chronisch-atrophische Gastritis → intestinale Metaplasie → Dysplasie), welche für die Entstehung eines Karzinoms des intestinalen-Typs beschrieben wurde, nur bei Wt-infizierten Tieren beobachtet werden. Bei diesen Tieren konnten ab einer Infektionsdauer von 16 Wochen fokale Dysplasien nachgewiesen werden. Bei der Analyse der Schleimproduktion durch eine spezielle PAS-Alcian-Blau-Färbung konnten *cag*-PAI-abhängige Veränderungen der Verteilung von saurem und neutralem Magenschleim beobachtet werden (Fig. 3B). Diese sogenannte Schleimdrüsenmetaplasie konnte nur bei Wt-infizierten Tieren ab acht Wochen nachgewiesen werden.

3.1.3 *cag*-PAI-abhängige immunologische Veränderungen

In diesem Abschnitt sollte die histologisch beobachtete, starke Infiltration von Entzündungszellen in die Lamina propria der Wt-infizierten Tiere untersucht werden. Hierfür wurde das Expressionsmuster der wichtigsten anti- und proinflammatorischen Zytokine mittels „real-time“ RT-PCR untersucht (IL-1β, IFN-γ, TNF-α, IL-6, KC, IL-10). Für die Detektion der Zytokine mussten spezifische Sonden und Primer entworfen werden. Da im Gegensatz zur Maus (*Mus musculus*) und zur Ratte (*Rattus norvegicus*) die Nukleotidsequenz vom Gerbil nicht über Genbanken zur Verfügung stand, mussten die zu analysierenden Gene zuerst mit Hilfe einer „cross-species“-Amplifikation isoliert und dann sequenziert werden. Die so erworbenen Oligonukleotid- und Sondensequenzen der anti- und proinflammatorischen Zytokine wurden in der RT-PCR eingesetzt. Zur Normalisierung der gemessenen Mediatoren wurde das 18S rRNA Gen verwendet.

Die Analyse ergab einen sprunghaften Anstieg der proinflammatorischen Zytokine in Wt-infizierten Tieren in der Antrum- und Korpusmukosa im Infektionszeitraum zwischen vier und acht Wochen. Im Gegensatz dazu konnte bei den B128Δ*cag*Y-infizierten Tieren nur ein geringer, auf die antrale Mukosa begrenzter kontinuierlicher Anstieg der proinflammatorischen Zytokine nachgewiesen werden (Fig. 5A-E). Da sowohl TNF-α als auch IL-1β starke Inhibitoren der Säuresekretion in der Korpusmukosa sind (Beales and Calam, 1998), stellen sie weitere *cag*-PAI-abhängige Faktoren dar, die am malignen Verlauf der *H. pylori* Typ I-Infektion eine essentielle Rolle spielen. Bei der Bestimmung der Expression des anti-inflammatorischen Zytokins IL-10 konnte ein gradueller Anstieg in der Antrum- wie auch in der Korpusmukosa von Wt- und B128Δ*cag*Y-infizierten Tieren über den gesamten Zeitverlauf gemessen werden. Interessanterweise zeigten jedoch nur Tiere, welche mit der Mutante infiziert worden waren, einen signifikanten Anstieg der IL-10 Expression in der Antrum- und Korpusmukosa nach acht Wochen Infektion. Im Gegensatz dazu zeigten Wt-infizierte Tiere nur in der Korpusmukosa einen signifikanten IL-10-Anstieg nach diesem Zeitraum (Fig. 5F). Diese Ergebnisse deuten daraufhin, dass IL-10 ein entscheidender Regulator bei der *H. pylori*-vermittelten chronischen Entzündung der Magenmukosa darstellt.

3.1.4 *cag*-PAI-abhängige physiologische Veränderungen

Im Weiteren sollte der Effekt der *H. pylori*-Infektion auf die physiologischen Parameter pH-Wert und Gastrinausschüttung analysiert werden. Die Messungen ergaben einen signifikant erhöhten pH-Wert (Hypochlorhydrie) nach 16 Wochen und eine ebenso signifikant erhöhte Gastrinkonzentration (Hypergastrinämie) nach 32 Wochen in Wt-infizierten Tieren (Fig. 6A,B). B128Δ*cag*Y-infizierte Tiere zeigten ähnliche pH- und Gastrinwerte wie die nicht-infizierte Kontrollgruppe. Aufgrund dieser Resultate konnte gezeigt werden, dass in *H. pylori* Typ I-infizierten Gerbils eine frühe Entzündung der Korpusmukosa, einhergehend mit einer fortschreitenden Parietalzellatrophie, der Grund für spätere physiologische Veränderungen ist. Diese scheinen für die präkanzerösen Transformationen der Magenmukosa verantwortlich zu sein.

3.2 *H. pylori*-induzierte Gastrinexpression

Originalarbeit:

Wiedemann T., *et al.* (2009) „A novel integrin-β_5-ILK signaling complex is jointly responsible for *Helicobacter pylori*-induced precancerous conditions"
(Cellular Microbiology, submitted)

In den letzten zwei Jahrzehnten ließen mehrere Studien vermuten, dass dem gastrointestinalen Hormon Gastrin eine bedeutende Schlüsselrolle in der Regulierung der normalen, aber auch malignen Entwicklung der gastrointestinalen Mukosa zukommt (Dockray *et al.*, 2001; Pritchard and Przemeck, 2004; Rozengurt and Walsh, 2001; Cui *et al.*, 2006; Przemeck *et al.*, 2008). Wie im Tiermodellexperiment (siehe 3.1) gezeigt werden konnte, verursacht die *H. pylori*-Infektion nur unter Verwendung des Wt-Stammes (B128) eine Hypochlorhydrie und eine Hypergastrinämie, die auf eine direkte oder indirekte Regulierung der Gastrinexpression durch ein intaktes T4SS schließen lässt. Um dieses näher zu untersuchen, verwendeten wir in dieser Teilarbeit eine humane Magenkarzinom-Zelllinie (AGS), welche stabil mit einem Gastrin-Luziferase-Reporterkonstrukt (AMO) transfiziert war. Als Kontrolle diente eine entsprechende Gastrinpromotormutante (AM4). Mit Hilfe dieser Zellen sollte der Effekt der *cag*-PAI von *H. pylori* Typ I-Stämmen auf den Gastrinpromotor analysiert werden. Vorversuche mit diesem *in vitro* Modell ergaben eine signifikante Erhöhung der Luziferaseaktivität durch die

Stimulierung mit *H. pylori* Typ I-Stämmen. Andere Pathogene des Gastrointestinaltraktes wie beispielsweise *Escherichia coli* und *Campylobacter jejuni* sowie *H. pylori* Typ II-Stämme waren nicht in der Lage den Gastrinpromotor zu induzieren (Rieder *et al.*, 2005).

3.2.1 Bakterielle Voraussetzungen für die Gastrinexpression

In diesem Teilabschnitt sollten die Voraussetzungen eines *H. pylori*-Stammes für die Induzierung des Gastrinpromotors identifiziert werden. Das Hauptaugenmerk lag hierbei auf der genetischen Ausstattung des Bakteriums sowie seiner Oberflächenproteine, die potentielle Bindungsstellen für die Wirtszelle darstellen könnten. Die erhaltenen Daten bestätigten unsere bisherigen Beobachtungen, dass im Gegensatz zu *H. pylori* Typ II-Stämmen nur *H. pylori* Typ I-Stämme mit einem funktionellen T4SS in der Lage sind die Gastrinexpression zu stimulieren (Rieder *et al.*, 2005). Des Weiteren wurde bestätigt, dass es sich bei der Induktion des Gastrinpromotors um ein *H. pylori*-spezifisches Phänomen handelt, da andere Pathogene des Gastrointestinaltraktes wie *Escherichia coli* und *Campylobacter jejuni* keine Stimulierung des Gastrinpromotors hervorrufen konnten (Fig. 1).
Bei der Untersuchung der Hauptvirulenzfaktoren VacA und CagA zeigte das Onkogen CagA nach seiner Überexpression keinen Effekt auf den Gastrinpromotor (Fig. 2A,B). Auch die Stimulierung der Zellen mit säureaktiviertem VacA ergab keine Induktion des Promotors (Fig. 2C). Eine wichtige Rolle bei der Besiedelung der Magenschleimhaut spielten die Adhäsine, eine Gruppe von äußeren Membranproteinen, die durch ihre Bindung an die Wirtszelle eine dauerhafte Infektion mit *H. pylori* ermöglichen (Linden *et al.*, 2002; Mahdavi *et al.*, 2002; Odenbreit *et al.*, 1999). Die Resultate der Untersuchung zeigten, dass die getesteten Adhäsine keinen Effekt auf die Gastrinpromotorinduktion haben (Fig. 3B), jedoch der direkte Kontakt eines intakten *H. pylori*-Stammes mit der Wirtszelle essentiell für die Promotorinduktion ist (Fig. 3A,C).

3.2.2 Essentielle Strukturen, Mechanismen und Signalwege in der Wirtszelle

In der 2007 erschienenen Studie von Kwock *et al.* konnte zum ersten Mal gezeigt werden, dass *H. pylori* $\alpha_5\beta_1$-Integrine als Wirtsrezeptor verwendet, um CagA mit Hilfe des T4SS in die Epithelzelle zu translozieren (Kwok *et al.*, 2007). Aufgrund der neuen Erkenntnis, dass die Proteinfamilie der Integrine als potentielle Wirtsrezeptoren bei der *H. pylori*-Erkennung in Betracht kommt, wurden diverse Integrine in Hinblick auf

die Gastrinexpression getestet. Hierfür wurde unter Verwendung von spezifischen anti-β-Integrin-Blockierungsantikörpern eine Beteiligung des vorher beschriebenen $\alpha_5\beta_1$-Integrins bei der *H. pylori*-induzierten Gastrinpromotorstimulierung ausgeschlossen (Fig. 4A,B). Im Gegensatz dazu konnte eine signifikante Reduktion der *H. pylori*-induzierten Promotoraktivität nach Präinkubation mit einem anti-β_5-Integrin-Blockierungsantikörper gezeigt werden (Fig. 4B). Mit dieser Studie gelang es uns erstmalig einen weiteren von *H. pylori* genutzten Integrinrezeptor auf der Wirtszelle zu beschreiben.

Nach Entdeckung des β_5-Integrins als wichtigen Rezeptor für die *H. pylori*-Pathogenese stellte sich die Frage, über welche inneren Zellstrukturen die Signale weitergeleitet werden. Die bis dato im Zusammenhang mit β-Integrin beschriebenen sogenannten „fokal adhesion“-Strukturen (FA), ILK (integrin-linked-kinase) und FAK (focal-adhesion-kinase) (Harburger and Calderwood, 2009) wurden mittels siRNA ausgeschaltet und die Gastrinpromotoraktivität bestimmt. Die Messungen ergaben, dass *H. pylori* einen neuartigen β_5-Integrin-ILK-Signalkomplex an der Zelloberfläche des Wirts benutzt, um die Signaltransduktions-kaskade für die Aktivierung der Gastrinexpression auszulösen (Fig. 6). Im weiteren Verlauf der Studie konnte durch die Verwendung von spezifischen Inhibitoren gegen die Src-, Abl-, PKC-, Raf-, Erk-, p38-, JAK- und EGF-R-Kinasen deren Involvierung bei der *H. pylori*-induzierten Gastrinexpression bewiesen werden (Fig. 5B+7A).

Aus den gewonnenen Daten dieser Teilarbeit konnte zusammenfassend ein hypothetischer Signaltransduktionsweg für die Aktivierung des Gastrinpromotors herausgearbeitet werden (Fig. 8). Hierbei bindet *H. pylori* über den β_5-Integrin-Rezeptor an die Wirtszelle und aktiviert ILK. Mit Hilfe der Kinasen PKC, Src und Abl kommt es zu einer aktivierenden Kopplung von ILK mit ADAM 10/17 (a disintegrin and metalloprotease). Aktivierte ADAM 10/17 schnüren den in der Membran sitzenden inaktiven EGF-R-Liganden, TGF (transforming growth factor)-α, ab. Freies aktives TGF-α bindet anschließend an seinen Rezeptor EGF-R und induziert die Gastrinexpression über den Ras-Raf-MEK-ERK-Signalweg.

Unter Verwendung von EGF und anti- EGF-Antikörpern konnte gezeigt werden, dass EGF einen synergistischen Effekt auf die *H. pylori*-induzierte Gastrinexpression hat, anscheinend aber nicht den oben beschriebenen Weg einschlägt (Fig. 5A,B).

3.3 *H. pylori* CagL-induzierte IL-8-Expression

Originalpaper:

Wiedemann T., *et al.* (2009) „A C-terminal coiled-coil region of CagL is responsible for *Helicobacter pylori*-induced IL-8-expression“
(Molecular Microbiology, submitted)

Das Pathogen *Helicobacter pylori* adhäriert an die Magenepithelzellen, wodurch Signaltransduktionskaskaden gestartet werden, die in der Induktion von Zytokinen, der Infiltration von immunreaktiven Zellen in die Lamina propria und letztendlich in einer chronischen Gastritis resultieren. Hierfür ist das T4SS von entscheidender Bedeutung. Wie auch im Tiermodell beobachtet (siehe 3.1.3), löst die Adhärenz des Bakteriums eine starke proinflammatorische Immunantwort in der Mukosa aus. Diese führt durch die Aktivierung des Transkriptionsfaktors NF-κB und über die Phosphorylierung der MKK4/JNK-Kinasen zur Sezernierung des Chemokins IL-8 (Foryst-Ludwig and Naumann, 2000; Keates *et al.*, 1997; Chu *et al.*, 2003). Die Sekretion von IL-8 durch die *H. pylori*-stimulierten Epithelzellen führt überwiegend zu einem erweiterten Einstrom von Neutrophilen und Lymphozyten in das betroffene Gewebe und spielt somit eine wichtige Rolle bei der *H. pylori*-vermittelten Immunopathogenese. Für die *in vitro* Untersuchung der *H. pylori*-induzierten IL-8-Expression wurde eine humane Magenkarzinom-Zelllinie (AGS) verwendet. Die Bestimmung der IL-8-Konzentration im Zellüberstand wurde mittels IL-8-ELISA durchgeführt. Um die *H. pylori*-induzierte IL-8-Promotoraktivität analysieren zu können, wurden die Zellen transient mit einem humanen IL-8-Promotor-Luziferase-Reporterkonstrukt transfiziert und anschließend stimuliert.
Die Analyse der *H. pylori*-induzierten Signaltransduktionskaskaden der Gastrin- und IL-8-Expression sollte die Rolle dieser Faktoren bei der Entstehung präkanzeröser Transformationen der Magenmukosa beleuchten. Da in einer früheren Studie das Stimulierungsverhalten des Gastrinpromoters mit *H. pylori* Typ I-Stämmen sowie den isogenen *cag*-Mutanten exakt dem Stimulierungsmuster der IL-8-Sekretion der AGS-Zellen entsprach (Rieder *et al.*, 2005), sollte in diesem Teilprojekt die Signaltransduktionskaskade der *H. pylori*-Infektion auf die IL-8-Expression näher analysiert werden.
Mit Hilfe von speziellen Filtereinsätzen konnte bestätigt werden, dass der direkte Kontakt von *H. pylori* Typ I-Stämmen an die Magenepithelzellen notwendig ist, um

die IL-8-Expression zu stimulieren (Fig. 1A-D) (Rieder *et al.*, 1997). Im weiteren Verlauf der Studie konnte durch Verwendung von diversen *H. pylori*-Adhäsinmutanten die Beteiligung der Hauptadhäsine BabA, SabA und AlpAB an der *H. pylori*-induzierten IL-8-Sekretion ausgeschlossen werden (Fig. 1E). Interessanterweise konnte bei der systematischen Untersuchung aller *cag*-Mutanten eine vollständige Blockierung der IL-8-Expression durch eine ΔCagL-Mutante beobachtet werden (Fig. 1A,B).

Das Protein CagL ist an der Spitze des T4SS lokalisiert und wurde erstmals durch Kwok *et al.* als essentiell für die CagA-Translokation beschrieben (Kwok *et al.*, 2007). Aufgrund dieser Erkenntnisse wurde die 3D-Struktur von CagL mit Hilfe von speziellen Proteinstruktur-Vorhersage-Programmen analysiert. Dabei wurde eine C-terminale „coiled-coil“ Region identifiziert, die eine potentielle Interaktion zwischen Bakterium und Wirtszelle darstellen könnte (Strauss and Keller, 2008; Gazi *et al.*, 2009). Messungen der IL-8-Expression nach Stimulierung mit speziell konstruierten Δ*cag*L-Mutanten (B128Δ*cag*L(1-206) bzw. B128Δ*cag*L(1-224)) ergaben, dass die C-terminale „coiled-coil“-Region essentiell für die *H. pylori* Typ I-induzierte IL-8-Expression ist (Fig. 2E,F). Durch Immunfloureszenzaufnahmen konnte im weiteren Verlauf der Arbeit gezeigt werden, dass die C-terminale „coiled-coil“-Region des CagL-Proteins eine wichtige Rolle bei der Adhärenz des Bakteriums an seine Wirtszellen spielt (Fig. 3). Bei der Inkubation von AGS-Zellen mit B128Δ*cag*L(1-206) bzw. B128Δ*cag*L(1-224) zeigte sich im Vergleich zum Wt-Stamm eine Reduktion der Adhärenz von circa 50%. Somit konnte ein neuer Wirtszellligand von *H. pylori* identifiziert werden, der sowohl bei der Induktion des proinflammatorischen Chemokins IL-8 als auch bei der Adhärenz des Keims an seine Wirtszelle eine bedeutende Rolle spielt.

Bei der Untersuchung der IL-8-Signalkaskaden innerhalb der Wirtszelle konnte wie schon bei der Gastrinpromotorinduktion (siehe 3.2.2) eine Beteiligung des EGF-R-Liganden TGF-α sowie dessen Rezeptor EGF-R nachgewiesen werden. Sowohl durch die Präinkubation der Zellen mit einem spezifischen anti-TGF-α-Antikörper als auch durch den Einsatz des EGF-R-Inhibitor Gefitinib konnte die *H. pylori*-induzierte IL-8-Sekretion signifikant blockiert werden (Fig. 4B).

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5. Darstellung des Eigenanteils

Teilarbeit A

Helicobacter pylori cag-pathogenicity island-dependent early immunological response triggers later precancerous gastric changes in Mongolian gerbils

Tobias Wiedemann, Eva Löll, Susanna Müller, Mechthild Stöckelhuber, Manfred Stolte, Rainer Haas, Gabriele Rieder

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Alle Arbeiten am Tier wurden von mir durchgeführt. Die Datenauswertung sowie die Durchführung der Experimente die zu den Abbildungen (Fig. 1; 5; 6A; 7 und Table S1) führten, stammen von mir. Alle Abbildungen und Tabellen wurden von mir erstellt bzw. bearbeitet (Fig. 1-7; Table 1; Table S1). Die Bilder für Abbildung 3 und 4 wurden von EL aufgenommen und von SM, MeS sowie MaS pathologisch untersucht (Fig. 2, Table 1). Das Manuskript wurde hauptsächlich durch GR verfasst.

Der Eigenanteil an der Entstehung dieses Manuskripts beträgt ~60%.

Teilarbeit B

A novel integrin-β_5-ILK signaling complex is jointly responsible for *Helicobacter pylori*-induced precancerous conditions

Tobias Wiedemann, Stefan Hofbaur, Gabriele Rieder

Submitted to Cellular Microbiology

Alle Versuchsdurchführungen, Optimierungen, Datenerhebungen sowie Datenauswertungen wurden von mir durchgeführt. Das Manuskript mit allen Abbildungen wurde von mir verfasst und durch GR ergänzt. Die Herstellung der B128Δ*cag*L Mutante wurde von SH im Rahmen seiner medizinischer Doktorarbeit durchgeführt.

Der Eigenanteil an der Entstehung dieses Manuskripts beträgt ~90%.

Teilarbeit C

A C-terminal coiled-coil region of CagL is responsible for *Helicobacter pylori*-induced IL-8-expression

Tobias Wiedemann, Stefan Hofbaur, Eva Löll, Gabriele Rieder

Submitted to Molecular Microbiology

Mein Beitrag zu dieser Arbeit war die Optimierung des Versuchsaufbaus sowie die Durchführung der IL-8 ELISA (Fig. 1B, 1D, 1E; 2F und 4). SH als medizinischer Doktorand wurde von mir in die Zellkulturtechnik eingeführt und mitbetreut. SH lieferte die Ergebnisse der IL-8 Promotorstudie (Fig. 1A, 1C; 2E). Die Fluoreszenzaufnahmen (Fig. 3) stammen von EL. Das Manuskript mit allen Abbildungen wurde von mir verfasst und durch GR ergänzt.

Der Eigenanteil an der Entstehung dieses Manuskripts beträgt ~45%.

6. Anhang

Teilarbeit A

Teilarbeit B

Teilarbeit C

OPEN ACCESS Freely available online

PLoS one

Helicobacter pylori cag-Pathogenicity Island-Dependent Early Immunological Response Triggers Later Precancerous Gastric Changes in Mongolian Gerbils

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Abstract

Infection with Helicobacter pylori, carrying a functional *cag* type IV secretion system (*cag*-T4SS) to inject the Cytotoxin associated antigen (CagA) into gastric cells, is associated with an increased risk for severe gastric diseases in humans. Here we studied the pathomechanism of *H. pylori* and the role of the *cag*-pathogenicity island (*cag*-PAI) for the induction of gastric ulcer and precancerous conditions over time (2–64 weeks) using the Mongolian gerbil model. Animals were challenged with *H. pylori* B128 (WT), or an isogenic B128ΔcagY mutant-strain that produces CagA, but is unable to translocate it into gastric cells. *H. pylori* colonization density was quantified in antrum and corpus mucosa separately. Paraffin sections were graded for inflammation and histological changes verified by immunohistochemistry. Physiological and inflammatory markers were quantitated by RIA and RT-PCR, respectively. An early *cag*-T4SS-dependent inflammation of the corpus mucosa (4–8 weeks) occurred only in WT-infected animals, resulting in a severe active and chronic gastritis with a significant increase of proinflammatory cytokines, mucous gland metaplasia, and atrophy of the parietal cells. At late time points only WT-infected animals developed hypochlorhydria and hypergastrinemia in parallel to gastric ulcers, gastritis cystica profunda, and focal dysplasia. The early *cag*-PAI-dependent immunological response triggers later physiological and histopathological alterations towards gastric malignancies.

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Introduction

The bacterial pathogen *Helicobacter pylori* colonizes the human gastric mucosa of about 50% of the world population to induce a chronic gastritis. As a consequence of an inflammation lasting for decades, gastric sequelae are developed like peptic ulcer and malignant diseases, such as gastric adenocarcinoma or MALT (mucosa-associated lymphoid tissue)-lymphoma [1,2]. Due to epidemiological studies the WHO declared *H. pylori* as a class I carcinogen in 1994 [3]. Furthermore, a *H. pylori*-induced chronic inflammation in the human body represents a risk factor for developing gastric cancer *per se*. A malignant transformation of cells is usually a multi-factorial process [4,5], which is also true for gastric carcinoma. In addition to *H. pylori* infection, environmental (diet, smoking) [6] and host factors (gene polymorphisms, e.g. interleukin (IL)-1β) [7] are certainly involved in its induction. Therefore the question remains what is the contribution of *H. pylori* for induction of gastric cancer.

H. pylori produces a number of important virulence factors inducing a local inflammation in the stomach. Two major virulence factors have been studied intensively, the vacuolating cytotoxin A (VacA) [8] and the cytotoxin-associated antigen A (CagA).

VacA is a secreted toxin that induces vacuoles in gastric epithelial cells, modulates cellular permeability, and enters immune cells via the β2 integrin receptor [8]. This is a possible mechanism for *H. pylori* to escape the adaptive immune system establishing a chronic inflammation. The cag-type IV secretion system (*cag*-T4SS), which is encoded on the *cag*-pathogenicity island (*cag*-PAI) [9] injects the effector protein CagA as well as peptidoglycan [10], resulting in activation of nuclear factor (NF)- κB and gastric inflammation. Translocated CagA is phosphorylated on certain tyrosine residues, leading to actin-cytoskeletal rearrangements, elongation, and the scattering phenotype of infected cells in vitro. The ''needle''-like structure of the T4SS interacts with the integrin α5β1-receptor on the gastric epithelial cells, to deliver CagA into the host cells [11], which finally leads to an activation of signal cascades inducing pro-inflammatory cytokines. Those *H. pylori* strains that express VacA and carry a complete and functional T4SS to translocate CagA into gastric host cells are designated as type I-strains, whereas type II-strains are defective in the *cag*-PAI and do not secrete functional VacA. Epidemiological studies have shown that type I-strains are associated with a more pronounced development of peptic ulcer and gastric cancer in humans [12,13].

To study the effect of *H. pylori* on the induction of gastroduodenal diseases different animal models have been established. *H. pylori* type I-strains are not fully virulent in mouse models, since they neither inject CagA, nor does VacA induce immunomodulation in murine T cells [8]. The mouse model is limited, since it cannot be used to recapitulate the *cag*-PAI dependent gastric carcinogenesis. It could be shown that infection of wild-type mice with cag^+ strains frequently leads to deletions within the *cag*-PAI [14,15]. Therefore, the Mongolian gerbil model has been established to study *H. pylori* pathogenesis towards gastric adenocarcinoma [16].

In earlier studies analyzing only a single time point of infection (seven month) we could demonstrate that only a chronic infection of *H. pylori* type I-strain was able to induce an atrophic corpus-dominant gastritis in Mongolian gerbils [17], which is a risk factor for developing gastric cancer. This observation was supported by human studies to be a precancerous condition, essential to be followed up tightly.

To gain more insight into the pathomechanisms of *H. pylori* and the role of the *cag*-PAI on the development of gastroduodenal diseases, we now performed a time course experiment. Mongolian gerbils were infected for 2, 4, 8, 16, 32, and 64 weeks with *H. pylori* B128 WT- (type I), or B128 Δ*cag*Y mutant-strain (type II). Drastic differences were observed in the capacity of the B128 WT and the ΔcagY-mutant strain to colonize the gastric antrum and corpus mucosa inducing severe inflammation over time.

Our data suggest that an early inflammation in the antrum and especially in the corpus mucosa at eight weeks of infection, which is a *cag*-PAI-dependent mechanism, is triggering several months later physiological (hypochlorhydria and hypergastrinemia) and histopathological changes towards precancerous conditions. In general, our long-term *in vivo*-study reveals early markers for late gastric diseases that represent potential risk factors for precancerous transformation with a possible clinical application.

Materials and Methods

Bacterial strains

Helicobacter pylori B128, a Mongolian gerbil-adapted type I-strain (CagA, VacA: s1m2) [18], and its isogenic mutant B128Δ*cag*Y (both streptomycin resistant) were used in this study as previous described [17]. Both strains carried a chromosomal streptomycin resistance that allowed quantitative recovery of *H. pylori* from the gerbil stomach by antibiotic selection (streptomycin 250 mg/L) [19]. Each antral and corpus tissue specimen was homogenized (glass homogenizer, Ochs, Bovenden, Germany) in 1 ml Brucella broth, appropriate dilutions were spread on selective serum plates (GC agar (Oxoid, Wesel, Germany) supplemented with horse serum (8%), vancomycin (10 mg/l), trimethoprim (5 mg/l), nystatin (1 mg/l)), and streptomycin (250 mg/l)), and incubated under microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) at 37^0C for up to five days. Numbers of colony forming units (CFU) were expressed per gram of gastric tissue. *H. pylori* reisolates were tested for urease (urea broth, Oxoid), oxidase (DrySlide, BBL), and catalase (3% hyperperoxid-solution) activity.

Animals and infection experiments

Outbred Mongolian gerbils (n = 167 females) from our own breeding colony were specific pathogen free (SPF) and housed in SEALSAFE IVC cages (H-Temp, Tecniplast, Hohenpeissenberg, Germany) in an air-conditioned biohazard room (room temperature, 23+/2^{0}C; relative humidity 55+/–5%; 12/12-h light/dark cycle) with free access to a commercial gerbil diet (ssniff Gerbil, SSNIFF, Soest, Germany) and sterile tap water. Animals at the age of 8–12 weeks were challenged orogastrically three-times over five consecutive days with approximately 10^9 viable *H. pylori*. Age-matched control animals were inoculated with identical volumes of sterile Brucella broth alone. All experiments and procedures carried out were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Regierung von Oberbayern (AZ 55.2-1-54-2531-41/04 and 55.2-1-54-2531-78/05). The animals were sacrificed after specified time of infection (2, 4, 8, 16, 32, and 64 weeks), the stomach opened along the greater curvature, and the gastric tissue conserved separately in antrum and corpus as previous described [17].

Histopathology

Paraffin embedded longitudinal sections of antrum and corpus were hematoxilin & eosin (H&E) stained for histomorphologic grading of gastritis and mucosal changes. The activity and chronicity of gastritis (scale 0–3) were analyzed double blind according to the updated Sydney System [20] and the intensity of inflammation, metaplasia, and ulcer development (scale 0–5) according to the grading scheme for rodents by Garhart *et al.* [21]. The presence of mucous gland metaplasia was confirmed by a periodic acid-Schiff (PAS)/Alcian blue stain (pH 2.5). The Cancer Risk Index was applied as previous described [22]. In brief, an increased carcinoma risk was shown if active and chronic gastritis in corpus tissue was greater than in the antrum and if metaplastic changes were present.

Immunohistochemistry

Parietal cells were detected immunohistochemically by applying anti-proton pump (PP)-antibody (Medical & Biological Laboratories, LTD, Naka-ku Nagoya, Japan). Positive-staining cells were visualized with diaminobenzidine (DAB) (Vectastain Elite ABC Kit; Vector Laboratories) and morphometrically analyzed with MetaMorph (Visitron, Puchheim Germany) software.

The evaluation of PP-positive cells as marker of the atrophy was performed using following score: 0, PP-positive cells distributed as in the non-infected control tissue; 1, small areas lacking PP-positive cells; 2, large areas without PP-positive cells; 3, no PP-positive cells in the entire tissue section.

pH measurement

The pH value of the gastric mucosa was measured at the corpus tissue using color-fixed indicator test sticks (pH-Fix 0.0-6.0, Macherey-Nagel, Dueren, Germany).

Gastrin and somatostatin radioimmunoassay (RIA)

Plasma was isolated by centrifugation (9,000 rpm, 15 min at 4^0C) from heparinized blood (2 ml) that was collected by cardiac puncture freshly after sacrifice of the animals. For the somatostatin RIA the plasma was extracted with Sep-Pak C18 cartridges (Waters GmbH, Eschborn, Germany). The gastrin- and somatostatin RIA were performed as described in the gastrin [^{125}I] (MP Biomedicals, Heidelberg, Germany) and somatostatin [^{125}I] (IBL, Hamburg, Germany) radioimmunoassay kits, respectively. Rabbit anti-human-gastrin (G-17) and -somatostatin (synthetic cyclic 14) antibodies in human serum albumin were used. The sensitivity of the gastrin and somatostatin assay was 3.3 pg/ml and 6.0 pg/ml, respectively.

RNA isolation and real-time RT-PCR

RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR) measurement were applied as described

previously [17]. cDNA was synthesised using 1 mg total RNA, random hexamer oligonucleotide primers, and TaqMan Reverse Transcriptase Kit (Roche). Oligonucleotide primer and probes specific for IL-1β, interferon (IFN)-γ, KC, somatostatin (Sst), IL-6, IL-10, tumor necrosis factor (TNF)-α, gastrin, histidine decarboxylase, and the housekeeping gene 18S rRNA ([17] and Table S1) were applied for real-time RT-PCR (ABI PRISM 7000, Applied Biosystems). All data were normalized with the corresponding 18S rRNA transcription level using a comparative delta Ct method.

Statistical Analysis

The results were statistically analyzed using the Mann-Whitney U-test for unpaired groups and the Fischer's Exact Test for inter-group differences. The p value < 0.05 was considered as significant.

Results

Colonization density of *H. pylori* wild type decreases in antrum and increases in corpus over time

Mongolian gerbils were orogastrically infected with *H. pylori* B128 wild type (WT) or *H. pylori* B128Δ*cag*Y-mutant strains in a time course experiment of 2, 4, 8, 16, 32, and 64 weeks. In WT-infected gerbils a medial reisolation rate of 85% was obtained, whereas all mutant-infected groups showed reisolation rates of 100% (Table 1). The reisolated *H. pylori* were selected by streptomycin to exclude growth of other gastric bacteria.

After two weeks both groups of infected animals started with a comparable colonization density of *H. pylori* in antrum and corpus of 10^5 and 10^3 CFU/g stomach, respectively (Figure 1A–B). The B128 WT strain increased its density in the corpus slowly but continuously. After 16 weeks of infection the WT bacteria decreased their number in the antrum and equalized with the corpus colonizing bacteria at 10^4 CFU/g stomach at 32 weeks of infection. This colonization rate remained stable until 64 weeks of infection (Figure 1A–B). The observed change in colonization density over time is clearly dependent on a functional *cag*-PAI, since the B128Δ*cag*Y mutant did not significantly change its bacterial density between 4 and 64 weeks, but maintained a constant difference (1–1.5 log stages) in bacterial load between antral and corpus tissue. It was interesting to observe that the colonization density in the antrum of the mutant-infected gerbils was increased by $\geq$1 log stage compared to the WT-infected groups.

Table 1. Macroscopic and histopathological findings of *Helicobacter pylori*-infected and non-infected Mongolian gerbils.

		2 weeks			4 weeks			8 weeks		
		Non-inf.	WT	Δ*cag*Y	Non-inf.	WT	Δ*cag*Y	Non-inf.	WT	Δ*cag*Y
		(n/n) %	(n/n) %	(n/n) %	(n/n) %	(n/n) %	(n/n) %	(n/n) %	(n/n) %	(n/n) %
Number of animals		6	9	4	8	12	10	5	7	9
Reisolation rate		(0/6) 0	(9/9) 100	(4/4) 100	(0/8) 0	(9/12) 75	(10/10) 100	(0/5) 0	(6/7) 86	(9/9) 100
Lymphoid aggregates	antrum	(0/6) 0	(0/9) 0	(0/4) 0	(0/8) 0	(1/9) 11	(0/10) 0	(0/5) 0	(6/6) 100	(5/9) 55
	corpus	(0/6) 0	(0/9) 0	(0/4) 0	(0/8) 0	(0/9) 0	(0/10) 0	(0/5) 0	(6/6) 100	(1/9) 11
Erosion		(0/6) 0	(0/9) 0	(0/4) 0	(0/8) 0	(1/9) 11	(0/10) 0	(0/5) 0	(6/6) 100	(4/9) 44
Ulcer		(0/6) 0	(0/9) 0	(0/4) 0	(0/8) 0	(0/9) 0	(0/10) 0	(0/5) 0	(1/6) 17	(0/9) 0
Atrophy corpus[A]		(0/6) 0	(0/9) 0	(0/4) 0	(0/8) 0	(0/9) 0	(0/10) 0	(0/5) 0	(6/6) 100	(4/9) 44
Metaplastic changes		(0/6) 0	(0/9) 0	(0/4) 0	(0/8) 0	(0/9) 0	(0/10) 0	(0/5) 0	(6/6) 100	(4/9) 44
Gastritis cystica profunda		(0/6) 0	(0/9) 0	(0/4) 0	(0/8) 0	(0/9) 0	(0/10) 0	(0/5) 0	(1/6) 17	(0/9) 0
Focal dysplasia		(0/6) 0	(0/9) 0	(0/4) 0	(0/8) 0	(0/9) 0	(0/10) 0	(0/5) 0	(0/6) 0	(0/9) 0
Increased carcinoma risk		(0/6) 0	(0/9) 0	(0/4) 0	(0/8) 0	(0/9) 0	(0/10) 0	(0/5) 0	(0/6) 0	(0/9) 0

		16 weeks			32 weeks			64 weeks		
		Non-inf.	WT	Δ*cag*Y	Non-inf.	WT	Δ*cag*Y	Non-inf.	WT	Δ*cag*Y
		(n/n) %	(n/n) %	(n/n) %	(n/n) %	(n/n) %	(n/n) %	(n/n) %	(n/n) %	(n/n) %
Number of animals		5	11	11	9	19	13	8	12	9
Reisolation rate		(0/5) 0	(10/11) 91	(11/11) 100	(0/9) 0	(17/19) 89	(13/13) 100	(0/8) 0	(8/12) 66	(9/9) 100
Lymphoid aggregates	antrum	(0/5) 0	(10/10) 100	(9/11) 82	(0/9) 0	(17/17) 100	(13/13) 100	(0/8) 0	(8/8) 100	(9/9) 100
	corpus	(0/5) 0	(7/10) 70	(0/11) 0	(0/9) 0	(15/17) 88	(4/13) 30	(0/8) 0	(8/8) 100*	(3/9) 33
Erosion		(0/5) 0	(10/10) 100	(3/11) 27	(0/9) 0	(17/17) 100	(6/13) 46	(0/8) 0	(8/8) 100	(8/9) 89
Ulcer		(0/5) 0	(3/10) 30	(0/11) 0	(0/9) 0	(8/17) 47	(0/13) 0	(0/8) 0	(6/8) 75*	(0/9) 0
Atrophy corpus[A]		(0/5) 0	(10/10) 100	(0/11) 0	(0/9) 0	(16/17) 94	(2/13) 15	(0/8) 0	(8/8) 100*	(4/9) 44
Metaplastic changes		(0/5) 0	(9/10) 90	(0/11) 0	(0/9) 0	(16/17) 94	(6/13) 46	(0/8) 0	(8/8) 100*	(4/9) 44
Gastritis cystica profunda		(0/5) 0	(0/10) 0	(0/11) 0	(0/9) 0	(4/17) 24	(1/13) 8	(0/8) 0	(6/8) 75*	(4/9) 44
Focal dysplasia		(0/5) 0	(1/10) 10	(0/11) 0	(0/9) 0	(1/17) 6	(0/13) 0	(0/8) 0	(2/8) 25*	(0/9) 0
Increased carcinoma risk		(0/5) 0	(3/10) 30	(0/11) 0	(0/9) 0	(12/17) 71	(1/13) 8	(0/8) 0	(6/8) 75*	(0/9) 0

[A]parietal cell atrophy.
*significant increase in comparison to mutant-infected group.
doi:10.1371/journal.pone.0004754.t001

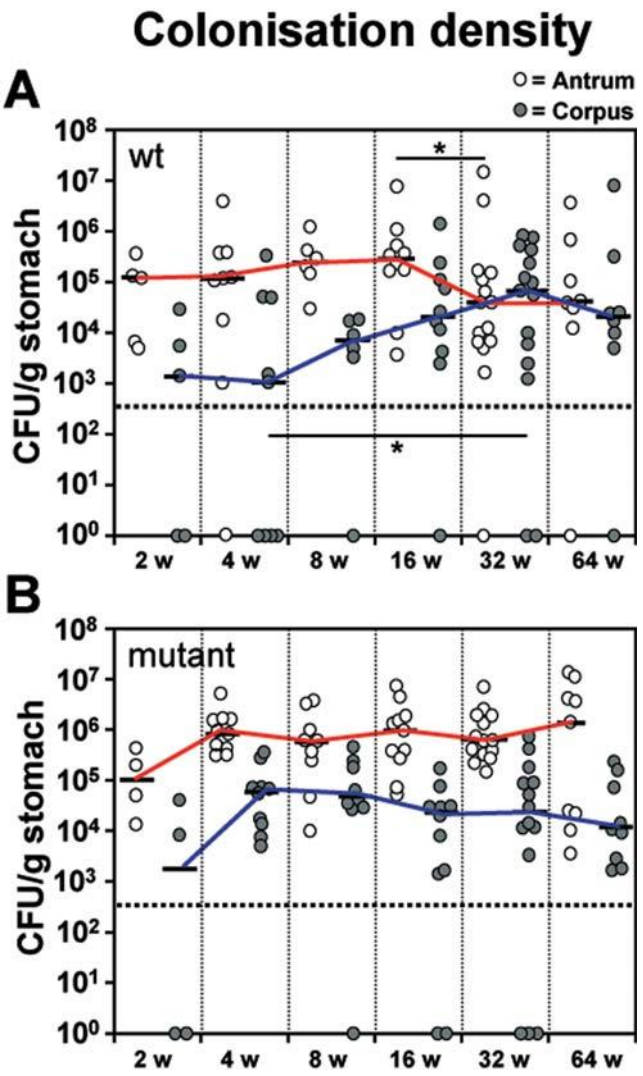

Figure 1. Increased Helicobacter pylori B128 WT colonization density shown in corpus mucosa over the time course experiment. Colonization density of antral (white circles) and corpus (gray circles) mucosa in orally challenged gerbils with *H. pylori* B128 WT (A) and B128Δ*cag*Y (B) isogenic mutant strain after 2, 4, 8, 16, weeks of infection. The interpolated lines connect the medians of the respective time-points. The detection limit was $<5x10^2$ colony-forming units (CFU) per gram of stomach (horizontal dotted line). Gastric tissue specimens without *H. pylori* reisolation are shown as null. (*p<0.05).
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An early severe active and chronic gastritis as well as severe histological changes are only induced in wild type infected Mongolian gerbils

To investigate the dynamics of the host response, we analyzed the induction of the innate immune system by *H. pylori*-infection using histology. The grade of active and chronic gastritis in Mongolian gerbils was assessed by the density of neutrophil granulocytes, as well as lymphocytes, macrophages, and plasma cells infiltrating the gastric mucosa, respectively. In WT-infected animals, a severe active and chronic gastritis was observed in H&E stained antral sections at eight weeks of infection (Figure 2A–B). However, in the corpus, active and chronic gastritis gradually increased over time. In contrast to the WT-infected animals, gerbils infected with the B128 Δ*cag*Y mutant revealed an attenuated progress of inflammation in antrum and corpus tissue. Only from 32 weeks of infection onwards the grading value in the antrum of mutant-infected animals reached the level of WT-infected animals (Figure 2A–B).

Applying the grading system by Garhart *et al.* [21], which includes the grade of metaplasia and ulcer development besides the intensity of inflammation, a significant increase at 8 weeks of infection was found in antral and corpus mucosa of WT-infected animals, but not in the corpus of mutant-infected gerbils (Figure 2C).

We conclude that in the antrum an early severe inflammation after 4 weeks of infection is dependent on a functional T4SS, inducing severe histological changes. After a delay of several months a strong antral inflammation is independent of CagA translocation, leading only to slight histological changes. In contrast, a severe inflammation in the corpus is only induced by a *H. pylori* type I-strain. Thus, our data clearly show a different pathomechanism in antrum and corpus mucosa.

Early precancerous conditions are observed at eight weeks of infection, but only with a *H. pylori* strain able to translocate CagA

Following the sequelae of *H. pylori*-associated chronic gastritis to gastric adenocarcinoma, several histological changes of the mucosa are usually involved, such as atrophy, metaplasia, and dysplasia. By means of H&E as well as Alcian blue/PAS staining, we assessed histopathological changes of the gastric mucosa. The time course experiment revealed an increasing disturbance of the differentiation of the gastric mucosa beginning at 8 weeks of infection with *H. pylori* B128 (Figure 3A). In antrum and corpus of infected gerbils, the severe gastritis is followed by multiple lymphocyte aggregates in mucosa and submucosa, extensive hyperplasia of antral mucosa, a high degree of atrophy (loss of parietal cells) (Figure 3A–B) in the corpus, and metaplastic changes (mucous gland metaplasia) in up to 100% of WT-infected animals. The disturbance of the glandular mucosa is dramatically increasing over the time course in antrum and corpus of WT-infected animals, resulting in the formation of a regenerated epithelium (data not shown), but also a significant increase of gastritis cystica profunda (75%), and focal dysplasia (25%) occurred at 64 weeks of infection (Figure 3A, Table 1). Severe neoplastic changes or gastric adenocarcinoma were not detected in any of the infected animals.

In contrast to WT-infected animals, mutant-infected groups revealed a less dramatic histopathological change of the antral mucosa over time. Although B128Δ*cag*Y colonizes the corpus mucosa very efficiently (Figure 1B), this strain was not able to induce major histopathological changes, since the epithelial and glandular architecture was mainly preserved (Figure 3A, Table 1). Thus, the histopathological changes seen in WT-infected gerbils are dependent on a functional T4SS.

A changing programme of mucus production in the gastric foveolae and gastric ulcer development due to infection with H. pylori type I-strain

By applying the Alcian blue/PAS stain, we determined changes in gastric mucus production during infection, staining paraffin sections red and blue for neutral and acid mucin, respectively (Figure 3B). Beginning at 8 weeks of infection, the neutral mucin secreting cells extended from the epithelium to the isthmus and down to the bottom of the foveolae of WT-infected antral and corpus mucosa (Figure 3B). In parallel, the red staining mucin at the top of the epithelium diminished considerably and the acid mucin secretion at the bottom of the antral glands disappeared

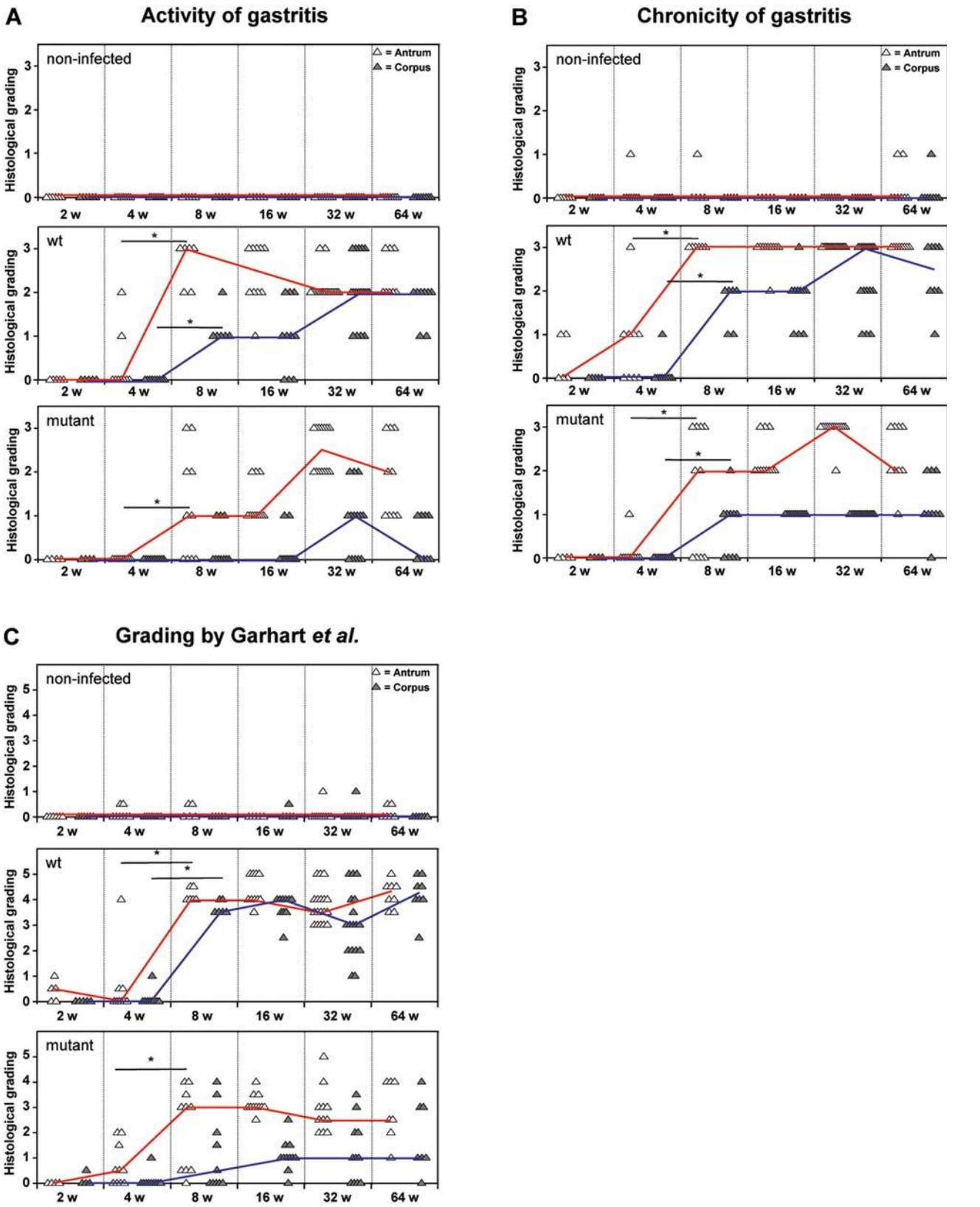

Figure 2. Induction of corpus dominant gastritis is B128 WT dependent. Histological grading of antral (white triangles) and corpus (gray triangles) mucosa. Gerbils were orally challenged with *H. pylori* B128 WT (middle panels) and B128Δ*cag*Y (bottom panels) isogenic mutant strain for 2, 4, 8, 16, 32, and 64 weeks compared with age-matched non-infected controls (upper panels). (A) Active gastritis and (B) chronic gastritis grading was performed according to the upgraded Sydney system, and (C) according to Garhart *et al.* The interpolated lines connect the medians of the respective time-points. (*p<0.05).

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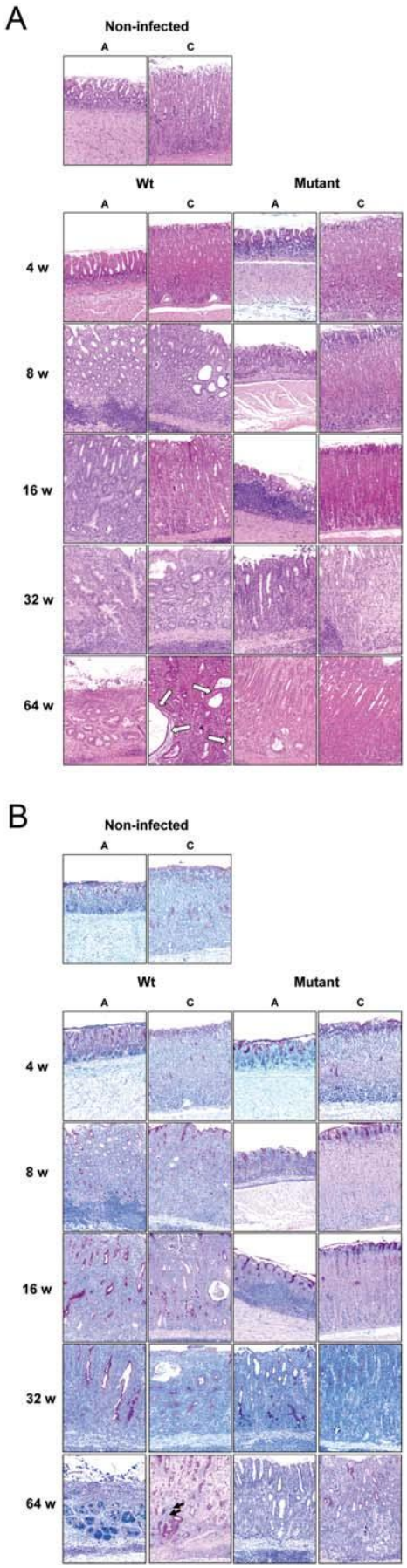

Figure 3. Increased severity of inflammation in *H. pylori* B128 WT infected gerbils. (A) H&E and (B) alcian blue-PAS stained paraffin-embedded antral [A] and corpus [C] tissue (original magnification x10). Mucous gland metaplasia (black arrows) and gastritis cystica profunda (white arrows) are shown. Gerbils were orally challenged with *H. pylori* B128 WT (left panels) and B128Δ*cag*Y isogenic mutant strain (right panels) for 2, 4, 8, 16, 32 and 64 weeks[w]. doi:10.1371/journal.pone.0004754.g003

entirely at 8 weeks of WT-infected gerbils. In contrast, the mutant-infected animals revealed less pronounced changes of the gastric mucin, indicating that a functional T4SS is important for reprogramming cells in terms of mucin production.

Another sequelae of chronic *H. pylori* gastritis is the development of gastric ulcer. Minor and large erosions of the gastric mucosa, which are precursor lesions of gastric ulcer, were present in 100% of WT-infected animals from 8 weeks onwards. The mutant-infected animals, in contrast, required an additional year of infection, to achieve in 89% of the animals a similar level of erosions (Table 1). Furthermore, gastric ulcers were observed first in WT-infected gerbils after 8 weeks of infection (17%) and increased in frequency up to 75% at 64 weeks of infection. The macroscopic observation of an open stomach of WT-infected gerbils at 64 weeks of infection often showed penetrating ulcers and a loss of the architecture of the gastric mucosa (data not shown). Such pathological changes were not found in any of the mutant- or non-infected animals. Taken together, these observations demonstrate that in contrast to a type II-strain, only a *H. pylori* type I-strain is able to induce severe gastric diseases, such as gastric ulcer or precancerous lesions.

Atrophy of parietal cells in the corpus is dependent on the H. pylori T4SS

Since atrophy of the glandular mucosa is defined as the loss of parietal cells, we first analyzed the distribution of parietal cells in a horizontal section of the gastric mucosa of a non-infected gerbil stomach by immunohistochemistry. Parietal cells were mainly detected in the corpus but some in the antrum, too (Figure 4A). Therefore, we applied the grading for the atrophy not only to the corpus, but also to the antral tissue. The atrophy was classified into three stages, a beginning, moderate, and complete loss of parietal cells (Figure 4B).

WT-infected gerbils revealed a significantly increased atrophy in antral and corpus mucosa after 4 weeks of infection (Figure 4C). However, mutant-infected animals showed only an atrophy in the antrum tissue but none in the corpus mucosa nearly until the end of the time-course experiment. These data suggest that in the antral mucosa atrophy is induced early after infection in a *cag*-PAI-independent manner, whereas the loss of huge numbers of parietal cells in the corpus is mainly dependent on the activity of the *cag*-T4SS of *H. pylori*.

Type I *H. pylori* increases the Cancer Risk Index for gastric carcinoma

The Cancer Risk Index [22] describes important histological factors involved in promoting gastric cancerogenesis in humans, such as an increased inflammation in the corpus (corpus dominant active and chronic gastritis) as well as metaplastic changes of the mucosa. Applying this index to the gerbil model, we observed that at 16 weeks of WT-infection 30% of the animals revealed an increased carcinoma risk index. This tendency further increased up to 75% at 64 weeks of infection. In contrast, most of the mutant-infected animals did not fulfil these criteria (Table 1). Therefore, mainly *H. pylori* type I-strains with a functional T4SS

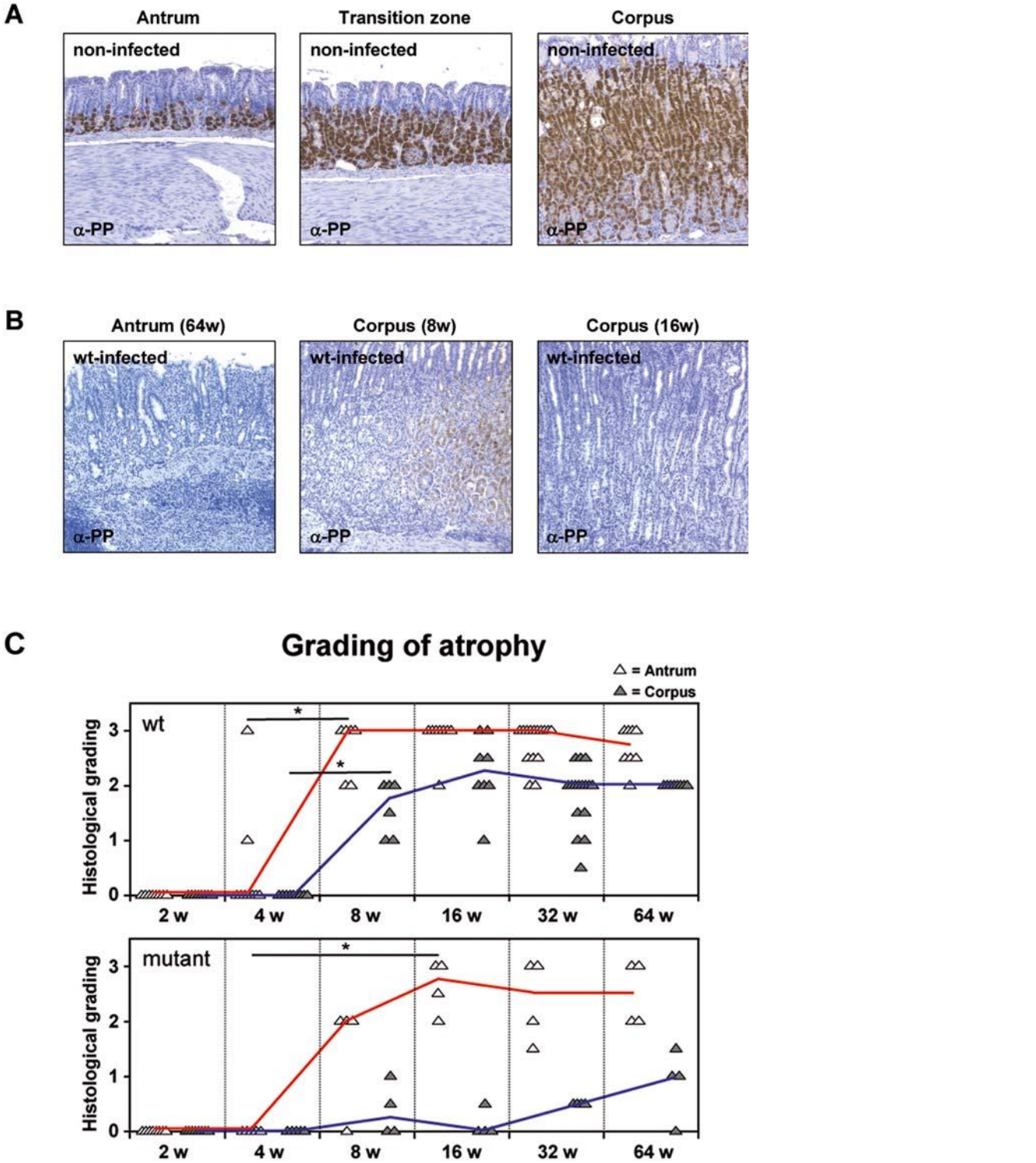

Figure 4. Distribution of parietal cells analyzed by immunohistochemical staining. (A) Parietal cells were detected with anti-proton pump antibody in antral tissue (left panel), transition zone (middle panel), and corpus tissue (right panel) of non-infected Mongolian gerbils (original magnification 10x). (B) Immunohistochemical staining with anti-proton pump antibody detecting complete parietal cell atrophy in antral tissue at 64 weeks (left panel) and beginning at 8 weeks (middle panel) as well as complete parietal cell atrophy in corpus tissue at 16 weeks (right panel) of *H. pylori* B128 WT-infected gerbils. (C) Histological parietal cell atrophy grading of antral (white triangles) and corpus (gray triangles) mucosa infected with *H. pylori* B128 WT (upper panel) and B128Δ*cag*Y isogenic mutant strain (bottom panel) over time. (*p<0.05).
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are able to induce precancerous conditions, such as a corpus dominant atrophic gastritis and metaplastic changes in a gerbil stomach.

Proinflammatory cytokine mRNA expression in antral and corpus tissue of WT-infected gerbils is drastically increased after four weeks of infection

The histological observed infiltration of inflammatory cells into the Lamina propria due to *H. pylori* infection was expected to be triggered by proinflammatory cytokines. To assess the level of cytokine mRNAs transcribed in the gastric tissue over the time course of infection, the cytokines IL-1β, TNF-α, IL-6, KC (IL-8 homologue), and IFN-γ were quantified by real time RT-PCR. Between four and eight weeks of WT-infection, the proinflammatory cytokine mRNA levels increased significantly in the antrum and corpus mucosa and remained relatively stable in the antrum until 64 weeks, but continually increased in the corpus up to 16 weeks (Figure 5A–E). Compared to basic expression levels in the non-infected control animal group the *H. pylori* WT-associated proinflammatory cytokine expression levels were induced about 25- to 200-fold in case of TNF-α, IL-6, KC, and IL-1β and even 700-fold for IFN-γ.

The mutant-infected animals revealed a gradual increase of these cytokine mRNAs in the antrum up to 32 or 64 weeks of infection. In general, the mRNA levels were about one log stage below that of WT levels, except for TNF-α. Interestingly, all analyzed proinflammatory cytokine mRNAs in the corpus of mutant-infected gerbils were comparable to the non-infected control groups, i.e. expressed basic levels over the whole infection period (Figure 5A–E).

In summary, the significant increase of cytokine mRNAs observed in antrum and corpus of WT-infected gerbils after 4 weeks clearly indicates a time-controlled *cag*-PAI-dependent mechanism of cytokine induction. At 8 weeks of infection there was a significant difference of mRNA levels in antral tissue between WT- and mutant-infected gerbils equalizing over time. These data suggest a two phase mechanism of cytokine induction in the antrum, an early *cag*-PAI dependent and a later *cag*-PAI-independent increase of proinflammatory cytokine mRNA expression by *H. pylori*.

IL-10, an anti-inflammatory cytokine, gradually increases over time of infection

To keep a chronic inflammation running, there has to be a balance of pro- and anti-inflammatory cytokines. The anti-inflammatory cytokine IL-10 is produced by T lymphocytes and regulatory T cells and is able to attenuate the inflammation of the gastric mucosa. Interestingly, the B128 type I-strain with a functional T4SS as well as the Δ*cag*Y-mutant strain, both were able to gradually induce IL-10 mRNA in the antral and corpus mucosa during the time course experiment (Figure 5F). In contrast

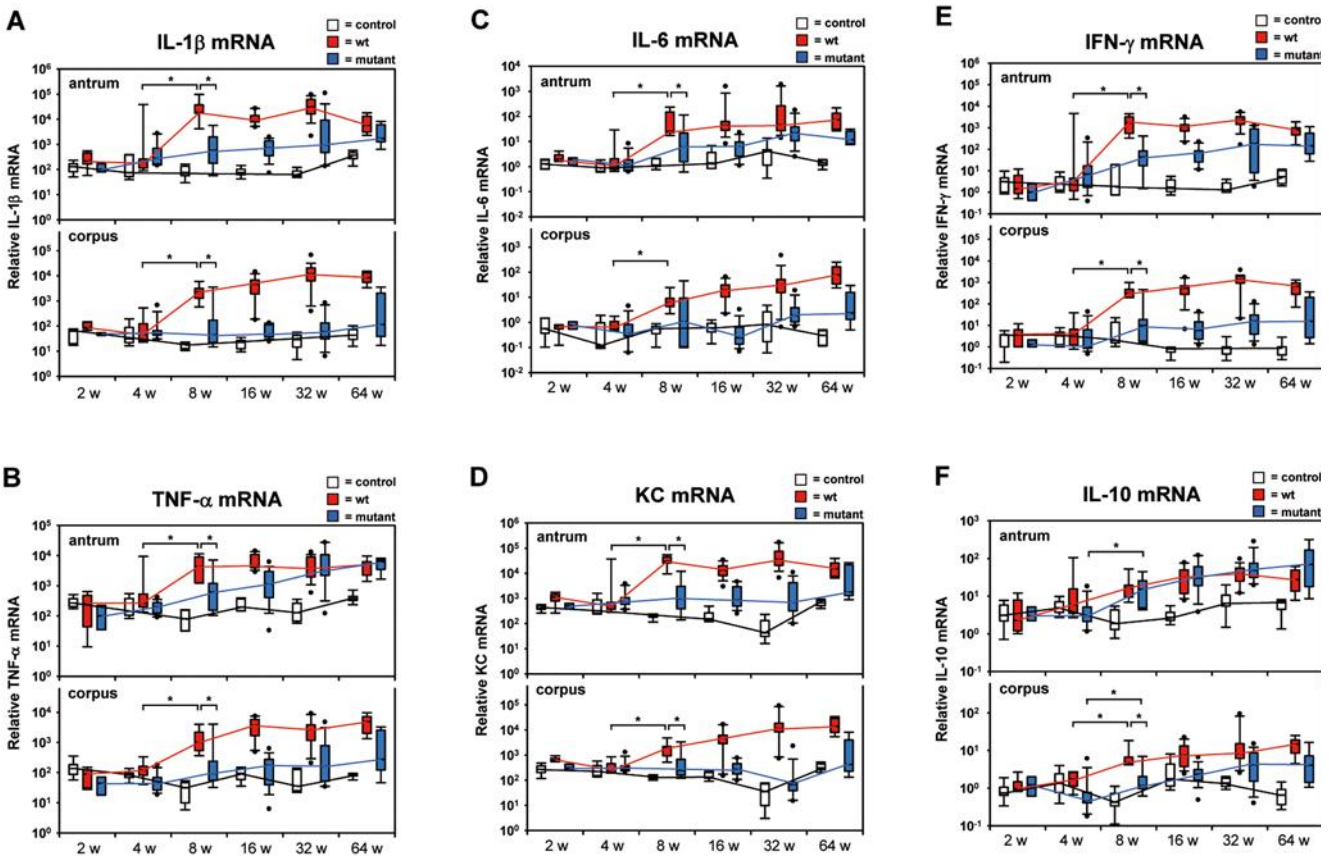

Figure 5. Early inflammatory events are reflected on mRNA level. (A) IL-1β, (B) TNF-α, (C) IL-6, (D) KC, (E) IFN-γ, and (F) IL-10 mRNA were measured by real-time RT-PCR normalized to 18S ribosomal RNA of antral and corpus mucosa of gerbils orally challenged with *H. pylori* B128 WT (red box) and B128Δ*cag*Y (blue box) isogenic mutant strain for 2, 4, 8, 16, 32, and 64 weeks compared with age-matched non-infected controls (white box). All box plots show 25th to 75th percentiles (box) and 5th to 95th percentiles (whiskers). Solid dots are outliers below 5% and above 95%. The line in the box represents the median. The interpolated lines connect the medians of the respective time-points. (*p<0.05).
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to the proinflammatory cytokines, the antral IL-10 mRNA level of WT-infected gerbils was not significantly induced after 4 weeks of infection, but constantly increased up to 15-fold after several months of infection. Whereas a significant increase of IL-10 mRNA expression was observed after 4 weeks infection in the corpus of WT-infected as well as in antrum and corpus of mutant-infected gerbils (Figure 5F). In conclusion we assume that the attenuated induction of an anti-inflammatory cytokine in the antral mucosa of WT-infected animals correlates with the significant increase of proinflammatory cytokines at 8 weeks of infection. This indicates IL-10 as a pivotal regulator of the *H. pylori*-induced chronic inflammation of the gastric mucosa.

Hypergastrinemia is following hypochlorhydria after 16 weeks of infection only in WT-infected gerbils

The effect of *H. pylori*-infection on physiological parameters (pH, gastrin) in the stomach was analyzed over time. In the control animals the gastric mucosa pH was on an average of pH 1.75. However in WT-infected gerbils, a significant increase of the pH value by 1.5 units was measured at 16 weeks. The pH values continued to rise gradually to pH 4.25 at 64 weeks of infection (Figure 6A). In contrast, the mutant-infected Mongolian gerbil stabilized their gastric pH during the long term infection at the level of the control group.

Since gastrin expression is regulated by the gastric pH, we expected an increase of the gastrin level as a direct consequence of the pH changes. In fact, the WT-infected animals revealed a significant increase of plasma gastrin level at 32 weeks following a rise of the pH at 16 weeks of infection (compare Figure 6A–B). In contrast, the non-infected control group and the B128Δ*cag*Y-infected animals showed very similar gastrin levels over time with a median of about 160 pg/ml. The data clearly show that the *H. pylori* WT-infection induces a relatively early increase of inflammatory markers (between 4 and 8 weeks) but a relatively late (at 16 and 32 weeks) increase in physiological parameters such as the

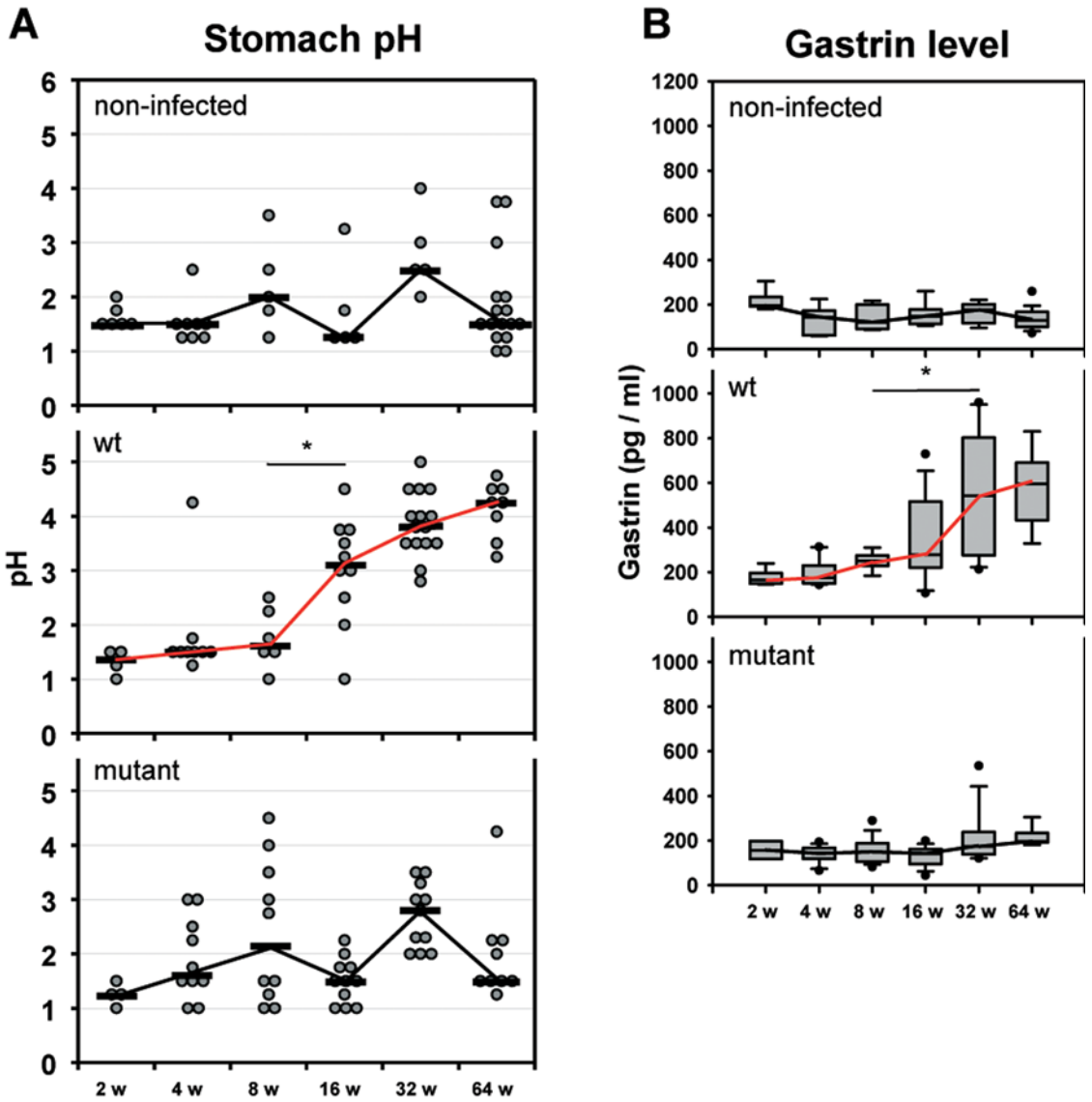

Figure 6. Early inflammatory events cause later physiological changes in *H. pylori* B128 WT-infected gerbils. The (A) pH and the (B) plasma gastrin level (detection limit <1 pmol/L) were determined of gerbils orally challenged with *H. pylori* B128 WT (middle panels) and B128Δ*cag*Y (bottom panels) isogenic mutant strain for 2, 4, 8, 16, 32, and 64 weeks compared with age-matched non-infected controls (upper panels). (*p<0.05). doi:10.1371/journal.pone.0004754.g006

gastric pH and the plasma gastrin level. Therefore we postulate that only an infection with a *H. pylori* type I-strain, carrying a functional T4SS able to interact with the host cells as well as translocate CagA and possibly other factors into the gastric cells, is a prerequisite to induce hypochlorhydria and hypergastrinema in the Mongolian gerbil model.

Gastric hormone mRNA concentrations are variably changed in type I H. pylori infections

Several gastric hormones are regulating the homoeostasis of the stomach. Gastrin produced in G-cells located in the antral stomach as well as histamine produced by the enzyme histidine decarboxylase in enterochromaffin-like (ECL) cells are inducing the acid secretion of parietal cells in the corpus mucosa, whereas somatostatin released from D-cells in the antral and corpus mucosa is an antagonist to gastrin and histamine down-regulating the acid secretion.

The gastrin and histidine decarboxylase mRNA concentration analyzed by real time RT-PCR decreased after 4 weeks significantly in antral tissue of WT-infected gerbils, but the concentrations in the corpus were constant over time (Figure 7A–B). Interestingly, the observed reduction was gradually reversed after 16 weeks of infection. A further increase of gastrin mRNA levels by one log stage was obtained at 64 weeks of infection. However, for somatostatin a significant decrease of mRNA levels was measured not only in antral, but also in corpus mucosa after 4 weeks of infection (Figure 7C). Measuring the plasma somatostatin concentration by RIA, there was a reduced plasma somatostatin concentration between 8 and 64 weeks of infection in WT-infected gerbils (data not shown).

Taken together our data reveal in WT-infected gerbils an early cag-PAI dependent down-regulation of hormones or enzymes involved in gastric homoeostasis on mRNA level, concomitantly with the early inflammation. However, those mRNA data are not directly correlating with the pH values and plasma gastrin levels observed after 16 weeks of infection. Thus, regulation of mRNA and protein levels is apparently separated by a time gap of several months and therefore possibly induced by two different mechanisms.

Discussion

The pathogenesis of *H. pylori*-associated gastritis involves immunological, histological, and physiological changes in the gastric mucosa. To get a better understanding about the role of *H. pylori* and its special virulence factors on the outcome of gastric disease, we used the Mongolian gerbil model in a long term infection experiment (2–64 weeks). This animal model was chosen instead of the mouse model, since a *H. pylori* type I-strain is instable in the mouse and rapidly switches off the *cag*-T4SS.

Our data demonstrate convincingly that an intact *cag*-PAI and thus, a functional T4SS is responsible for nearly all of the observed pathological changes in the animals. The T4SS is essential for the induction of an early and severe corpus inflammation, associated with an increased expression of cytokines and histopathological changes. These cytokines seem to operate in a regulatory way on the physiology of the stomach, resulting in severe histological de-differentiation of the gastric mucosa towards the intestinal type. Therefore, this study elucidates mucosal atrophy and metaplasia as parts of the early pathomechanism due to *H. pylori* infection. We assume that an early reprogramming of gastric target cells via the cag-T4SS, probably by bacteria-host cell interaction and/or translocation of virulence factors, is responsible for a number of gastric changes. These are starting with an induction of severe chronic and atrophic gastritis, induction of pro-inflammatory cytokine gene transcription, finally leading to hypochlorhydria and hypergastrinemia, and late severe or malignant gastric diseases. The Mongolian gerbil model turns out to be an excellent model, to study these effects in detail. It very well mimics the human situation, with the advantage to interfere at defined stages of the process in order to eventually prevent these diseases. In a recent publication Ohnishi et al. revealed that a transgenic expression of *H. pylori* CagA induces gastrointestinal neoplasms in mouse [23] which supports our *in vivo*-data.

The difference in colonization density of *H. pylori* WT-strain in the stomach of Mongolian gerbils during the time course experiment revealed two *cag*-PAI dependent procedures: first, a reduced *H. pylori* density in antral mucosa and second, an increasing density in corpus mucosa, which both equalize after 32 weeks of infection. The first scenario might be explained by the development of gastric ulcers in the antrum, which correlates reciprocally with the density of the type I-strain in the gerbil stomach (Figure 1A), since *H. pylori* is unable to colonize ulcerous tissue. The second mechanism, explaining the procedure in the corpus, indicates that *H. pylori* type I-strain is continuously able to increase its load in parallel to the up-rise of the pH in the corpus mucosa of WT-infected gerbils. This reflects the destruction of parietal cells (atrophy), which is a *cag*-PAI dependent process.

Following the time course of our study, the first outstanding time point was between 4 and 8 weeks of infection. During these four weeks, the WT-infected gerbils presented already macroscopically an enlargement of the stomach size. At this early time point we assume that the translocated CagA might be active in inhibiting apoptosis, a process described recently in the Mongolian gerbil model *in vivo* [24].

Applying the grading system of Garhart *et al.* [21], a significant increase of inflammation and histological changes were already seen after four weeks of *H. pylori* type I-infection. Interestingly, at 8 weeks 100% of the infected animals developed numerous lymphoid aggregates and erosions, as well as an atrophy of the parietal cells and mucous gland metaplasia. These early observations are essential steps of the cancer pathway defined by Correa [25] indicating that a functional T4SS is a pivotal risk factor for developing gastric cancer. Some scientists argue that gastric atrophy appears to be a better indicator of gastric cancer risk than intestinal metaplasia [26]. Patients with gastric cancer of the intestinal type demonstrated a prevalence of atrophic gastritis (loss of oxyntic glands containing parietal cells) of 100% [27] that is not associated with intestinal metaplasia, but strongly associated with a mucous metaplasia (pseudopyloric metaplasia), and therefore a further important precancerous stage [28]. These observations are in accordance with our data presented here. A possible explanation is that the loss of parietal cells leads to a reduction of the secreted signal peptides that modulate the growth and differentiation of gastric progenitor cells, finally resulting in an increased proliferation of undifferentiated cells [26].

In contrast to the WT-infected animals, the mutant-infected gerbils, showed only a gradually increasing inflammation in the antral mucosa and minor histological changes (mainly erosions) over time. These changes are independent of the *H. pylori cag*-PAI. Thus, our data strongly support that a corpus predominant gastritis with a marked atrophy, as seen only in WT-infected gerbils, is associated with an increased risk for developing gastric adenocarcinoma [4].

We and others have shown that a hypochlorhydria is associated with a hypergastrinemia in *H. pylori* WT-infected animals, since gastrin is regulating the acid homeostasis in the stomach via histamine in response to an alkaline environment [17,26,29]. A complete atrophy of parietal cells in the corpus mucosa, in parallel to a stable G-cell differentiation in the antrum of the WT-infected

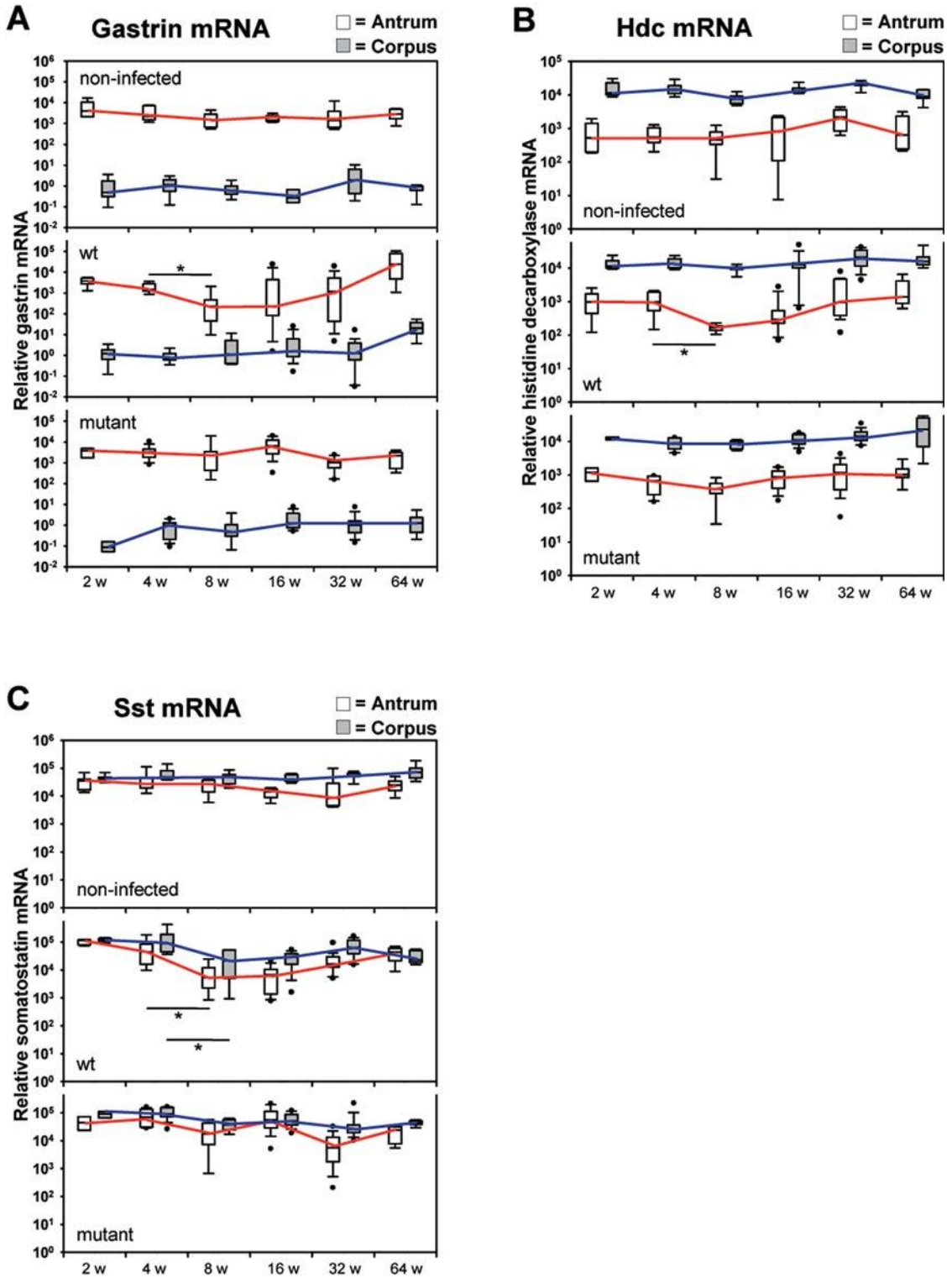

Figure 7. Decrease in gastrin-, histidine decarboxylase-, and somatostatin mRNA due to the *H. pylori* B128 WT-infection. (A) Gastrin-, (B) histidine decarboxylase-, and (C) somatostatin mRNA were measured by real-time RT-PCR normalized to 18S ribosomal RNA of antral (white box) and corpus (gray box) mucosa of gerbils orally challenged with *H. pylori* B128 WT (middle panels) and B128Δ*cag*Y (bottom panels) isogenic mutant strain for 2, 4, 8, 16, 32, and 64 weeks compared with age-matched non-infected controls (upper panels). All box plots show 25th to 75th percentiles (box) and 5th to 95th percentiles (whiskers). The line in the box represents the median. Solid dots are outliers below 5% and above 95%. The interpolated lines connect the medians of the respective time-points. (*p<0.05).
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gerbils supports the release of gastrin, resulting in a hypergastrinemia [30]. Therefore, it can be assumed that an important role of *H. pylori* in gastric carcinogenesis is the induction of atrophic gastritis, resulting in hypochlorhydria.

Gastrin over-expressed in an insulin-gastrin transgenic mouse model (INSGAS) induces corpus atrophy in those mice, spontaneously progressing to gastric adenocarcinoma with advanced age [31,32]. Furthermore, a parallel infection of those mice with *Helicobacter* spp. synergises with the effect of hypergastrinemia to accelerate the malignant transformation [33]. We assume that a strong interaction of an early induced atrophy of the parietal cells together with a significantly increased stimulation of the G-cells in the antrum to release gastrin after 32 weeks of WT-infection, is responsible for severe precancerous transformations of the gastric cells. Thus, after 64 weeks of infection, gerbils challenged with a *H. pylori* type I-strain developed precancerous gastric changes, resulting in a statistically significant increase of gastritis cystica profunda (75%) and focal dysplasia (25%). Furthermore, WT-infected animals revealed an increased carcinoma risk index [22] as compared to the mutant-infected ones, but none of the infected gerbils developed gastric adenocarcinoma. Our data are in agreement with several studies of western research groups [7,34], but contradicting published data from Japanese groups and others, that demonstrated a *H. pylori*-induced gastric cancer in Mongolian gerbils [16,35,36]. Thus, our data support a multifactorial process of gastric cancer induction, as it is suggested to occur in humans.

H. pylori induces a Th1 immune response characterized by an infiltration of T-cells releasing proinflammatory cytokines [37]. The *H. pylori* colonization of the gastric mucosa results in expression of proinflammatory cytokines such as TNF-α, IL-1β, IL-8, and IFN-γ, which attract leukocytes infiltrating the Lamina propria. This early rise of an inflammatory response is specific for a *H. pylori* type I-strain, whereas a late *cag*-PAI independent mechanism in the antral mucosa was described in mutant-infected gerbils, too. An IFN-γ null mouse challenged with *H. pylori* shows no inflammatory response, even after more than one year of infection [38]. Thus, it has been suggested that those released proinflammatory cytokines, but especially IFN-γ, possess a pivotal role in triggering cellular changes that contribute to gastric mucosal damage [38,39]. Our data support this observation for early time points of 4 to 8 weeks of infection with a *H. pylori* type I-strain. But how does an early rise of cytokines relate to the later physiological and histological changes seen in WT-infected gerbils ? We could show that the chronic gastritis is a precursor of the active gastritis in WT-infected gerbils (Figure 2B), since a mild inflammatory response was already observed by infiltrating of IFN-γ expressing T-cells after two weeks of infection. IFN-γ itself stimulates neutrophils to release proinflammatory cytokines such as TNF-α, IL-1β, and KC. Those cytokines together with IFN-γ stimulate the G-cells in the antrum to release the hormone gastrin, but they also inhibit D-cells to express somatostatin, an inhibitor of gastrin-stimulated acid secretion. [7,40] IL-1β and TNF-α are potent inhibitors of parietal cells blocking acid secretion [41], and IL-1β decreases histamine release from enterochromaffin-like (ECL-) cells [42]. Furthermore, it was shown that TNF-α induces apoptosis of parietal cells [43] and gastrin stimulates the hyperproliferation of the gastric epithelial cells, [44] thus an enhanced inflammation, as induced by a functional *cag*-PAI positive *H. pylori*-strain, is responsible for the progression of gastric preneoplastic lesion, such as atrophy and dysplasia, to adenocercinoma [45]. In humans, carrying a *H. pylori* CagA-seropositive infection, an elevated IFN-γ concentration was also detected that accounts for an increased plasma gastrin level [46].

In contrast, the anti-inflammatory Th2 cytokine IL-4 stimulates the secretion of somatostatin [47], thereby decreasing the development of gastric atrophy [48]. *H. pylori* type II-strain infected gerbils revealed a significant increase of the anti-inflammatory cytokine IL-10 mRNA in antral and corpus tissue after four weeks of infection whereas, gerbils challenged with the type I-strain only gradually increased the IL-10 mRNA level in antral mucosa without any significance. Due to the reduced IL-10 expression in WT-infected gerbils, the Th1/Th2 equilibrium, which sustains a chronic inflammation, is deranged resulting in a hypergastrinemia and atrophy of the parietal cells in the oxyntic mucosa. Therefore, our results indicate a *H. pylori* type I-strain dependent early modulation of the immune response towards a Th1 response that is dominated by an IFN-γ regulating cascade. This includes the stimulation of further proinflammatory Th1 cytokines that in the following regulate the gastric physiology resulting in severe histological changes such as precancerous lesions.

In conclusion, the Mongolian gerbil model is the only suitable rodent model available, mimicking the human situation, for analyzing the effect of major *H. pylori* virulence factors on the pathogenesis of the *H. pylori*-infected gastric mucosa. As our study reveals, we could standardize the gerbil model for investigating the complex interaction of immunological, physiological, and histological parameters in antral and corpus mucosa separately during a long-term *H. pylori*-infection experiment. Further clinical aspects of the pathogenesis of *H. pylori* towards gastric adenocarcinoma can now be addressed. In future, these results might be of therapeutic relevance.

Supporting Information

Table S1

Found at: doi:10.1371/journal.pone.0004754.s001 (0.08 MB DOC)

Author Contributions

Conceived and designed the experiments: GR. Performed the experiments: TW EL MStoeckelhuber. Analyzed the data: TW EL SM MStoeckelhuber MStolte GR. Contributed reagents/materials/analysis tools: SM MStoeckelhuber MStolte RH. Wrote the paper: RH GR.

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A novel integrin-β_5-ILK signaling complex is jointly responsible for *Helicobacter pylori*-induced precancerous conditions

Submitted to Cellular Microbiology

Short Title: *H. pylori* induced gastrin signaling

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Summary

One of the most important hormones in the human stomach is the peptide gastrin. It is mainly required for the regulation of the gastric pH, but also involved in the growth and differentiation of gastric epithelial cells. In *Helicobacter pylori*-infected patients gastrin regulation can be disrupted by the human pathogen, resulting in hypergastrinemia. *Helicobacter pylori*-induced hypergastrinemia is described as being a major risk factor for developing of gastric adenocarcinoma. In this study we investigated the signal cascades involved in *Helicobacter pylori*-induced gastrin gene expression in gastric epithelial cells. For this purpose gastric adenocarcinoma cells (AGS) stably transfected with a human gastrin promoter luciferase reporter construct were used. Interestingly, a novel β_5-integrin-ILK (integrin-linked kinase) signaling complex could be identified as responsible for *Helicobacter pylori* induced gastrin expression. Upon the binding of *Helicobacter pylori* to β_5-integrin, we resolved a downstream signaling cascade via TGF-α (transforming growth factor alpha) and EGF-R (epidermal growth factor receptor). This receptor signaling complex plays a central role in *Helicobacter pylori* gastrin induction. The newly discovered recognition receptor and the subsequent signal cascade could be useful targets in treating precancerous conditions triggered by *Helicobacter pylori*-induced hypergastrinemia.

Introduction

Gastrin is one of the most important peptide hormones in the human stomach. It was first described in 1906 as a tissue factor which stimulates acid secretion in the corpus mucosa (Edkins, 1906). Gastrin is released into the gastric vasculature from endocrine G-cells in the antrum in response to food intake. Gastrin binds to CCK-2 receptors on enterochromaffin-like (ECL) cells to stimulate histamine release and also binds to CCK-2 receptors on parietal cells to activate acid secretion in corpus tissue (Hakanson and Liedberg, 1970; Schmitz *et al.*, 2001). Histamine stimulates adjacent parietal cells through histamine 2 (H2) receptors and thus activates acid secretion.

Diverse animal models, the usage of various human gastric cancer cell lines, and the exploration of human biopsies elucidate gastrin as a kind of growth factor which affects proliferation, apoptosis, migration, invasion, tissue remodeling, and angiogenesis *in vitro* and *in vivo* (Cui *et al.*, 2006; Kidd *et al.*, 2000; Noble *et al.*, 2003; Przemeck *et al.*, 2008; Subramaniam *et al.*, 2008; Sun *et al.*, 2008; Willems *et al.*, 1972; Wroblewski *et al.*, 2002). INS-GAS and GAS-KO mice models are two animal models frequently used to investigate the physiological and pathological role of gastrin. INS-GAS mice which overexpress human amidated gastrin show initial gastric mucosal hypertrophy and excess gastric acid secretion. After 5 months the mice display progressive changes in histology and physiology and after 20 months they are essentially achlorhydric and all develop gastric dysplasia. If these mice are treated with *Helicobacter pylori* ($CagA^+$ / $VacA^+$) they develop gastric adenocarcinoma after 7 months of infection (Wang *et al.*, 1993; Wang *et al.*, 1996).

In contrast to the INS-GAS mice, GAS-KO mice are gastrin deficient. They are achlorhydric with reduced parietal cell numbers and predisposed to bacterial colonization and, therefore, often overgrown by multiple bacteria species (Friis-Hansen *et al.*, 1998; Koh *et al.*, 1997; Sun *et al.*, 2003; Zavros *et al.*, 2002). When these mice are infected with *H. pylori* ($CagA^+$ / $VacA^+$), there is an alteration in acid secretion, thought to be stimulated by a vagal response mechanism, but no increased risk of tumor development is observed after 6 months (Zhao *et al.*, 2003). The absence of increased tumor development observed could be attributed to the lack of gastrin and its mitogenic function.

H. pylori is a gram-negative, spiral-shaped bacterium that colonizes the gastric mucosa of approximately 50 % of the world's population. This pathogen causes gastritis, peptic ulcer, and in some cases gastric adenocarcinoma and MALT (mucosa-associated lymphoid tissue)-lymphoma (Kuipers *et al.*, 1995; Suerbaum and Michetti, 2002). Gastric cancer is the fourth leading cause of death worldwide. In 1994 the WHO (World Health Organization) declared *H. pylori* as class I-carcinogen due to epidemiological studies.

In our recently published Mongolian gerbil study, one group of the animals were infected with a *H. pylori* B128 ($CagA^+$ / $VacA^+$) (WT) strain that expresses a functional type IV secretion system (T4SS) (type I-strains) and another group with an isogenic mutant strain B128Δ*cagY* which is not able to translocate the major virulence factor CagA into the host cells (type II-strains). The results of our animal study revealed early (4-8 weeks) *cag*-T4SS-dependent inflammation only in WT-infected animals followed by severe active and chronic gastritis, mucous gland metaplasia, and dysplasia. In contrast to mutant-infected animals, WT-infected ones exhibited hypochlorhydria at 16 weeks and hypergastrinemia at 32 weeks post infection. To summarize these observations, we assert that the early proinflammatory response (increase of IL-1β, IFN-γ, TNF-α, IL-6, and KC) triggers later physiological changes (hypochlorhydria and hypergastrinemia) (Rieder *et al.*, 2005; Wiedemann *et al.*, 2009).

Hence, in the present study we investigated the mechanisms of how *H. pylori* stimulates the expression of the human hormone gastrin. Our main interests were to identify the binding partner of *H. pylori* on the cell surface as well as the involved signaling network inside the gastric cells that induces the human gastrin promoter. To explore the regulation of the human gastrin gene by *H. pylori*, we used an *in vitro* approach with AGS cells stably transfected with a human gastrin promoter luciferase reporter construct (GAS-Luc).

Results

H. pylori type-I strains specifically induce the gastrin promoter

Several years ago, it was published by several groups (Levi *et al.*, 1989; Smith *et al.*, 1990) that *H. pylori* infected patients showed elevated serum gastrin concentrations relative to a control population. After *H. pylori* eradication they observed a reduction in fasting gastrin concentration. To better understand how gastrin expression is influenced by *H. pylori*, we started an *in vitro* gastrin promoter study. We used AGS cells that were stably transfected with a luciferase reporter construct regulated by the human gastrin WT gene promoter (AMO) to examine the human gastrin expression. These cells were stimulated with different bacteria and, subsequently, gastrin expression was determined. As an internal control, epidermal growth factor (EGF), a natural agonist for stimulation of gastrin gene expression, was utilized (Godley and Brand, 1989). Only pathogenicity island (PAI)-positive *H. pylori*-strains with a functional T4SS (type I-strains) (*H. pylori* P12, 7.13, and P1) showed equal gastrin promoter activation compared to the positive control *H. pylori* B128 type I-strain. The B128Δ*cag*Y mutant strain (type II-strain), which served as a negative control in this study, demonstrated only slight basal expression of the gastrin promoter due to the non-functional T4SS. *Campylobacter jejuni* and *Escherichia coli*, two other bacterial species usually resident in the gastrointestinal tract, could not activate the gastrin promoter. Thus, transcriptional activation of the gastrin promoter *in vitro* seems to be a specific feature of *H. pylori* type I-strains that express a functional T4SS (Fig. 1).

Figure 1

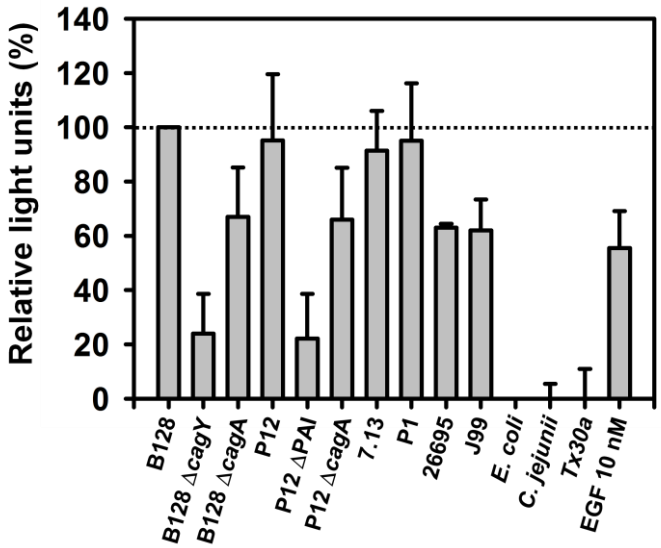


Figure 1. Gastrin promoter activation is *H. pylori* type-I strain specific. AMO cells were stimulated with *H. pylori* strains, *Escherichia coli*, and *Campylobacter jejuni*. After infection cells were harvested and luciferase activity was determined. Luciferase activities in relation to *H. pylori* B128 WT (represents 100%; interpolated dotted line) were plotted. The B128Δ*cag*Y mutant strain serves as negative control. The mean values ± SEM of at least three independent experiments are shown.

H. pylori major virulence factors are not required for gastrin induction

The cytotoxin-associated gene A (CagA) oncoprotein of *H. pylori* is currently the only known T4SS effector protein. CagA is injected into host cells, resulting in the activation of pro-inflammatory chemokines and leading to actin-cytoskeletal rearrangements (Backert and Meyer, 2006; Covacci and Rappuoli, 2000; Hatakeyama, 2006; Kwok *et al.*, 2007). To test the influence of the CagA oncoprotein on gastrin promoter stimulation, CagA was overexpressed in AMO cells. We could show that CagA was present and phosphorylated in samples stimulated with *H. pylori* B128 and in samples transfected with the CagA overexpression plasmid pSP65SR^{+}*cag*A in AMO cells. AMO cells without any stimulation (control) or AMO cells transfected with the control plasmid pSP65SR^{+} displayed no detectable CagA (Fig. 2B). The luciferase measurement revealed that CagA had no effect on the gastrin promoter activation (Fig. 2A).

Another major *H. pylori* virulence determinant that was tested is the vacuolating cytotoxin A (VacA) which induces cytoplasmic vacuolation in cultured epithelial cells. Targeting the adapted immune system, VacA is also a potent immunomodulatory toxin (Cover and Blaser, 1992; Gebert *et al.*, 2003; Sewald *et al.*, 2008). AMO cells were stimulated with purified acid-activated VacA, but no promoter induction could be detected (Fig. 2C).

Figure 2

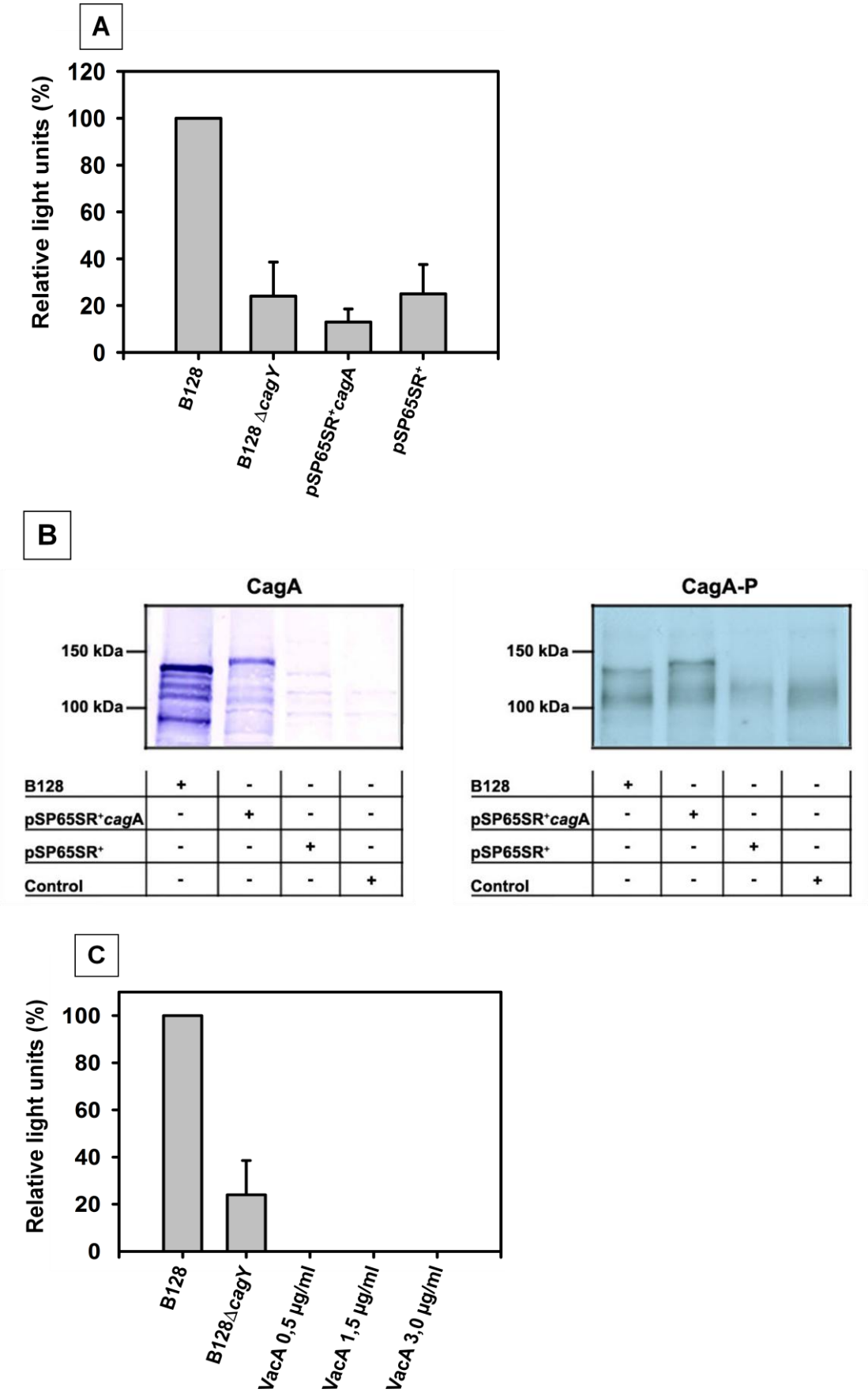

A
Relative light units (%)
120
100
80
60
40
20
0
B128
B128 ΔcagY
pSP65SR+cagA
pSP65SR+
B
CagA
150 kDa
100 kDa
B128 + - - -
pSP65SR+cagA - + - -
pSP65SR+ - - + -
Control - - - +
CagA-P
150 kDa
100 kDa
B128 + - - -
pSP65SR+cagA - + - -
pSP65SR+ - - + -
Control - - - +
C
Relative light units (%)
100
80
60
40
20
0
B128
B128ΔcagY
VacA 0,5 µg/ml
VacA 1,5 µg/ml
VacA 3,0 µg/ml

Figure 2. CagA and VacA play no role in promoter activation. (A + B) AMO cells were either transfected with a CagA overexpression plasmid (pSP65SR$^+$*cag*A) or the respective control plasmid without CagA (pSP65SR$^+$) for 24 h. (A) Luciferase activities in relation to *H. pylori* B128 WT (represents 100%) were plotted. (B) Western blot analyses were performed to test the CagA overexpression and phosphorylation. (C) AMO cells were incubated with several amounts of acid activated VacA and luciferase activities in relation to *H. pylori* B128 WT (represents 100%) were plotted. The mean values ± SEM of at least three independent experiments are shown.

Binding of *H. pylori* strains is essential for gastrin promoter activation

H. pylori causes persistent infection in the gastric mucosa, which is the major colonization site for the bacterium. The binding of the bacteria to the epithelial cell is mainly mediated through its major adhesins BabA, SabA, and AlpAB (Linden *et al.*, 2002; Mahdavi *et al.*, 2002; Odenbreit *et al.*, 1999). To explore whether contact of the bacteria with epithelial cells is necessary to induce the gastrin promoter, filter inserts were used to prevent contact between the bacteria and the cells. When there was no contact of *H. pylori* B128 with the AMO cells, promoter induction was not present at measurable levels (Fig. 3A).

In a second step we investigated whether the gastrin promoter was activated upon the binding of *H. pylori* to the epithelial cell via the aforementioned adhesins. Therefore, *H. pylori* J99 and isogenic single and double adhesine mutant-strains were used to stimulate AMO cells. No differences in gastrin promoter induction between the J99 WT and its isogenic mutant strains were observed (Fig. 3B). These results indicate that binding of *H. pylori* via the major adhesines BabA, SabA, and AlpAB does not activate the gastrin signaling pathway. Therefore we investigated whether an intact and viable *H. pylori* is essential for gastrin promoter induction. Stimulating AMO cells with either cooked or sonificated *H. pylori* B128 lysates addressed this issue. The results of these experiments supported the previous findings that viable *H. pylori* must be in contact with the cells because neither the cooked nor the sonificated lysates were present at levels significantly different from the basal B128Δ*cag*Y induction level (Fig. 3C). To confirm these results, we prepared metabolically inactive *H. pylori* cells by adding them to a solution of sodium azide (NaN_3). These structurally intact but metabolically inactive *H. pylori* cells were no

longer able to actively secrete or translocate any bacterial factors. The metabolically inactive *H. pylori* B128 exhibited the same induction pattern as the untreated *H. pylori* B128 strain (Fig. 3D). Therefore we conclude that contact of a metabolically inactive but viable *H. pylori* type I-strain with epithelial cells is sufficient to induce the gastrin promoter.

Figure 3

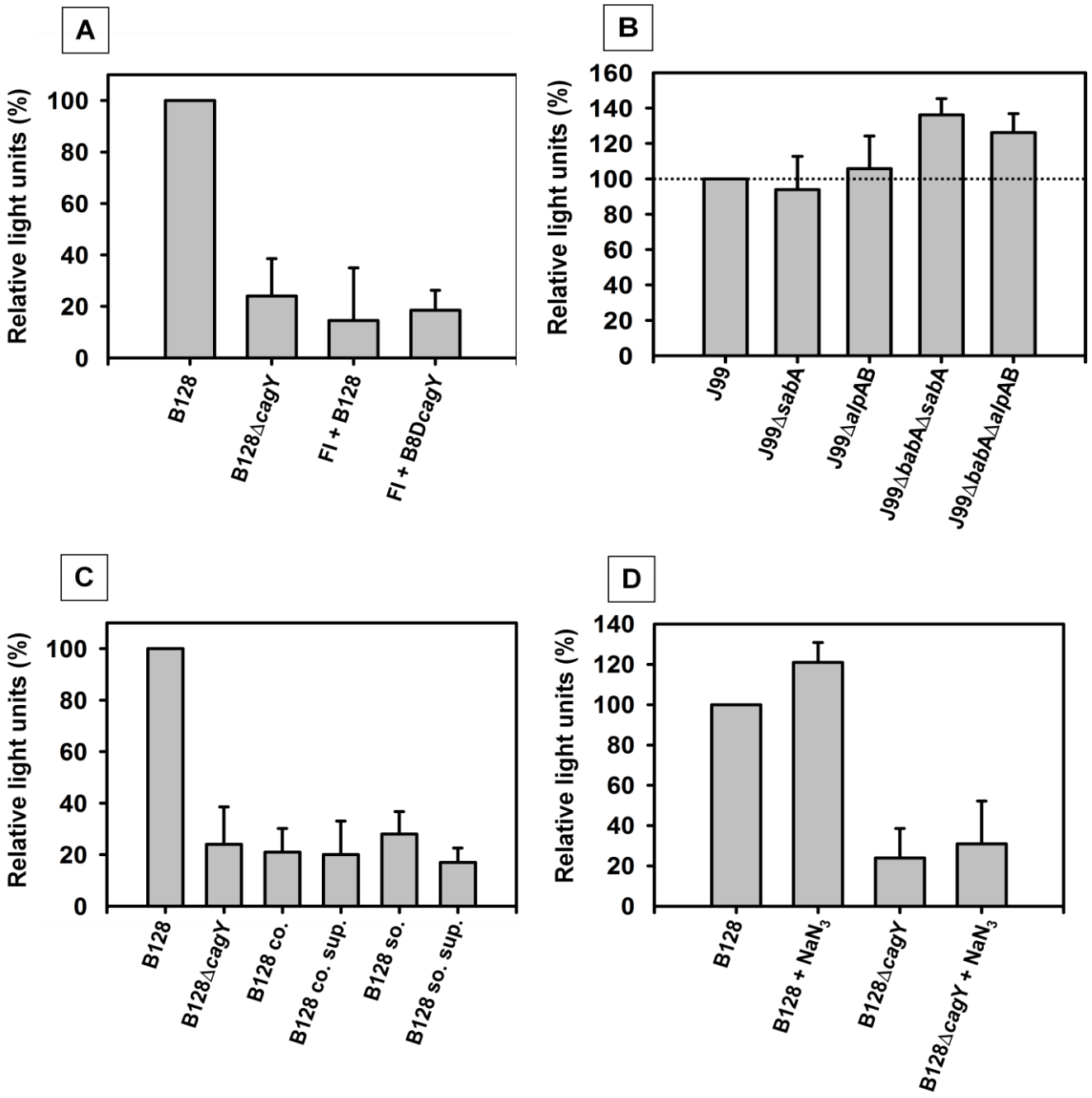


Figure 3. Contact of viable *H. pylori*, independent of BabA, SabA and AlpAB, is essential for gastrin induction. (A) Filter inserts (FI) were placed in each well containing AMO cells. *H. pylori* B128 WT or B128Δ*cag*Y mutant strain were added. (B) AMO cells were stimulated with *H. pylori* J99 and isogenic adhesine mutant strains. (C) Cooked (co), cooked supernatant (co.sup), sonificated (so), and sonificated supernatant (so.sup) *H. pylori* B128 lysates were used to stimulate AMO cells. (D) Metabolic inactive *H. pylori* B128 WT or B128Δ*cag*Y were used to stimulate AMO cells. Luciferase activities in relation to (A, C, D) *H. pylori* B128 WT or (B) J99 (represents 100%; interpolated dotted line) were plotted. The mean values ± SEM of at least three independent experiments are shown.

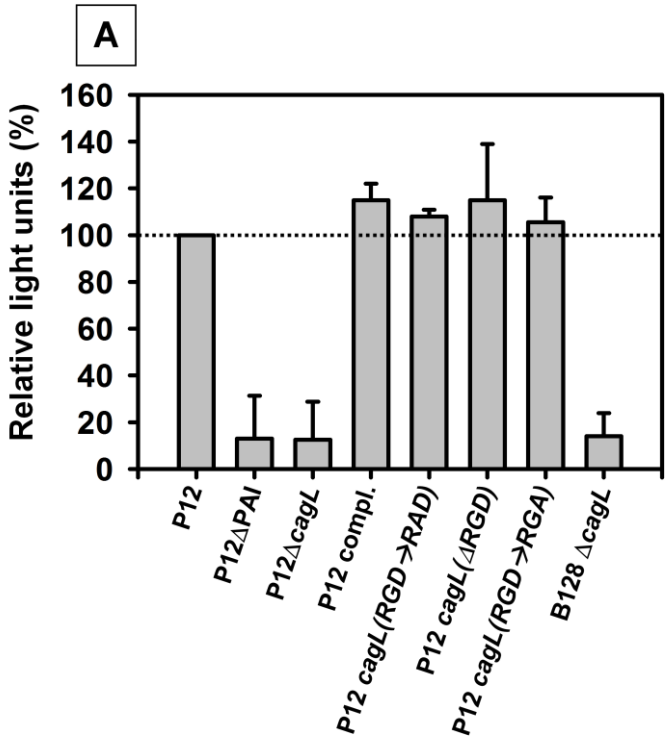
Proinflammatory cytokines induce gastrin promoter activation

It has been shown that pro-inflammatory cytokines such as TNF-α and IL-1β, released during gastric inflammation, can stimulate the G-cells to produce gastrin (Suzuki *et al.*, 2001; Zavros and Merchant, 2005). Conversely, the anti-inflammatory cytokine IL-4 increases somatostatin levels and effectively suppresses gastrin expression and secretion (Zavros *et al.*, 2003). Based on these observations we wanted to examine the effect of pro-inflammatory and anti-inflammatory cytokines on gastrin expression in epithelial cells (AMO). Our data revealed that only IL-1β and TNF-α but not IFN-γ and IL-8 could stimulate the gastrin promoter of AMO cells (Fig.S1A). To exclude the possibility that AMO cells could secrete IL-1β or TNF-α upon stimulation with *H. pylori* B128, these cells were pre-incubated with IL-1β or TNF-α catching antibodies. There was no secretion of either IL-1β or TNF-α in AMO cells (data not shown). To test the potential suppressive effectiveness of anti-inflammatory cytokines on gastrin promoter expression, *H. pylori* B128 or TNF-α stimulated AMO cells were pre-incubated with IL-4 and/or IL-10. No suppressive effect of IL-4 and IL-10 could be measured on the stimulated gastrin promoter (Fig. S1B+C). Thus, the data suggest that anti-inflammatory cytokines did not suppress *H. pylori* and pro-inflammatory cytokine related induction of the gastrin promoter.

H. pylori induces the gastrin promoter via binding to β_5-integrin

A recent study indicated that *H. pylori* uses an integrin $\alpha_5\beta_1$ receptor to bind to gastric epithelial cells through an Arg-Gly-Asp (RGD)-motive present on the *H. pylori* CagL protein, located at the pilus of the T4SS. This binding enables the major virulence factor CagA to translocate into the epithelial cell (Kwok *et al.*, 2007). To investigate the role of this binding complex on gastrin promoter stimulation, the *H. pylori* P12 WT-strain and several isogenic *cag*L-mutant strains were used to stimulate AMO cells. These data reveal that the RGD-motive plays no substantial role in the gastrin expression pathway. Supportive to our previous observation, there was an abolished gastrin expression only in the B128 Δ*cag*L-and P12Δ*cag*L-mutant strains (Fig. 4A) (Rieder *et al.*, 2005). To find the crucial binding receptor for induction of the gastrin promoter, several anti-β-integrin blocking antibodies were applied. Only the anti-β_5-Integrin blocking antibody could significantly decrease gastrin promoter stimulation by about 50 % (Fig. 4B). Therefore we conclude that β_5-integrin is a potential binding receptor for *H. pylori* on gastric epithelial cells. *H. pylori* binds to this novel receptor in a CagL-dependent, but RGD-independent manner.

Figure 4



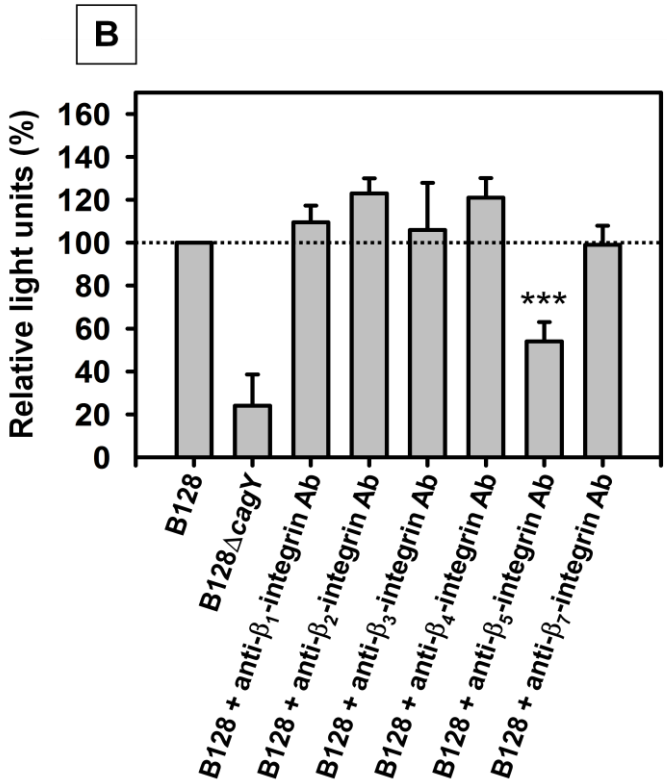


Figure 4. *H. pylori* binds to the epithelial cell via β_5-integrin. (A) AMO cells were stimulated with *H. pylori* P12, isogenic P12 *cag*L-mutant strains with a deleted or mutated RGD-motif and *H. pylori* B128Δ*cag*L. (B) AMO cells were pre-incubated with anti-β-integrin blocking antibodies (Ab) [10 µg/ml] and stimulated with *H. pylori* B128. Luciferase activities in relation to *H. pylori* B128 WT (represents 100%; interpolated dotted line) were plotted. The mean values ± SEM of at least three independent experiments are shown. Statistically analysis (Student`s *t* test) was applied using SigmaStat software. ****P* ≤ 0.001

H. pylori induces TGF-α membrane shedding

EGF and transforming growth factor (TGF)-α are known stimuli of gastrin release. They belong to the group of epidermal growth factor receptor (EGF-R) ligands (Ford *et al.*, 1997). All ligands of the EGF-R are synthesized as membrane-anchored precursors, which must be released from their membrane tether by ADAM`s (A Disintegrin And Metalloproteinase) to activate the EGF-R in a paracrine manner (Blobel, 2005).

To investigate the role of EGF and TGF-α on *H. pylori* induced gastrin promoter stimulation, we performed a further *in vitro* stimulation assay. Our data demonstrate

that *H. pylori* induced gastrin expression is an indirect mechanism, because there was a significant reduction of promoter activity ($P \leq 0.001$) when AMO cells were pre-incubated with an EGF-R tyrosine kinase inhibitor (Fig. 5B). To determine which EGF-R ligand is the most important in *H. pylori* triggered gastrin gene induction, *H. pylori* B128 stimulated AMO cells were pre-incubated with anti-TGF-α or anti-EGF catching antibodies. There was no decrease in promoter stimulation with the anti-EGF-, but a 60 % suppression with the anti-TGF-α-antibody (Fig. 5B). This indicates that *H. pylori* induces TGF-α shedding in AMO cells, and then TGF-α binds to the EGF-R and stimulates the gastrin expression pathway. Additionally, we investigated whether *H. pylori* B128 activates the gastrin pathway synergistically to EGF stimulation (Fig. 5A). Consequently, AMO cells were pre-incubated with EGF and then stimulated with *H. pylori* B128. The results showed that there was an approximately 70 % increase of promoter activation in the samples stimulated with EGF and *H. pylori* B128.

Figure 5

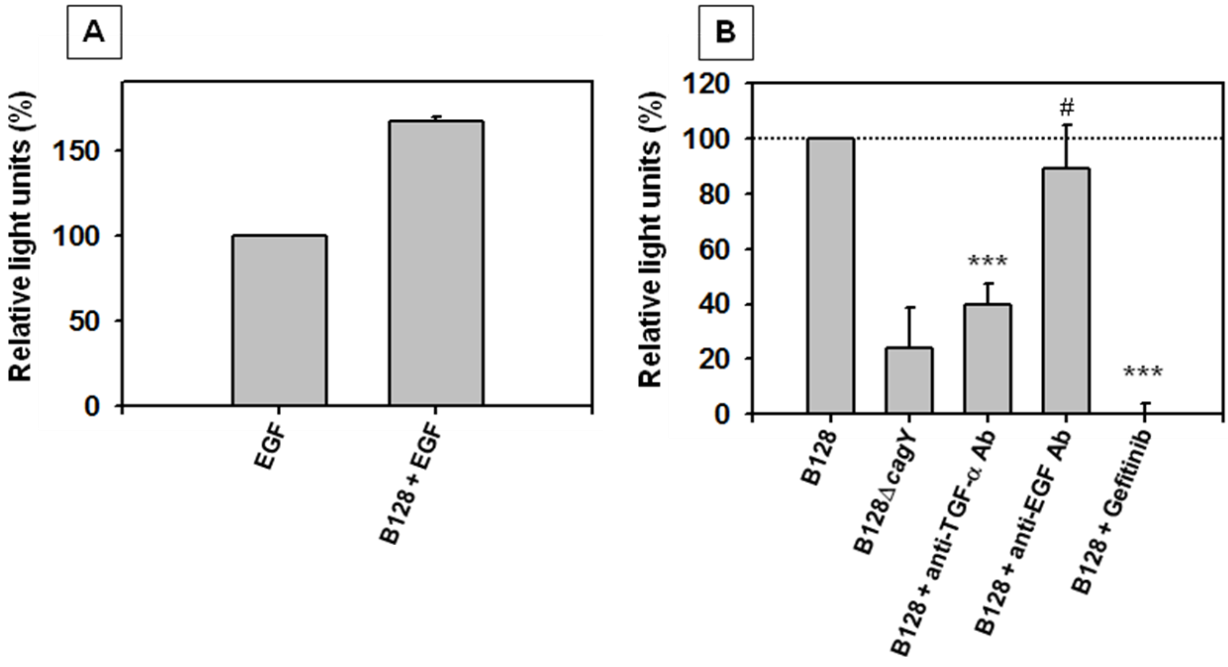


Figure 5. *H. pylori* induced gastrin expression is mediated via TNFα and EGF-R but not by EGF. (A) AMO cells were stimulated either with EGF [10 nM] or with EGF [10 nM] and *H. pylori* B128. Luciferase activities in relation to EGF (represents 100%) were plotted. (B) AMO cells were pre-incubated with anti-TGF-α [10 µg/ml] and anti-EGF [10 µg/ml] antibodies or EGF-R-inhibitor gefitinib [5 µM], and stimulated with *H.*

pylori B128. Luciferase activities in relation to *H. pylori* B128 WT (represents 100%; interpolated dotted line) were plotted. The mean values ± SEM of at least three independent experiments are shown. Statistically analysis (Student`s *t* test) was applied using SigmaStat software. ***$P \leq 0.001$; # $P \geq 0.05$

H. pylori-host communication is mediated through integrin-linked kinase

Focal adhesions (FAs) are specialized extracellular matrix (ECM) attachments and signaling organelles. Integrin linked kinase (ILK) and focal adhesion kinase (FAK) are the most important FA components in mediating signals upon β-integrin binding (Harburger and Calderwood, 2009).

To analyze whether the binding of *H. pylori* B128 to β_5-integrin (Fig. 4B) triggers integrin signaling via FAK or ILK and eventually induces gastrin promoter stimulation, an RNA interference (RNAi) approach was established. Small interfering RNA (siRNA) was applied to achieve knockdown of endogenous ILK and FAK levels in AMO cells. Real-time PCR analyses revealed a knockdown efficiency of 80 % – 90 % on the mRNA level for FAK and ILK siRNA (Fig. 6A). A subsequent step was to confirm the knockdown of FAK and ILK on the protein level by Western blot. Following optimization, after 72 h of siRNA transfection, neither ILK nor FAK could be detected which verified an effective knockdown (Fig. 6B). AMO cells were transfected with FAK or ILK siRNA for 72 h, and then stimulated with *H. pylori* B128. Our results established that FAK played no pivotal role in gastrin signaling. However, all samples transfected with ILK siRNA showed a significant decrease in gastrin promoter induction of approximately 50 % (Fig. 6C). Therefore, we assume that gastrin expression is initiated via a β_5-integrin-ILK signaling complex.

Figure 6

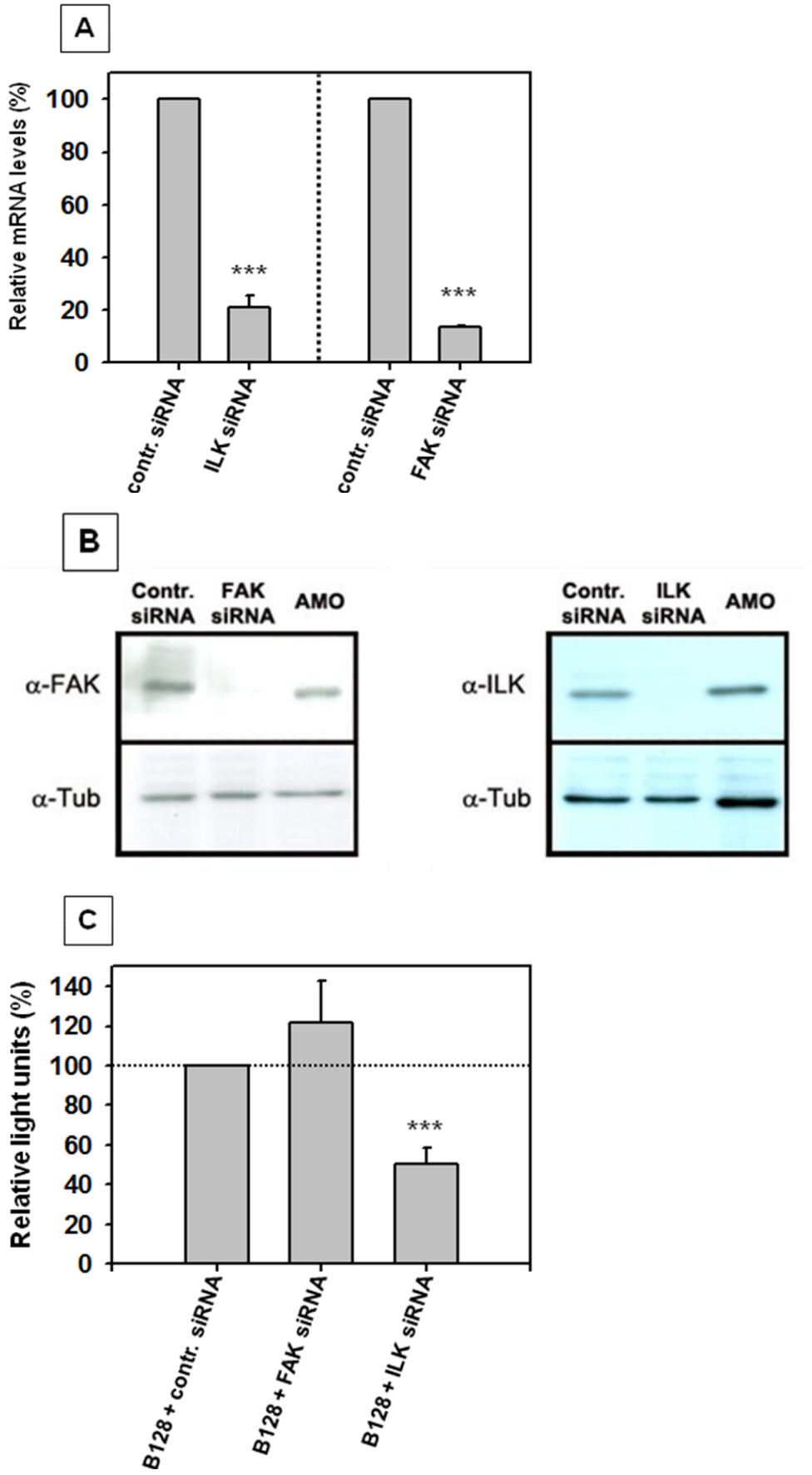

A
Relative mRNA levels (%)
100
80
60
40
20
0
***
***
contr. siRNA
ILK siRNA
contr. siRNA
FAK siRNA
B
Contr. siRNA
FAK siRNA
AMO
α-FAK
α-Tub
Contr. siRNA
ILK siRNA
AMO
α-ILK
α-Tub
C
Relative light units (%)
140
120
100
80
60
40
20
0
***
B128 + contr. siRNA
B128 + FAK siRNA
B128 + ILK siRNA

Figure 6. ILK is involved in *H. pylori*-induced gastrin gene expression. (A) AMO cells were transfected with either ILK siRNA [50 nM] or FAK siRNA [50 nM] for 24 h. RNA was isolated and RT-PCR was performed. Control siRNA [50 nM] with the same GC-content like the ILK siRNA or FAK siRNA was used as knock down negative control (represents 100%). (B) Western blot analyses were performed to confirm ILK or FAK silencing on protein level. α-Tubulin served as loading control. (C) AMO cells were transfected with ILK siRNA [50 nM], FAK siRNA [50 nM] or control siRNA [50 nM] for 72 h and subsequently stimulated with *H. pylori* B128. Luciferase activities in relation to *H. pylori* B128 WT transfected with control siRNA (represents 100%; interpolated dotted line) were plotted. The mean values ± SEM of at least three independent experiments are shown. Statistically analysis (Student`s *t* test) was applied using SigmaStat software. ***$P \leq 0.001$

Intracellular signaling cascades involved in gastrin gene expression

Observations in various cell systems revealed that different intracellular signaling pathways (MAP, ERK, JNK and p38) can be involved in the transmission of gastrin-triggered cellular effects (Hocker, 2004). In contrast to the signaling pathways involved in gastrin gene induction, the signaling pathways activated by gastrin are well characterized (Chupreta *et al.*, 2000). In this part of the study we investigated the signaling cascades involved in *H. pylori* induced gastrin gene expression. Western blot analysis showed that, compared to the *H. pylori* B128Δ*cag*Y-mutant strain, *H. pylori* B128 activates the p38 and JNK signaling cascades to a higher extent.. Erk signaling in AMO cells was induced comparably in *H. pylori* B128 and *H. pylori* B128Δ*cag*Y-mutant strains. The phosphorylation of Erk was no longer detectable when the cells were pre-incubated with the Mek1 kinase inhibitor PD98059 (Fig. 7B).
To ascertain more about the signaling kinases involved in *H. pylori*-induced gastrin promoter activation, AMO cells were pre-incubated with several kinase inhibitors and subsequently infected with *H. pylori.* The results indicate that Src-, Abl-, PKC-, Raf-, Erk-, p38-, and JAK-kinases play a crucial role in gastrin promoter induction. The samples pre-incubated with the JNK-inhibitor showed only a maximal inhibition of about 50 % (Fig. 7A).

Figure 7

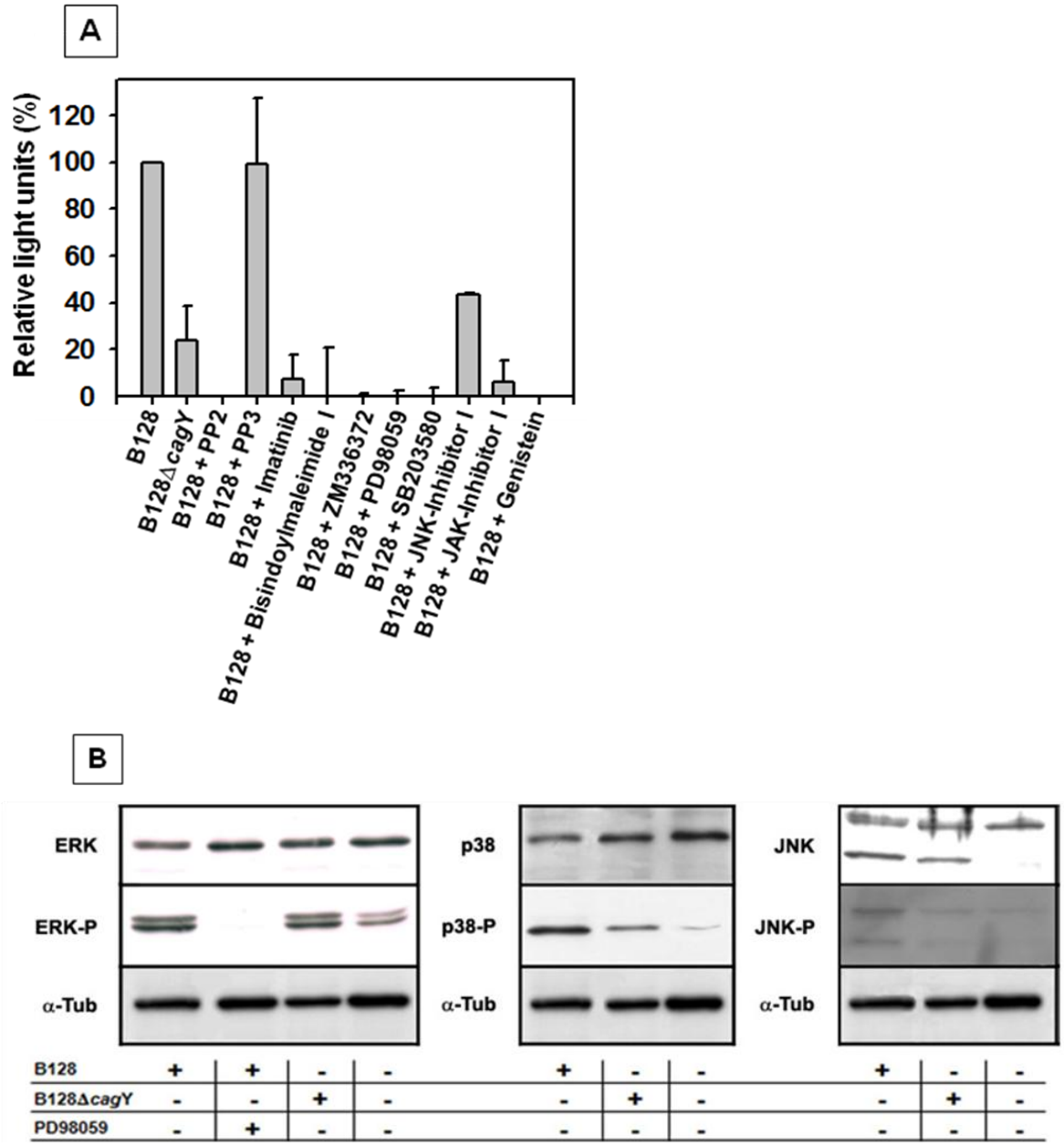


Figure 7. Several protein kinases are involved in *H. pylori* induced gastrin promoter activation. (A) AMO cells were pre-incubated with PP2 [25 µM], PP3 [25 µM], imatinib [10 µM], bisindoylmaleimide I [2 µM], ZM336372 [50 µM], PD98059 [100 µM], JNK-inhibitor I [5 µM], JAK-inhibitor [10 µM] or Genistein [100 µM] and stimulated with *H. pylori* B128. Luciferase activities in relation to *H. pylori* B128 WT (represents 100%) were plotted. The mean values ± SEM of at least three independent experiments are shown. (B) Western blot analyses were performed to show *H. pylori*-induced phosphorylation of Erk, p38, and JNK. Erk signaling was inhibited by using PD98059 [100 µM]. α-Tubulin served as loading control.

Discussion

Gastrin regulates not only the pH of the stomach but also the growth and differentiation of gastric epithelial cells (Dockray, 1999). *H. pylori*-induced hypergastrinemia was postulated to be a major risk factor promoting the development of gastric cancer (Fox *et al.*, 2003; Rieder *et al.*, 2005; Wang *et al.*, 2000; Wiedemann *et al.*, 2009). To date the mechanism by which *H. pylori* induces the gastrin signaling pathway is poorly understood. To elucidate how *H. pylori* induces gastrin gene expression, an *in vitro* promoter study was performed.

Data from this study demonstrate for the first time that *H. pylori* uses a β_5-integrin-ILK signaling complex to induce gastrin expression (Fig. 4B + 6C). We show that β_5-integrin is a novel human receptor exploited by a human bacterial pathogen to induce precancerous conditions in the gastric epithelial cells. In 2007 *Kwok et al*. found that β_1-integrin functioned as a receptor for *H. pylori* binding to the host cells in a CagL RGD-dependent manner. The authors demonstrated this interaction triggers CagA translocation into the host cells and activates the downstream cascade via Fak and Src (Kwok *et al.*, 2007). In our study we established that neither the β_1-integrin receptor nor the RGD-motif of the *H. pylori* surface protein CagL plays a major role in gastrin promoter induction (Fig. 4 A+B). By contrast, a newly discovered binding interaction could be identified between *H. pylori* B128 and β_5-integrin that was followed by an ILK-dependent downstream signaling cascade.

Furthermore, our data suggest that *H. pylori* mainly induces the gastrin promoter via TGF-α and not via the natural agonist EGF (Fig. 5B). TGF-α is proteolytically cleaved by the specific metalloprotease-activity of ADAM 10/17 which leads to the rapid phosphorylation of EGF-R and the subsequent intracellular signaling results in gastrin gene expression (Ford *et al.*, 1997; Higashiyama *et al.*, 2008; Le Gall *et al.*, 2009). These results are supported by previous data showing an increased ADAM 10/17 expression in the antral mucosa of *H. pylori* infected patients compared to biopsies of uninfected patients (Yoshimura *et al.*, 2002). The activation mechanisms of ADAMs are still largely unknown but there are some indications of how the novel β_5-integrin-ILK signaling complex could interact with ADAMs and activate TGF-α shedding. Several ADAMs have been already described to have potential phosphorylation sites for serin-threonin and / or tyrosine kinases which might regulate ADAM function

directly or provide ligands for SH2-domain containing proteins (Duffy *et al.*, 2009; Higashiyama *et al.*, 2008; Seals and Courtneidge, 2003). Therefore we hypothesize that the β_5-integrin-ILK signaling complex is interconnected with the EGF-R signaling via the kinases PKC, Abl, and Src. These activators are possibly direct binding partners of ADAMs (Fig. 8). Our investigations applying specific inhibitors against PKC, Src, and Abl indicate that these kinases play a pivotal role in gastrin promoter induction. Several publications have described an interaction between Src and PKC with the β-subunit of integrins (Besson *et al.*, 2002; Cox *et al.*, 2003; Liliental and Chang, 1998; Playford and Schaller, 2004) which strengthens the integrin aspect of this hypothesis.

Our data reveal that the major *H. pylori* virulence factors CagA and VacA play no crucial role in inducing gastrin gene expression. But interestingly enough, the binding of a *H. pylori* type-I strain with an intact T4SS is necessary to induce the gastrin promoter via β_5-integrin binding. This observation raises the question: Which bacterial factor is essential for gastrin gene induction upon binding to β_5-integrin? Our results indicate that CagL has to play a crucial role in the binding of *H. pylori* B128 to β_5-integrin because the *H. pylori* B128Δ*cag*L isogenic mutant strain could not induce the gastrin promoter. Apart from the fact that CagL seems to be involved in β_5-integrin binding, our study suggests that β_5-integrin binding has to be an RGD-independent mechanism because diverse *H. pylori* P12 isogenic mutant strains, which have a total deleted or mutated RGD-motif in the *cag*L-gene, exhibited WT gastrin induction. Our results from the filter-assay and the adhesine mutant-strain experiments strongly support that CagL plays a major role in gastrin induction. These data demonstrate the contact of the bacteria, independent of the tested adhesines, is essential for gastrin promoter induction. A further approach using bacterial lysates was performed to exclude that any bacterial factors could stimulate the gastrin promoter without contact of a viable *H. pylori* B128 to the epithelial cells.

Pro-inflammatory cytokines play an important role in the progression of precancerous conditions. In our recently published animal study it was shown that only *H. pylori* type-I strains can induce an early and severe inflammation in antral and corpus tissue that is associated with an increased expression of pro-inflammatory cytokines (Wiedemann *et al.*, 2009). This early inflammation is a part of a pathomechanism which causes later precancerous physiological changes (hypergastrinemia). Our data confirm previous findings regarding G-cells that the pro-inflammatory cytokines IL-1β

and TNF-α can induce the gastrin promoter *in vitro* (Suzuki *et al.*, 2001). By contrast, the pro-inflammatory cytokines IL-8 and IFN-γ have no effect on gastrin expression. These observations assume that different receptors are involved in the induction of gastrin expression.

In this report, we present evidence for the first time that *H. pylori* type I-strains are able to induce the gastrin promoter via TGF-α transactivating EGF-R upon binding to β_5-integrin. This seems to be the main *H. pylori*-associated pathway in gastrin gene induction, yet pro-inflammatory cytokines IL-1β and TNF-α also stimulate the gastrin promoter. It is known that *H. pylori* type I-strains activate NF-κB, regulating the release of IL-8 (Blackwell and Christman, 1997; Brandt *et al.*, 2005). IL-8 is the major mediator of inflammatory response and causes chemotaxis in its target cells (Th_1-cells). These Th_1-cells secrete pro-inflammatory cytokines such as IL-1β and TNF-α which are capable of inducing gastrin expression.

In conclusion, we elucidate a novel pathway for gastrin gene induction via the binding of *H. pylori* type I-strains to β_5-integrin and propose this as a potentiating pathway in addition to previously established pathways. The newly discovered interaction of *H. pylori* with β_5-integrin and the subsequent pathway via TGF-α and the activation of EGF-R could be useful targets in treating gastric precancerous conditions triggered by *H. pylori*-induced hypergastrinemia.

Figure 8

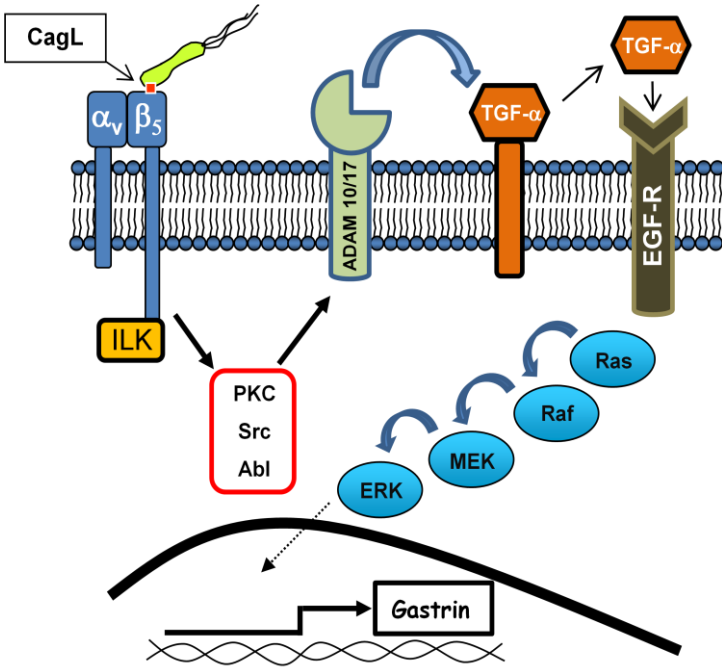


Figure 8. *H. pylori* uses a β_5-integrin-ILK signaling complex to induce gastrin expression. *H. pylori* binds to β_5-integrin and transmits the signal via the integrin-linked kinase (ILK). PKC, Src, and Abl might be involved in the activation of ADAM 10/17. Then ADAM 10/17 is shedding the inactive EGF-R ligand TGF-α. Soluble TGF-α subsequently binds to EGF-R and transactivates the Ras-Raf-MEK-ERK-pathway to induce gastrin promoter activation.

Experimental procedures

Bacterial strains and culture condition

H. pylori strains were grown on GC agar plates (Oxoid) supplemented with horse serum (5%), vancomycin (10 µg/ml), trimethoprim (5 µg/ml) and nystatin (1 µg/ml)(serum plates) and incubated for 2-3 days in a microaerobic atmosphere (85% N_2, 10% CO_2, 5% O_2) at 37^0C. *E. coli* strain DH5α (BRL) was grown on Luria-Bertani (LB) agar plates under oxic conditions at 37^0C. *Campylobacter jejuni* C64 (Gebert *et al.*, 2003) was grown at 37^0C on Columbia blood agar plates with sheep blood plus (Oxoid) under microaerobic conditions.

In vitro gastrin promoter stimulation assay

AM0 cells are AGS cells stably transfected with a plasmid containing 240 base pairs of the human gastrin promoter expressing the luciferase reporter (240 GasLuc) (Ford *et al.*, 1997). AM4 cells express the 240 GasLuc construct with a 4–base pair mutation within the GC-rich element preventing epidermal growth factor (EGF) responsiveness (gERE) to the gastrin promoter (Shiotani and Merchant, 1995). AM4 cells equally treated served as negative control. Both cell lines were cultured in RPMI 1640 (Gibco) supplemented with horse serum (10%) under standard conditions. Cells at 70% confluence and starved for 24 hours in Nutrient Mixture F12 (HAM; GIBCO) without supplements were stimulated with bacterial suspensions of WT and mutant strains at a multiplicity of infection of 100:1 (bacteria per cell) for 5 hours. Cells were harvested in lysis buffer (14 g/l K_2HPO_4, 2,67 g/l KH_2PO_4, 0,74 g/l EDTA, 1 g/l Triton X-100, 1nM DTT), and luciferase activity was measured (MicroLumat Plus LB 96 V; Berthold Technologies, Bad Wildbad, Germany) and normalized to total cell protein. Blocking antibodies, purified VacA and inhibitors were pre-incubated 45 minutes before bacterial stimulation. To generate metabolic inactive bacteria there was a pretreatment with 0,15% NaN_3 for 20 minutes.

Materials, Reagents and Antibodies

Anti-TNF-α, anti-$ß_2$-integrin, anti-$ß_3$-integrin, anti-$ß_4$-integrin, anti-$ß_5$-integrin antibodies and cell culture inserts, were purchased from Millipore. Anti-IL1ß, anti-ILK and IL-8 were purchased from Sigma-Aldrich. Anti-TGF-α and the inhibitors

Bisindolylmaleimide I, ZM336372, PD98059, JNK-Inhibitor I, SB203580, PP2, PP3 and JAK-Inhibitor I were purchased from Merck. The inhibitors Gefitinib and Imatinib were purchased from LC laboratories. The phospho-tyrosine-specific antibody PY99, anti-Erk, anti-p38, anti-$ß_1$-integrin and anti-Fak were purchased from Santa Cruz. Pro- and anti-inflammatory cytokines IL-1ß, IFN-γ, TNF-α, IL-4 and IL-10 were purchased from ImmunoTools. Antibodies against phospho-p38, phospho-erk, phospho-JNK and JNK were purchased from Cell Signaling. The antibody against CagA (Odenbreit *et al.*, 2000) and the purified VacA (Sewald *et al.*, 2008) were produced in the laboratory of Rainer Haas (Max-von-Pettenkofer Institute, Munich). The anti-EGF antibody was purchased from ProSci and the anti-$ß_7$-Integrin antibody from BioLegend. As Western blot loading control an anti-α-Tubulin antibody (Upstate) was used.

Plasmid transfection

Low passage cells with 40-50% confluency were transfected with either $pSP65SR^+$ or $pSP65SR^+$CagA for 24 hours using Lipofectamine 2000 (Invitrogen) according to manufacturer`s instructions. The CagA overexpression plasmid $pSP65SR^+$CagA contains the *cag*A gene of *H. pylori* strain NCTC11637 which was cloned into basic vector pSP65SRα (Higashi *et al.*, 2002).

RNA interferenz

Low passage cells with 20-30% confluency were transfected with 50 nM siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen) according to manufacturer`s instructions. SiRNA`s against FAK, ILK and control siRNA (with an equal GC-content) were purchased from Invitrogen. Three days after transfection, cells were harvested and subjected to immunoblotting and luciferase measurement.

RNA isolation and real-time RT-PCR

Gastric epithelial cells were harvested 24 h post transfection with FAK or ILK siRNA in RLT-buffer (Qiagen RNeasy Mini Kit) + 1% ß-mercaptoethanol. RNA isolation was performed as described in the Qiagen RNeasy Mini Kit protocol. Using the TaqMan Reverse Transcription Reagents (Roche) with random primers according to the kit

protocol, 1 µg mRNA was transcribed into cDNA. Oligonucleotide primers specific for FAK, ILK and the housekeeping gene 18S rRNA were applied for real-time RT-PCR (ABI PRISM 7000, Applied Biosystems). For amplification, the FastStart Universal SYBR Green Master (ROX) kit (Roche) was used according to manufacturers instructions. All data were normalized with the corresponding 18S rRNA transcription level using a comparative delta Ct method.

Immunoblotting

Cells were harvested in lysis buffer (PhosSTOP (Roche) phosphatase inhibitor cocktail tablets resolved in DPBS (Gibco)) by scraping. These cell lysates were boiled in Laemmli sample buffer for 10 minutes. Boiled samples were subjected to SDS-PAGE using a minigel apparatus (Bio-Rad) and blotted onto a PVDF-membrane using a semi-dry blot system (Biotec Fischer). The membranes were blocked with either 5 % milk-powder or 3 % BSA in TBS-buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated overnight with the respective primary antibody at 4^0 C followed by secondary anti-rabbit IgG or anti-mouse IgG conjugated HRP for 1 hour at room temperature. Immunoreactive bands were detected by ECL and radiography.

Statistics

Data are presented as mean ± SEM. The results were statistically analyzed using the Student`s *t*-test with SigmaStat statistical software. (***, *P* value ≤ 0,001 was considered as significant; #, P value ≥0,05 was considered not significant).

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Supporting material

Figure S1

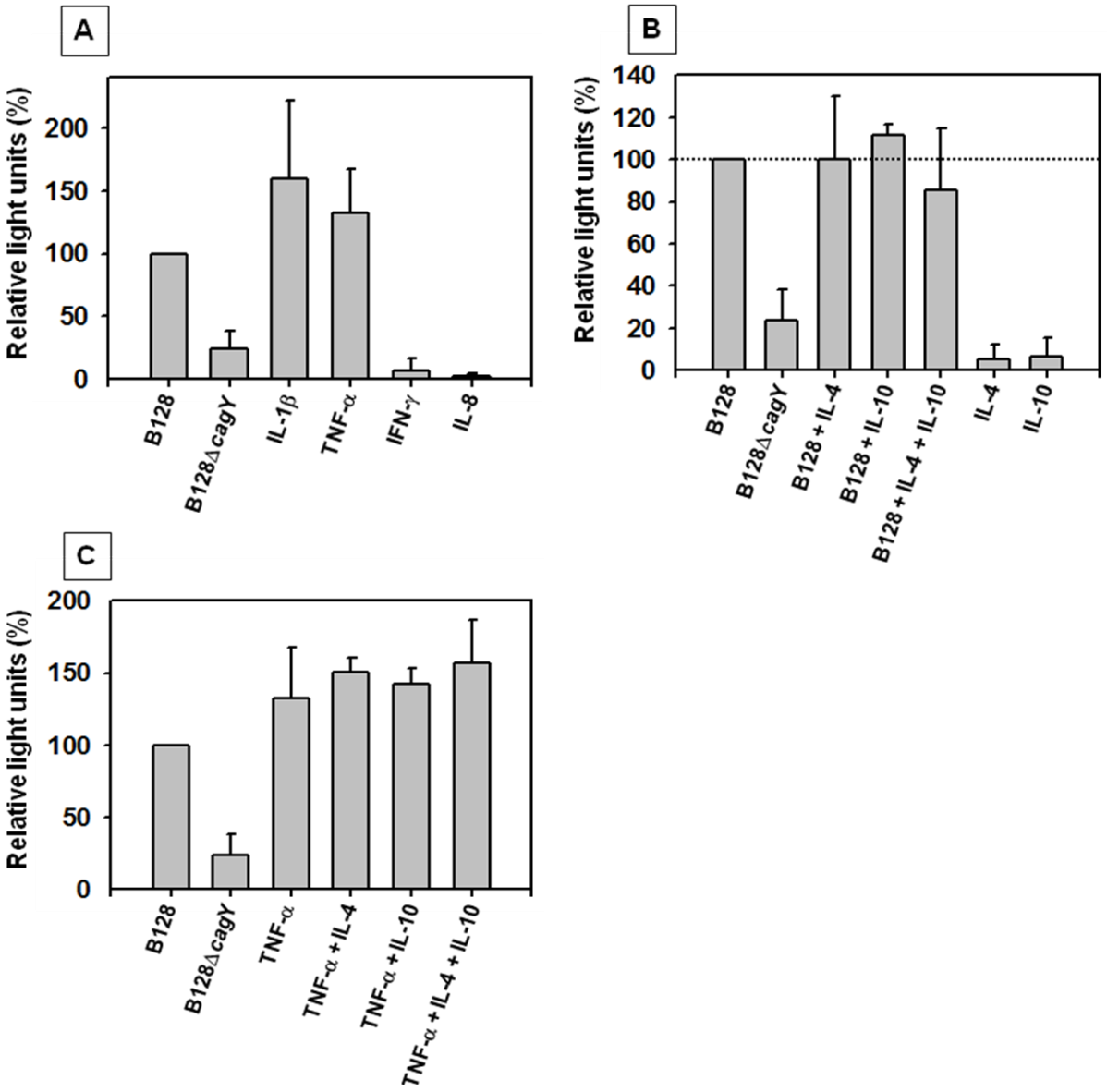

Figure S1. TNF-α and IL-1β are potent stimulators of the gastrin promoter. (A) AMO cells were stimulated with several pro-inflammatory cytokines (IL-1β [50 ng/ml], TNF-α [5 ng/ml], IFN-γ [50 ng/ml], and IL-8 [10 ng/ml]), and (B) the suppressive effect of anti-inflammatory cytokines (IL-4 [10 ng/ml] and / or IL-10 [10 ng/ml]) on *H. pylori* B128 stimulated AMO cells was tested. (C) AMO cells were pre-incubated with anti-inflammatory cytokines (IL-4 [10 ng/ml] and / or IL-10 [10 ng/ml]) and stimulated with the pro-inflammatory cytokine TNF-α [5 ng/ml]. Luciferase activities in relation to *H. pylori* B128 WT (represents 100%; interpolated dotted line) were plotted. The mean values ± SEM of at least three independent experiments are shown.

A C-terminal coiled-coil region of CagL is responsible for *Helicobacter pylori*-induced IL-8 expression

Short Title: *H. pylori* CagL-induced IL-8 expression

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Summary

Interleukin-8 (IL-8) is a potent neutrophil-activating chemokine which triggers the infiltration and migration of neutrophils into areas of bacterial infection. *H. pylori*-infected patient studies as well as animal models have revealed that *H. pylori* type I-strains carrying an intact *cag*-PAI (cytotoxin associated gene-pathogenicity island) with a functional type IV secretion system (T4SS) induce IL-8 expression and secretion in gastric mucosa. This gastric mucosal IL-8 expression correlates with severe histological changes due to *H. pylori*-infection.

In the present study we explored new bacterial factors and recognition patterns inducing IL-8 expression in *H. pylori*-infected host cells. To analyze the secreted IL-8 concentration, we performed IL-8 ELISA (enzyme-linked immunosorbent assay). To investigate the *H. pylori*-induced IL-8 expression on the transcriptional level, we transiently transfected gastric epithelial cells (AGS) with a human IL-8 luciferase reporter construct.

The results of this study demonstrate that specifically the C-terminal coiled-coil region of the *H. pylori* CagL-protein, a protein described to be located on the tip of the T4SS-pilus, is responsible for *H. pylori*-induced IL-8 secretion. This novel bacterial-host recognition sequence allows a new insight into how *H. pylori* induces the inflammatory response in gastric epithelial cells and facilitates the development of precancerous conditions.

Introduction

H. pylori is a gram-negative, spiral-shaped, microaerophilic and highly motile bacterial pathogen which colonizes the human stomach in more than 50% of the world population. In about 10 - 20% of the infected individuals, *H. pylori* causes chronic gastric inflammation and severe sequelae such as gastroduodenal ulcers, MALT (mucosa-associated lymphoid tissue)-lymphoma, and gastric adenocarcinoma (Houghton and Wang, 2005; Lee *et al.*, 1993). The outcome of severe forms of disease is dependent on bacterial factors produced by *H. pylori* type I-strains as well as specific host susceptibility (El Omar *et al.*, 2000; Rieder *et al.*, 2005a). In the *H. pylori* infection model of Mongolian gerbils only animals treated with *H. pylori* type I-strains developed severe gastrointestinal inflammation. It has been demonstrated that only *H. pylori* type I-strains characterized by harboring an intact *cag*-PAI with a functional T4SS induce a severe outcome of gastric diseases. In parallel, this results in a strong induction of proinflammatory cytokines (e.g. IL-1β, IL-6, TNF-α, IFN-γ, and IL-8). Animals infected with an isogenic mutant strain carrying a non-functional T4SS did not display severe inflammatory responses in the corpus mucosa (Ogura *et al.*, 2000; Rieder *et al.*, 2005b; Wiedemann *et al.*, 2009).

IL-8 belongs to a superfamily of secreted proteins, the chemokines (chemoattractant cytokines), that specialize in mobilizing leukocytes to areas of immune challenge (Baggiolini *et al.*, 1995). The function of this protein family member is to potently stimulate leukocyte migration along the chemotactic gradient, subsequently leading to the adhesion and infiltration of inflammatory cells into the affected tissues.

During *H. pylori*-infection, the increased IL-8-concentration causes a significant infiltration of neutrophils and lymphocytes into the gastric mucosa, resulting in chronic gastritis (Crabtree and Lindley, 1994). *In vivo* studies demonstrated a correlation between the gastric mucosal IL-8 levels and the histological severity of *H. pylori*-infected gastritis patients (Ando *et al.*, 1996).

Furthermore, *in vitro* data showed that *H. pylori*-induced IL-8 secretion under serum-free conditions is dependent on a functional T4SS (Fischer *et al.*, 2001) but not on the translocated CagA-protein because isogenic *cag*A-mutant-strains were not able to eliminate the ability of the bacterium to stimulate IL-8 expression (Nozawa *et al.*, 2002; Owen *et al.*, 2003). Also, adhesion studies have revealed that direct contact of the bacterium with the epithelial cell is required for IL-8 induction (Rieder *et al.*,

1997). To date, the exact mechanism how *H. pylori* induces IL-8 expression is only partially understood. So far it has been shown that the IL-8 promoter binding sites for both transcription factors, NF-κB- and AP (activating protein)-1, are required for an optimal transcription induced by *H. pylori* infection (Aihara *et al.*, 1997). In response to *H. pylori*-infection, the activated transcription factors NF-κB and AP-1 attach to their DNA binding sites within the IL-8 promoter and induce its expression (Chu *et al.*, 2003; Seo *et al.*, 2004; Naumann *et al.*, 1999; Sharma *et al.*, 1998).

In this study we identified a *H. pylori* factor that is essential for inducing IL-8 expression. Since direct contact to the host cell and a functional T4SS is required to induce IL-8 expression, we focused on the *H. pylori* surface proteins that could be potential binding partners to the host cell. To investigate the *H. pylori*-induced IL-8 promoter activity, we used AGS cells transiently transfected with a human IL-8 promoter luciferase reporter construct. In parallel, IL-8 secretion of stimulated gastric epithelial cells was quantified by applying a specific IL-8-ELISA. Our data reveal for the first time that a specific C-terminal coiled-coil region of CagL plays a crucial role in *H. pylori* adherence to the epithelial cells as well as IL-8 expression. The latter was determined by applying isogenic *H. pylori* CagL-mutant strains lacking the specific C-terminal coiled-coil region. These mutants were completely unable to induce IL-8 expression and secretion.

Results

H. pylori-specific CagL-dependent IL-8-induction

Previous studies have described that *H. pylori* type I-strains carrying a *cag*-PAI with a functional T4SS are able to induce IL-8 secretion in gastric epithelial cells (Selbach *et al.*, 2002; Naumann, 2005). In 2001 Fischer *et al.* analyzed the importance of each single protein of the *cag*-PAI on IL-8 induction using a systematic mutagenesis approach (Fischer *et al.*, 2001). In contrast to the importance of the T4SS, it could be shown that several major *H. pylori* virulence factors, such as CagA and VacA, play no crucial role in IL-8 induction (Huang *et al.*, 1995; Fischer *et al.*, 2001; Owen *et al.*, 2003; Nozawa *et al.*, 2002).

Our study supports the findings that *H. pylori*-induced IL-8 secretion (Fig. 1B) and transcriptional up-regulation (Fig. 1A) are not dependent on CagA but dependent on an intact T4SS, since there was only a small basal IL-8 expression in wells stimulated with the isogenic B128Δ*cag*Y-mutant strain. The mutant strain carries a deletion in the *cag*Y-locus which is essential for assembly of the T4SS. We also transfected AGS cells with a CagA-overexpression plasmid in serum-free media and could not measure any secreted IL-8 in the supernatant (data not shown).

To evaluate whether the increased IL-8 secretion is *H. pylori*-specific, we stimulated cells with another gastrointestinal pathogen *Campylobacter jejuni*. The results verified a *H. pylori*-specific IL-8 induction because *Campylobacter jejuni* was not able to stimulate IL-8 expression.

Furthermore, we have elucidated whether the direct contact of the bacterium to the host-cell is necessary to induce the IL-8 promoter activity as well as IL-8 secretion. The results demonstrated that the induction of the IL-8 promoter and the IL-8 secretion is dependent on a direct contact to the host-cell. Parallel to that, by applying filter inserts, we were able to exclude that soluble *H. pylori* factors induce IL-8 expression without binding the bacterium to the epithelial cells (Fig. 1C+D). To establish persistent infection in the gastric mucosa, *H. pylori* mainly binds to the epithelial cell via the major bacterial adhesins BabA, SabA, and AlpAB (Linden *et al.*, 2002; Mahdavi *et al.*, 2002; Odenbreit *et al.*, 1999). We tested these adhesins on the ability to induce IL-8 secretion and revealed that *H. pylori*-induced IL-8 expression is independent of the major adhesins BabA, SabA, and AlpAB (Fig. 1E).

It was most interesting to discover that CagL, a protein localized on the tip of the T4SS-pilus (Kwok *et al.*, 2007), seems to play an essential role in the induction of IL-8 expression, since AGS cells stimulated with a B128Δ*cag*L-mutant strain did not express and secret IL-8.

Figure 1

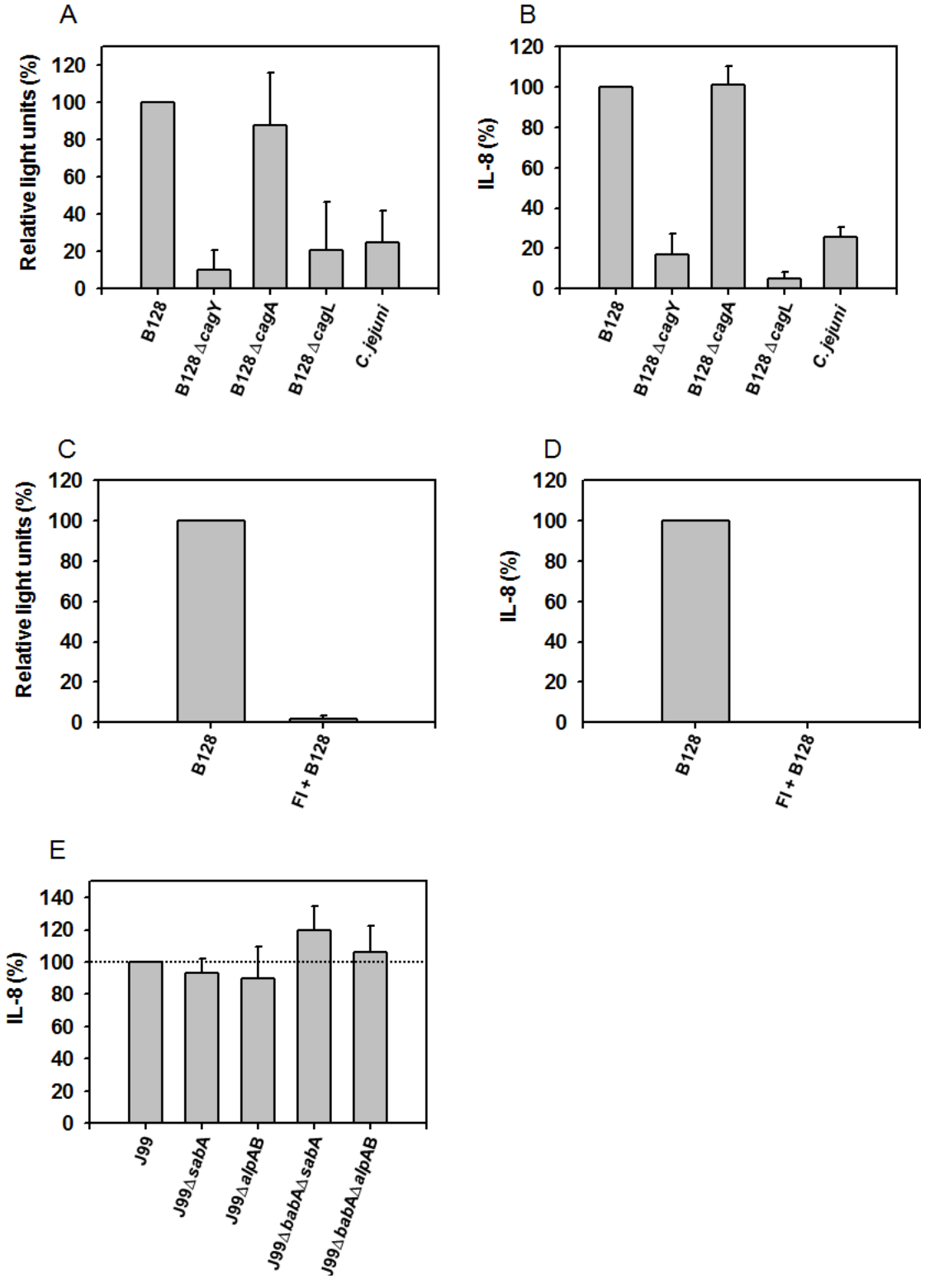


Fig. 1 *H. pylori* type-I strain specific IL-8-expression. (A+B) AGS cells were stimulated with *H. pylori* B128 WT, isogenic mutant-strains, and *Campylobacter jejuni*. (A, C) IL-8-promoter induction and (B, D) IL-8-secretion were determined in relation to *H. pylori* B128 WT (represents 100%). (C+D) Filter inserts (FI) were placed in each well and AGS cells were stimulated with *H. pylori* B128 WT. (E) AGS cells were stimulated with *H. pylori* J99 WT and isogenic adhesin-mutant-strains. IL-8-secretion was determined in relation to *H. pylori* J99 WT (represents 100%; interpolated dotted line). The mean values ± SEM of at least three independent experiments are shown.

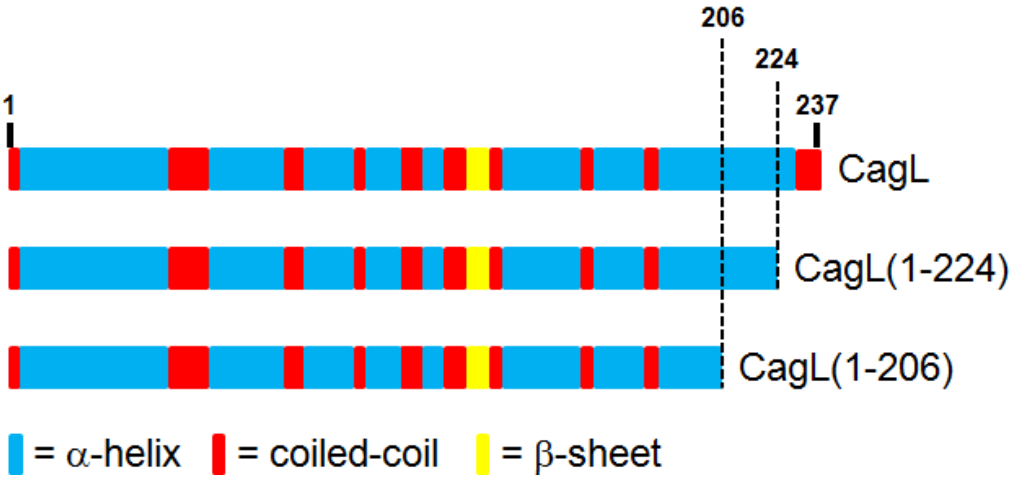
CagL C-terminal coiled-coil region is essential for IL-8 induction

CagL is a protein located at the tip of the T4SS that is proposed to be necessary for CagA translocation (Kwok *et al.*, 2007). To predict the 2D- and 3D-structure of this important protein, we submitted the CagL amino acid sequence of *H. pylori* B128 to the Expasy program (www.expasy.org) for analysis by the Phyre (Protein Homology/analogY Recognition Engine) software. The selected “Predict Secondary Structure” (PSIPRED) method of the protein structure prediction server at the UCL Bioinformatics Group, Bloomsbury Centre for Bioinformatics, London, UK (www.sbg.bio.ic.ac.uk/phyre/), is a highly accurate method for protein secondary structure prediction because it uses a very stringent cross validation method to evaluate the method’s performance (Jones, 1999). The PSIPRED analysis of the CagL protein sequence determined a basic α-helix structure separated by eight coiled-coil motifs and one β-sheet region as well as an N- and C-terminal coiled-coil motif (Fig. 2A). Considering the predicted 3D-structure obtained from the same program with the highest estimated precision of 95 % (Fig. 2B), we concentrated on the C-terminal end of the protein with its distant α-helix arm ending in a coiled-coil structure. To identify a putative IL-8-inducing domain of the CagL protein, we constructed two isogenic mutant strains: one deprived of the whole C-terminal protein arm, named *H. pylori* B128 Δ*cag*L(1-206) (Fig. 2A+D) and the other mutant strain shortened by 13 amino acids covering the C-terminal coiled-coil region, named *H. pylori* B128 Δ*cag*L(1-224) (Fig. 2A+C). Both mutant strains, still expressing 206 and 224 amino acids of the CagL protein (data not shown), were used together with the WT strain and an isogenic Δ*cag*L knock-out mutant for stimulating AGS cells directly

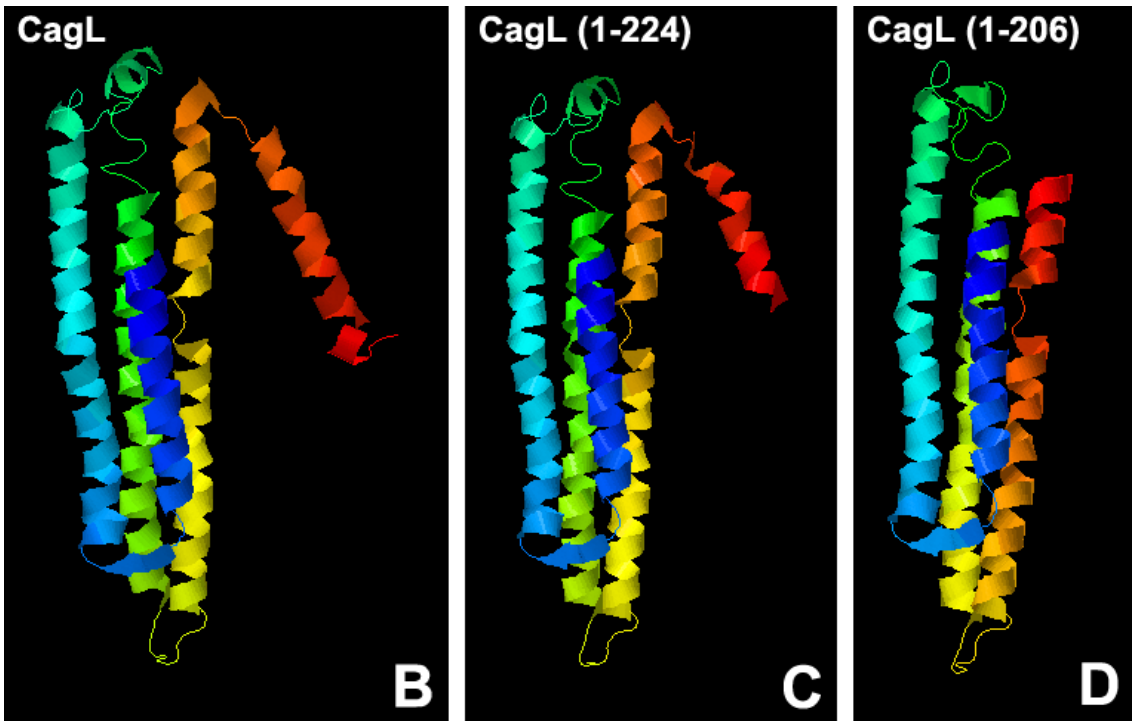
or transiently transfected with the IL-8 promoter luciferase reporter construct. All three Δ*cag*L-mutant strains revealed a highly significant reduction of the IL-8 promoter expression (Fig. 2E) as well as IL-8 secretion (Fig. 2F) compared to the WT-strain. Our data suggest that the C-terminal coiled-coil structure of the CagL protein is essential for IL-8 induction.

Figure 2

A



B-D



E+F

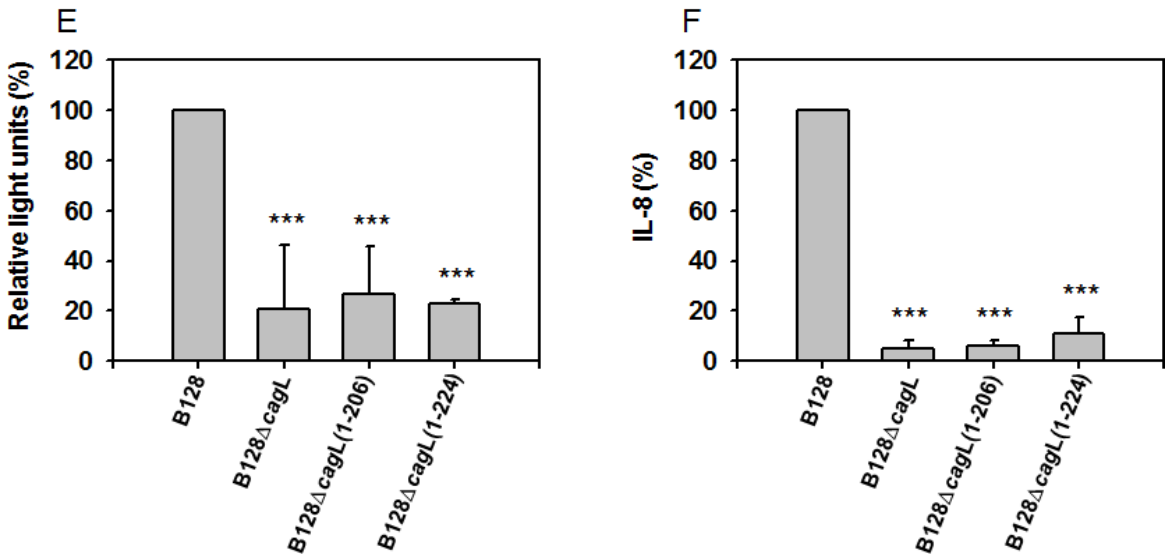


Fig. 2 *H. pylori*-induced IL-8-expression is dependent on a C-terminal coiled-coil region of CagL. (A) Schematic view of the *H. pylori* CagL-protein and its isogenic mutants ΔCagL(1-224) and ΔCagL(1-206). The proteins are drawn to scale as bars, with the number of amino acid residues from the genome sequence of strain *H. pylori* B128 WT. Blue color represents predicted α-helices, red color for coiled-coils, and yellow color for β-sheets analyzed by PSIPRED (www.sbg.bio.ic.ac.uk/phyre/). The numbers above the bars indicate the respective amino acids. (B-D) 3-dimensional structure of the *H. pylori* (B) CagL-protein and its isogenic mutants (C) ΔCagL(1-224) and (D) ΔCagL(1-206) predicted by Phyre software with 95% estimated precision. (E, F) AGS cells were stimulated with *H. pylori* B128 WT and isogenic *H. pylori* B128Δ*cag*L, B128Δ*cag*L(1-206), and B128Δ*cag*L(1-224)-mutant strains. (E) IL-8-promoter induction and (F) IL-8-secretion were determined in relation to *H. pylori* B128 WT (represents 100%). The mean values ± SEM of at least three independent experiments are shown. Statistical analysis (Student`s *t* test) was applied using SigmaStat software. ****P* ≤ 0.001; # *P* ≥ 0.05.

CagL C-terminal coiled-coil region is crucial for *H. pylori*-adherence

To examine whether the CagL protein and especially the C-terminal coiled-coil region play a crucial role in *H. pylori* adhesion to the epithelial cells, we tested the previously described CagL-mutant strains on their ability to adhere to gastric epithelial cells

(AGS). Therefore, AGS cells were stimulated with the *H. pylori* B128 WT-strain and its isogenic mutant strains B128Δ*cag*L, B128Δ*cag*L(1-224), and B128Δ*cag*L(1-206). The adherence patterns of the bacteria to the cell surface were analyzed via immunofluorescence. To visualize the cell-bound bacteria and the epithelial cells, we applied a FITC-labeled anti-*H. pylori* antibody and the actin-staining phalloidin, respectively. It could be demonstrated that all three CagL-mutant strains showed similar adherence patterns with a reduced binding capability of approximately 50%, compared to the *H. pylori* B128 WT-strain (Fig.3). Additionally, the CagL-mutant strains lost their ability to induce cell-elongations („hummingbird-phenotype") in comparison to the WT-strain. These data reveal that the C-terminal coiled-coil region of the CagL protein is sufficient to considerably reduce *H. pylori*-binding efficiency to the epithelial cells.

Figure 3

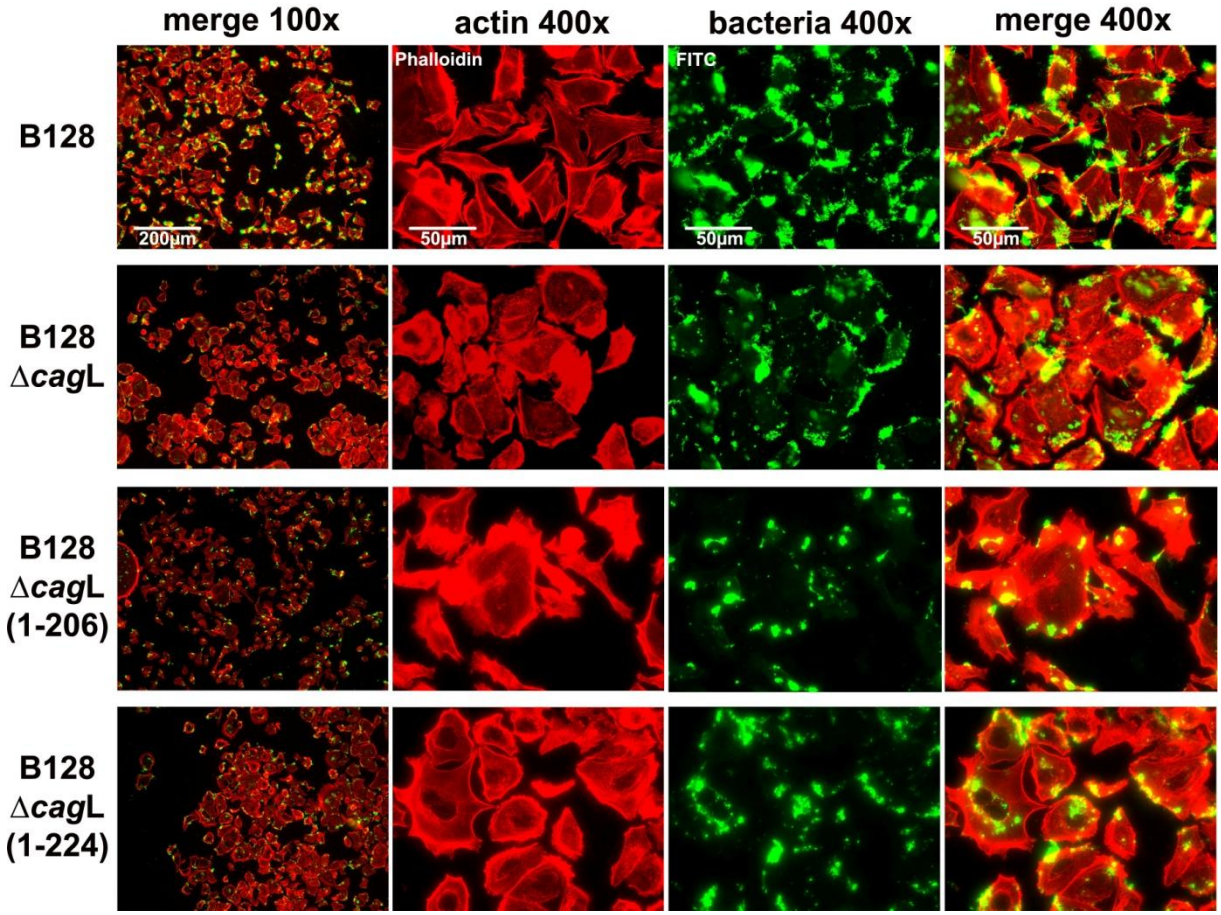


Fig. 3 CagL C-terminal coiled-coil region is involved in *H. pylori*-adhesion.
AGS cells grown on chamber slides were stimulated with *H. pylori* B128 WT and isogenic *H. pylori* B128 Δ*cag*L, B128Δ*cag*L(1-206) and B128Δ*cag*L(1-224) mutant strains. The AGS cells were stained with phalloidin (red), the bacteria detected with anti-*H. pylori*-antibody stained with FITC-labelled secondary antibody (green), and merged at 1:10 and 1:40 magnification (yellow: overlap of bacteria and cells).

β_1-integrin-independent IL-8-induction via TGF-α and EGF-R

Upon binding of *H. pylori* to the host-cell, clustered integrins assemble into actin-rich structures called focal-adhesions (FAs), where integrin signaling is mediated predominantly by Src and the focal adhesion kinase (FAK) (Mitra and Schlaepfer, 2006). In 2007, Kwok *et al.* were able to demonstrate that the translocation of CagA is dependent on binding of the *H. pylori* CagL-protein to the β_1-integrin-receptor of the host-cell (Kwok *et al.*, 2007). Hence, we examined whether the binding to β_1-integrin and the signal transduction via FAK play a central role in *H. pylori* CagL-mediated IL-8 induction.

To elucidate the role of FAK and β_1-integrin in *H. pylori*-activated IL-8 expression, we applied a siRNA-approach. The obtained data revealed a FAK- and β_1-integrin-independent IL-8 induction mechanism (Fig. 4A). In parallel, a specific Src-kinase inhibitor was not able to inhibit *H. pylori*-stimulated IL-8 secretion (data not shown). With the knowledge that neither the β_1-integrin-host-receptor nor FAK is involved in *H. pylori*-IL-8-induction, we focused on host-cell signaling, especially on epidermal growth factor (EGF)-receptor (EGF-R) and its ligands activated upon *H. pylori*-binding. Therefore, the EGF-R and its major ligands EGF and TGF (transforming growth factor)-α were tested as potential candidates involved in the *H. pylori*-induced IL-8 signal transduction pathway. Measurements with the EGF-R tyrosine kinase inhibitor Gefitinib and a specific TGF-α catching antibody showed a significant decrease in *H. pylori*-induced IL-8 secretion. These data established the involvement of TGF-α and its receptor EGF-R in *H. pylori*-induced IL-8 signaling (Fig. 4B). In contrast the natural agonist EGF was not important for *H. pylori*-induced IL-8 secretion.

Figure 4

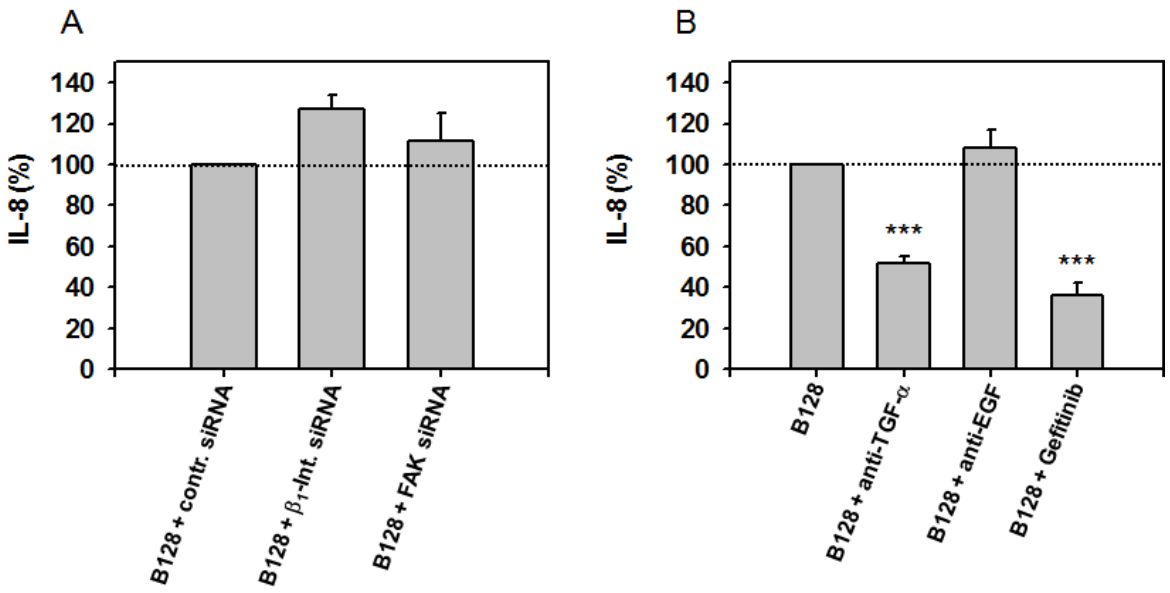


Fig. 4 *H. pylori*-induced IL-8-secretion is EGF-R dependent. (A) AGS cells were transfected with β_1-integrin siRNA [50 nM], FAK siRNA [50 nM] or control siRNA [50 nM] for 72 h and subsequently stimulated with *H. pylori* B128 WT. (B) AGS cells were pre-incubated with anti-TGF-α [10 µg/ml] and anti-EGF [10 µg/ml] antibodies or EGF-R-inhibitor gefitinib [5 µM] and stimulated with *H. pylori* B128 WT. IL-8-secretion was determined in relation to *H. pylori* B128 WT (represents 100%; interpolated dotted line). The mean values ± SEM of at least three independent experiments are shown. Statistical analysis (Student`s *t* test) was applied using SigmaStat software.
***$P \leq 0.001$; # $P \geq 0.05$.

Discussion

This study examined the role of the *H. pylori* CagL protein in bacterial triggered IL-8 expression of gastric epithelial cells. Our findings support prior reports which demonstrate only *cag*-PAI positive *H. pylori* strains with direct contact to gastric epithelial cells are capable of inducing IL-8 expression (Fig. 1A-D) (Selbach *et al.*, 2002; Fischer *et al.*, 2001; Rieder *et al.*, 1997; Nilsson *et al.*, 2003). The systematic mutagenesis screen of the *H. pylori cag*-PAI revealed an abolished IL-8 secretion in Δ*cag*L-mutants (Fischer *et al.*, 2001). A recently published protein interaction study by Kutter *et al.* described predicted coiled-coil regions in nearly all T4SS-proteins and speculated that their interactions are mediated by these domains (Kutter *et al.*, 2008). Based on the following three facts: (I) CagL is located on the tip of the T4SS (Kwok *et al.*, 2007), (II) Δ*cag*L-mutant strains are unable to induce IL-8 (Fischer *et al.*, 2001), and (III) CagL contains a predicted C-terminal coiled-coil region (Fig. 2A+B), we decided to investigate CagL as bacterial-ligand involved in *H. pylori*-induced IL-8 induction. In our study we identified for the first time a C-terminal coiled-coil region in CagL which is responsible for *H. pylori* induced IL-8 expression as well as host-cell adhesion. Our data prove that a deletion of the last 13 C-terminal amino acids of CagL is sufficient to block *H. pylori*-induced IL-8 expression (Fig. 2E+F).

The coiled-coil motif is a common and highly versatile assembly motif found in a wide range of structural proteins with functions ranging from the assembly of macromolecular complexes, signal transduction and vesicular trafficking up to molecular recognition (Strauss and Keller, 2008; Lupas, 1996). In several gram-negative bacteria (e.g. *Shigella flexneri, Salmonella spp., Yersinia spp., Pseudomonas aeroginosa*) the presence and functions of coiled-coils in structural and regulatory proteins have been thoroughly explored (Gazi *et al.*, 2009; Delahay and Frankel, 2002). This is in sharp contrast to *H. pylori* where little is presently known about coiled-coil motifs and its functions.

To determine the host-cell receptor recognizing the CagL coiled-coil motif, we primarily focused on the described interaction of CagL with the β_1-integin host receptor. This interaction was demonstrated to be essential for CagA translocation into the gastric epithelial cell and activation of downstream cascades via FAK and Src (Kwok *et al.*, 2007). It was also reported that other gram-negative pathogenic bacteria

(e.g *Yersinia enterocolitica*) use the β_1-integin host-receptor to induce IL-8 expression (Grassl *et al.*, 2003). In contrast, our data revealed that neither β_1-Integin nor FAK is involved in *H. pylori*-associated IL-8 induction. Additionally, several β-integrin-blocking antibodies were applied (β_1, β_2, β_3, β_4, β_5, and β_7) to screen for a putative CagL coiled-coil receptor on the host-cell surface which could be involved in IL-8 induction (data not shown). Consequently, it could be demonstrated that all tested integrins play no remarkable role in *H. pylori*-triggered IL-8 expression.

Coiled-coil associations, with crucial roles in many physiological and pathological processes, structural simplicity and a reversible nature, are promising targets for pharmacological interference, as already successfully established by botulinum toxins and viral fusion inhibitors (Strauss and Keller, 2008).

Infection with *H. pylori* induces the chemotactic cytokine IL-8 that is associated with severe gastric inflammation demonstrated by recruitment, migration, and activation of neutrophils into the sites of infection. Therefore it is of great interest to clarify the molecular mechanisms involved in *H. pylori* CagL-triggered IL-8 induction. In the future, the major focus should concentrate on the elucidation of the host-receptor for the CagL-mediated IL-8-induction via coiled-coil structures. The interference of this protein-protein interaction might offer an auspicious therapeutical approach to impair *H. pylori* binding to the gastric epithelial cells and to prevent *H. pylori* CagL-induced IL-8 secretion.

Experimental procedures

Bacteria and cell lines

H. pylori strains were grown on GC agar plates (Oxoid, Wesel, Germany) supplemented with horse serum (5%), vancomycin (10 µg/ml), trimethoprim (5 µg/ml) and nystatin (1 µg/ml) (serum plates) and incubated for 2-3 days under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) at 37^0C. *Campylobacter jejuni* C64 (Gebert *et al.*, 2003) was grown on Columbia blood agar plates (Oxoid) under microaerobic conditions.

Human gastric adenocarcinoma cells AGS (ATCC CRL 1739) were obtained from the American Type Culture Collection (Rockville, Md). The cells were cultured in RPMI 1640 (Invitrogen, Germany) supplemented with horse serum (10%) under standard conditions. Cells at 70% confluence were starved for 12 hours in Nutrient Mixture F12 (Invitrogen) and then infected with a multiplicity of infection (MOI) of 100 for 5 hours. The cell culture supernatants were preserved at -70^0 C for quantification of IL-8.

Construction of Δ*cag*L-mutants

Standard techniques were used for routine DNA manipulation, subcloning, and plasmid construction as previously described (Sambrook *et al.*, 1989). To produce a *H. pylori* B128 Δ*cag*L mutant, the plasmid pSH6 was constructed. pSH6 is carrying a kanamycin cassette in a *Xba*I site between two adjacent nucleotide regions of about 1000 bp up- and down-stream of the *cag*L gene. The PCR-products were generated by using genomic DNA from *H. pylori* B128 strain applying the primers for 5'-*cag*L fragment: FP 5'-CGGAGCTCAGGTTCAGACATCTTGCTTGG-'3 and RP 5'-GCTCTAGAGCATCTTCTTCACCCATTTC-'3 and 3'-*cag*L fragment: FP 5'-CCTCTA GAGCCAATTTTGAAGCGAATGAG-'3 and RP 5'-CGGAATTCGACAACACTTGAGT GGTTTAAAAC-'3. The amplified products were cloned into the restriction sites ScaI and EcoRI of pBluescript SK+ (Stratagene, La Jolla, CA).

To construct the *H. pylori* B128 Δ*cag*L(1-206) and Δ*cag*L(1-224) mutants C-terminal shortened by 31 and 13 amino acids, respectively, PCR-products were amplified covering the remaining 206 and 224 amino acids followed by an additional stop codon. For amplifying the Δ*cag*L(1-206) and Δ*cag*L(1-224) following primers were used: FP for both, 5'-GATCGAGCTCGGGATCAATGGAGAAATCAAAACC-'3 and

RP Δ*cag*L(1-206): 5'-GATCGCATGCCTAATTTAAAAAGACCTTGTTGTGAGC-'3, and RP Δ*cag*L(1-224): 5'-GATCGCATGCCTATTGCCGCTTACTTTGTTCTAGG-'3. For both constructs the PCR-product down-stream of the *cag*L gene were amplified with following primer set: FP 5'-GATCGCATGCTATGATTTCTAACCTTTGGGA-'3 and RP 5'-GATCGAATTCGCCACTAACGCTTTGAGAG-'3. The additional *Sph*I site between the two PCR fragments is located in the non-coding region between the *cag*L and *cag*N genes. This restriction site was used for inserting the kanamycin cassette. All PCR-products were cloned into the pBluescript SK+ vector opened with *Sac*I and *Eco*RI to generate the plasmids pGR-LD1 (1-224), and pGR-LD2 (1-206).
The resulting plasmids were transformed into *E. coli* DH5α and the reisolated plasmids (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) subjected to DNA sequencing to verify sequence integrity.
The deletion mutants were obtained by transforming the constructed plasmids pSH6, pGR-LD1, and pGR-LD2 into the natural competent *H. pylori* B128 cells kept in liquid culture medium (brucella broth plus 10% FCS). The transformed bacteria were selected on serum-plates supplemented with kanamycin.

Quantification of IL-8

The concentration of IL-8 in the cell culture supernatants was determined by sandwich ELISA as described previously (Yasumoto *et al.*, 1992).

IL-8 promoter assays

Cells, grown to a confluency of 90% in RPMI 1640 (Gibco, Karlsruhe, Germany) supplemented with horse serum (10%), were transfected with the human IL-8 promoter (gift of A. Sing) and pE1 (beta-Galactosidase) at a rate of 10:1 using the Lipofectamine 2000 reagent (Invitrogen, Karlsruhe, Germany) according to manufacturer`s protocol. After incubation with the transfection solution, cells were washed and then starved for 12 hours in Nutrient Mixture F12 (HAM; GIBCO). Following stimulation with H. pylori for 5 hours, cells were harvested in lysis buffer (14 g/l K_2HPO_4, 2,67 g/l KH_2PO_4, 0,74 g/l EDTA, 1 g/l Triton X-100, 1nM DTT) and luciferase activity was measured (MicroLumat Plus LB 96 V; Berthold Technologies, Bad Wildbad, Germany). The results were normalized to beta-galactosidase levels

measured using the beta Gal Assay Kit (Invitrogen) according to manufacturer`s instructions.

RNA interference

AGS cells were transfected with 50 nM siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen) according to manufacturer`s instructions. siRNA`s against FAK, β_1-integrin, and control siRNA (with equal GC-content) were purchased from Invitrogen. Cells were harvested 24 h post-transfection with FAK, β_1-integrin or control siRNA in RLT-buffer (Qiagen RNeasy Mini Kit) + 1% ß-mercaptoethanol. RNA isolation was performed as described in the Qiagen RNeasy Mini Kit protocol. Using the TaqMan Reverse Transcription Reagents (Roche, Germany) with random primers according to the kit protocol, we transcribed 1 µg mRNA into cDNA. Oligonucleotide primers specific for FAK, β_1-integrin and the housekeeping gene 18S rRNA were applied for real-time RT-PCR (ABI PRISM 7000, Applied Biosystems). For the amplification step, the FastStart Universal SYBR Green Master (ROX) kit (Roche) was used according to manufacturer's instructions (data not shown). Immunoblotting was performed to verify the FAK- and β_1-integrin-knockdown rate on protein level.

Immunoblotting

To prepare the cell lysates for immunoblotting, cells were harvested in DPBS-buffer (Invitrogen) and boiled in Laemmli sample buffer for 10 minutes (Laemmli, 1970). The lysates were subjected to SDS-PAGE using a minigel apparatus (Bio-Rad, Germany) and blotted onto a PVDF-membrane using a semi-dry blot system (Biotec Fischer, Reiskirchen, Germany). The membranes were blocked with 3 % bovine serum albumin (BSA) in TBS-buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated overnight with a polyclonal anti-CagL-antibody (AK271) (Fischer *et al.*, 2001). Alkaline phosphatase-coupled protein A was used to visualize bound antibodies.

Immunfluorescence

AGS cells were infected with *H. pylori* at OD 0.2 for five hours. The cells were fixed by 1% PFA. After washing the cells with PBS and blocking with goat-serum, we applied an α-*H. pylori*-antibody (AK 175) (Odenbreit *et al.*, 2000). Alexa488-conjugated secondary antibody (Molecular Probes, Germany) was used to visualize

rabbit antibodies. To depict the whole cells, a counter stain with Phalloidin (Sigma Aldrich, Germany) was applied. The stained specimens were examined with Olympus BX61 microscope.

Statistics

Data are presented as mean ± SEM. The results were statistically analyzed using the Student`s *t* test with SigmaStat statistical software. (***, *P* value ≤ 0,001 was considered as highly significant; #, P value ≥0,05 was considered not significant).

Acknowledgments

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Wiedemann,T., Loell,E., Mueller,S., Stoeckelhuber,M., Stolte,M., Haas,R., and Rieder,G. (2009) *Helicobacter pylori cag*-Pathogenicity island-dependent early immunological response triggers later precancerous gastric changes in Mongolian gerbils. *PLoS One* **4**: e4754.

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7. Zusammenstellung aller eigenen Publikationen

Originalarbeiten

Wiedemann T., Loell,E., Mueller,S., Stoeckelhuber,M., Stolte,M., Haas,R., and Rieder,G. (2009) *Helicobacter pylori cag*-Pathogenicity island-dependent early immunological response triggers later precancerous gastric changes in Mongolian gerbils. *PLoS One* **4**: e4754.

Wiedemann T., Hofbaur S., and Rieder,G. (2009) A novel integrin-β_5-ILK signaling complex is jointly responsible for *Helicobacter pylori*-induced precancerous conditions. (Submitted to Cellular Microbiology).

Wiedemann T., Hofbaur S., Loell,E., and Rieder,G. (2009) A C-terminal coiled-coil region of CagL is responsible for *Helicobacter pylori*-induced IL-8-expression. (Submitted to Molecular Microbiology)

Abstracts

[#]Wiedemann T., *et al.*(2007) Time course analysis of *Helicobacter pylori* colonization in Mongolian gerbils: Role of the *cag*-pathogenicity island on physiological and immunological markers. IJMM 2007; 297S1:58

Wiedemann T., *et al.* (2007) Analysis of Physiological and Immunological Markers over a 14 month Colonization Study in Mongolian Gerbils: Role of *H. pylori cag*PAI. Helicobacter 12:475

[#]Wiedemann T., *et al.*(2008) Role of *H. pylori cag*PAI over a 14 months colonization study in Mongolian gerbils: Analysis of physiological and immunological markers. Zoonoses Public Health 2008; 54 (Suppl. 1) p.87

[#]Wiedemann T., *et al.*(2008) *Helicobacter pylori cag*PAI dependent two-step pathomechanism in Mongolian gerbils. IJMM 2008; 298S2:28

Hofbaur S., Wiedemann T., *et al.* (2009) *Helicobacter pylori cag*-pathogenicity island-dependent down-regulation of the anti-inflammatory response in gastric cells. *Helicobacter* 14:317

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[#]Wiedemann T., *et al.*(2009) *Helicobacter pylori* cagPAI-mediated carcinogenesis in Mongolian gerbils. IJMM 2009; 299S1:24

Buch K., Wiedemann T. *et al.*(2009) Severe inflammation triggers phenotypical changes of *Helicobacter pylori* passaged through the Mongolian gerbil stomach. IJMM 2009; 299S1:23

[#]Orale Präsentationen

Nicht publizierte orale Präsentationen

A 14 month *H. pylori* colonization study in Mongolian gerbils: The role of the *cag*PAI in physiological and immunological changes

6. *Helicobacter pylori*-Workshop 2007 (Herrsching; Germany)

Early CagA-mediated gastric cell reprogramming by *Helicobacter pylori* induces later precancerous changes in Mongolian gerbils

8th International Workshop on Pathogenesis and Host Response in *Helicobacter* Infections 2008 (Helsingor; Denmark)

Auszeichnungen

International award for young scientists 2008

Early CagA-mediated gastric cell reprogramming by *Helicobacter pylori* induces later precancerous changes in Mongolian gerbils

8th International Workshop on Pathogenesis and Host Response in *Helicobacter* Infections 2008 (Helsingor; Denmark)

8. Erklärung

Hiermit erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Tobias Wiedemann

Bayreuth, den 23.11.2009