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Specific high affinity interaction of *Helicobacter pylori* CagL with integrin $\alpha_V\beta_6$ promotes type IV secretion of CagA into human cells

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CagL is an essential pilus surface component of the virulence-associated type IV secretion system (T4SS) employed by *Helicobacter pylori* to translocate the oncogenic effector protein CagA into human gastric epithelial cells. CagL contains an RGD motif and integrin $\alpha_5\beta_1$ is widely accepted as its host cell receptor. Here, we show that CagL binds integrin $\alpha_V\beta_6$ with substantially higher affinity and that this interaction is functionally important. Cell surface expression of $\alpha_V\beta_6$ on various cell lines correlated perfectly with cell adhesion to immobilized CagL and with binding of soluble CagL to cells. We found no such correlation for $\alpha_5\beta_1$. The purified $\alpha_V\beta_6$ ectodomain bound CagL with high affinity. This interaction was highly specific, as the affinity of CagL for other RGD-binding integrins was two to three orders of magnitude weaker. Mutation of either conserved leucine in the CagL RGDLXXL motif, a motif that generally confers specificity for integrin $\alpha_V\beta_6$ and $\alpha_V\beta_8$, lowered the affinity of CagL for $\alpha_V\beta_6$. Stable expression of $\alpha_V\beta_6$ in $\alpha_V\beta_6$-negative but $\alpha_5\beta_1$-expressing human cells promoted two hallmarks of the functional *H. pylori* T4SS, namely translocation of CagA into host cells and induction of interleukin-8 secretion by host cells. These findings suggest that integrin $\alpha_V\beta_6$, although not essential for T4SS function, represents an important host cell receptor for CagL.

Introduction

The human-specific gastric pathogen *Helicobacter pylori* has been ranked as class I carcinogen by the world health organization, because it can cause gastric cancer [1]. While *H. pylori* colonizes about half the world population, the majority of infections remain asymptomatic. Whether a symptomatic disease manifests depends on nutritional parameters, host genetics and on the *H. pylori* strain. High virulence of *H. pylori* strains correlates with the presence of a genomic insertion, the cytotoxin-associated gene pathogenicity island (*cag*PAI) that encodes a type IV secretion system (T4SS) [1]. The *cag*T4SS translocates CagA, the

Abbreviations
CagA, cytotoxin-associated gene A; *cag*PAI, cytotoxin-associated gene pathogenicity island; CEACAM, carcinoembryonic antigen-related cell adhesion molecule; EC_{50}, half maximal effective concentration; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; FAK, focal adhesion kinase; FP, fluorescence polarization; HRP, horse radish peroxidase; IL-8, interleukin-8; ILK, integrin-linked kinase; LAP, latency-associated peptide; SPR, surface plasmon resonance; T4SS, type IV secretion system; TGF-β, transforming growth factor-β.

product of the cytotoxin-associated gene A, into host cells [2]. CagA, the only known *cag*T4SS effector protein so far, exerts an oncogenic effect by subversion of host cell signalling [3]. Translocation of CagA into host cells, activation of the pro-inflammatory transcription factor NF-κB and increased secretion of interleukin-8 (IL-8) by host cells are hallmarks of a functional *cag*T4SS [4].

The *cag*T4SS is a macromolecular assembly consisting of a core complex spanning both bacterial membranes and an extracellular secretion pilus that makes contact to host cells [5–7]. The *cag*PAI protein CagL is an essential component of the *cag*T4SS. A *cagL* deletion resembles a complete *cag*PAI knockout in many respects [4,8]. This is, at least in part, due to the fact that the mutant no longer forms T4SS pili in response to host cell contact [9,10] and, therefore, cannot translocate CagA or other molecules like peptidoglycan [11], DNA [12] or precursors of lipopolysaccharide [13,14] into host cells. In addition to its role in pilus formation, CagL directly influences host cells by binding to cell surface receptors. Due to its location at the pilus surface, CagL may serve as T4SS adhesin [9]. The role as adhesin is supported by sequence and biochemical similarity to VirB5 proteins that function as adhesins in other T4SSs [15,16]. However, it is still controversial whether CagL actually is a VirB5 homolog. Based on sequence [10,17] and structure [18], CagL was instead proposed to be a *H. pylori*-specific protein.

Cell assays with recombinantly produced, purified CagL showed that the protein binds to human cells [9], mediates cell spreading [19] or cell adhesion [16,20–23], stimulates transcription from the gastrin promoter [24] and induces IL-8 secretion that is independent of CagA [25,26]. Purified CagL induces the phosphorylation of various kinases like Src, focal adhesion kinase (FAK) [19] or integrin-linked kinase (ILK) [24], and activates epidermal growth factor receptor (EGFR) and its downstream signalling cascade [19,24,26].

Several cellular effects induced by purified CagL were shown to depend on integrin $\alpha_5\beta_1$ [9,25]. Specific cellular phenotypes upon infection with *H. pylori* like CagA translocation [9,27] and IL-8 induction in gastric epithelial or biliary cells [25,28] also depend on β_1 integrins or $\alpha_5\beta_1$ in particular. Integrin $\alpha_5\beta_1$ is a heterodimeric cell surface receptor that interacts with ligands, mostly from the extracellular matrix, by binding to an Arg-Gly-Asp (RGD) tripeptide motif [29]. Of the 24 human integrin heterodimers, eight act as RGD receptors, namely $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$ and $\alpha_{IIb}\beta_3$ [30]. While the RGD motif is generally important for high affinity integrin binding, features outside of or adjacent to the RGD motif like the synergy region in fibronectin [31] or an LXXL/I motif in pro-forms of transforming growth factor-β (TGF-β) [32] also contribute to binding and are not recognized equally well by all RGD-binding integrins. Hence, RGD motif-containing ligands can show selectivity for individual RGD-binding integrins and vice versa.

Many cellular effects of recombinant CagL or CagL expressed by *H. pylori* depend on an intact RGD motif [9,18,19,22,23,25,26,33]. This RGD dependence is not reflected by the effect that mutations in the RGD motif have on the affinity of purified $\alpha_5\beta_1$ for purified CagL. Surface plasmon resonance (SPR) revealed a modest 4-fold increase of the dissociation constant (K_d) for the CagL RGA variant [9]. Some reports showed that a *H. pylori* Δ*cagL* strain complemented with a *cagL* gene with mutations in the RGD motif (e.g. RAD or RGA) or a deletion of the RGD motif (ΔRGD) behaved like bacteria expressing wild-type CagL upon infection of AGS cells with regard to CagA translocation, IL-8 induction or induction of the gastrin promoter [16,24,27]. Instead, functional importance was assigned to regions outside the RGD motif based on experiments with *H. pylori* Δ*cagL* strains harbouring mutated *cagL* genes [16] or with purified CagL variants [16,21].

The effect of function-blocking antibodies specific for individual integrins indicated that CagL may additionally act through integrins $\alpha_V\beta_3$ or $\alpha_V\beta_5$ [20,24] and spot blotting indicated binding of these integrins to purified CagL [34]. CagL binding to $\alpha_V\beta_5$ was additionally shown by pull-down assays from *H. pylori* infection experiments and by SPR with purified CagL [24]. Surprisingly, the binding of CagL to integrin $\alpha_V\beta_5$ was RGD-independent, although $\alpha_V\beta_5$ is a typical RGD receptor. Very recently, purified CagL was shown to induce IL-8 secretion in endothelial cells of the gastric submucosa [26]. This effect was RGD-dependent but independent of integrins $\alpha_5\beta_1$ and $\alpha_V\beta_5$ suggesting that another unidentified RGD receptor, most likely another integrin, acts as cellular CagL receptor.

We previously showed that expression of integrin $\alpha_V\beta_6$ in $\alpha_V\beta_6$-negative cells was sufficient to render these cells adherent to CagL [22]. In addition, a function-blocking antibody against $\alpha_V\beta_6$ abrogated adhesion of two human gastric epithelial cell lines to CagL, while a function-blocking antibody against $\alpha_5\beta_1$ had no effect. However, we had not been able to determine the expression levels of integrins $\alpha_5\beta_1$ and $\alpha_V\beta_6$ in these and several other cell lines that we analysed [22]. Moreover, we had not been able to show direct

binding of CagL to integrins but had to resort to a competitive assay, in which CagL and fibronectin or the high-affinity $\alpha_V\beta_6$ ligand latency-associated peptide 1 (LAP-1) competed for binding to $\alpha_V\beta_6$. Thus, many questions remained open with regard to what role individual RGD-binding integrins play as cellular receptors for CagL.

Here we present a systematic and detailed study of CagL binding to various integrins in the cellular context and *in vitro*. We analysed the expression of integrins $\alpha_5\beta_1$ and $\alpha_V\beta_6$ on eight cell lines and correlated it to adhesion of these cells to CagL. We quantitatively compared binding strength of CagL to various integrins *in vitro* and tested whether integrin $\alpha_V\beta_6$ has an influence on IL-8 secretion and CagA translocation upon infecting cultured cells with *H. pylori*.

Results

Soluble CagL specifically binds to various human cell lines

Previously, we had tested eight human cell lines for the adhesion to immobilized recombinant CagL [18,22]. Three adenocarcinoma cell lines of gastric (23132/87, MKN-45) or colorectal origin (HT-29) adhered to CagL in an RGD-dependent manner. We additionally had observed RGD-dependent adhesion to CagL for the colorectal carcinoma cell line SW480β_6 that is stably transfected to express integrin $\alpha_V\beta_6$. The control-transfected SW480 mock cells (also just called SW480 from now on), the lung carcinoma A-549, the human embryonic kidney 293T (HEK) and the melanoma WM-115 cells did not adhere or adhered only weakly to CagL. In this cell adhesion assay, avidity effects could occur, as individual cells can, at least in principle, simultaneously bind to several or many immobilized CagL molecules. In order to visualize the direct binding of soluble CagL molecules to these cell lines, we set up a flow cytometry approach, where the cell lines of interest were first incubated with biotinylated CagL and afterwards with phycoerythrin (PE)-labelled NeutrAvidin™ for detection (Fig. 1). A clear shift in fluorescence showed binding of CagL to the cell lines 23132/87, HT-29, MKN-45 and SW480β_6. CagL did not bind to HEK, mock-transfected SW480 and WM-115. A-549 cells showed a small fluorescence shift in the flow cytometry assay, although they did not adhere to CagL. Overall, the binding of soluble CagL, which should reflect true affinity independent of avidity effects, agreed well with cell adhesion to immobilized CagL (Table 1). Thus, cell adhesion assays presumably constitute a reliable read-out for binding of CagL to cell surface receptors. In addition, these results confirm that the binding of CagL is cell line specific and they raise the question which receptor on the cell surface is addressed by CagL. Previously, this role was assigned to integrin $\alpha_5\beta_1$ [9,25]. However, $\alpha_5\beta_1$ expression was reported both for HEK [35] and for untransfected SW480 cells [36], although CagL did not bind to these cell lines in our assays. Instead, binding of CagL to SW480β_6 cells suggested integrin $\alpha_V\beta_6$ to act as CagL receptor. Therefore, we analysed the expression of $\alpha_5\beta_1$ and $\alpha_V\beta_6$ in all cell lines, for which we had tested binding of soluble CagL.

Binding of CagL to cells does not depend on integrin $\alpha_5\beta_1$

To establish a reliable detection system for integrins, we first analysed $\alpha_V\beta_6$ expression in SW480 cells as negative control and SW480β_6 as positive control. Western blots with several antibodies failed to consistently reveal the expected expression profile. In contrast, flow cytometry gave reproducible results for $\alpha_V\beta_6$ with these two cell lines. Therefore, we analysed expression of $\alpha_5\beta_1$ and $\alpha_V\beta_6$ by flow cytometry in all cell lines. Integrin $\alpha_5\beta_1$ was detected with an antibody directed against the α_5 subunit and a PE-coupled secondary antibody (Fig. 2). 23132/87 cells expressed $\alpha_5\beta_1$ only weakly and HT-29 cells were $\alpha_5\beta_1$ negative. As these cell lines adhered to immobilized CagL [22] and bound soluble CagL despite the low level or absence of $\alpha_5\beta_1$ expression, $\alpha_5\beta_1$ cannot be the only cellular receptor for CagL. The other six cell lines clearly showed $\alpha_5\beta_1$ expression. Thus, the four cell lines A-549, HEK, SW480, and WM-115 exposed $\alpha_5\beta_1$ on their surface, but neither adhered to immobilized CagL [22] nor bound soluble CagL except for the very low level binding of CagL to A-549. These results indicate that $\alpha_5\beta_1$ does not act as high affinity receptor for CagL. Hence, another RGD-binding integrin might be the main receptor for CagL. We proposed that this receptor could be $\alpha_V\beta_6$.

Binding of CagL to cells correlates with expression of integrin $\alpha_V\beta_6$

Integrin $\alpha_V\beta_6$ was detected with a function-blocking antibody directed against native $\alpha_V\beta_6$ [37]. $\alpha_V\beta_6$ was expressed on 23132/87, HT-29, MKN-45 and SW480β_6 cells, but not on HEK, SW480 and WM-115 cells (Fig. 3). The $\alpha_V\beta_6$ expression correlated well with the ability of the seven cell lines to adhere to CagL [18,22] and to bind soluble CagL (Table 1). For A-549 cells,

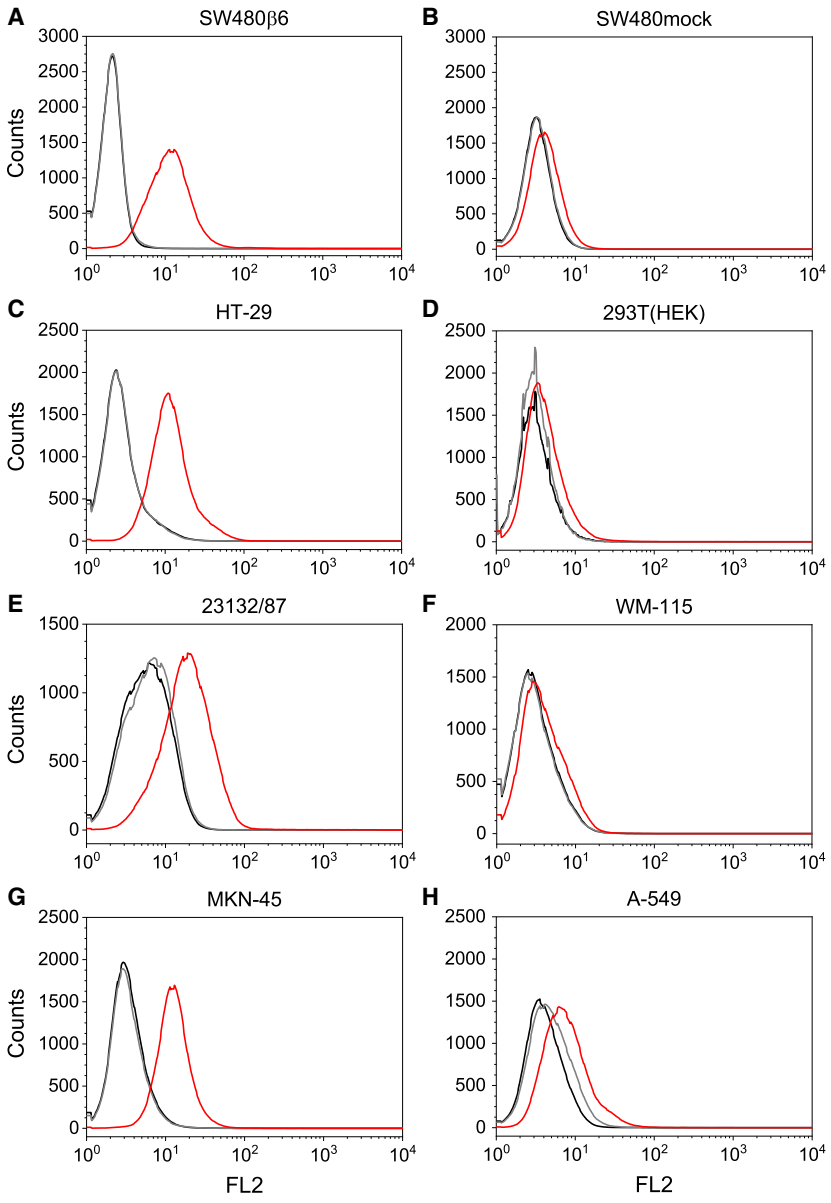

Fig. 1. Binding of biotinylated CagL to eight human cell lines. (A–H) Cell lines were incubated with biotinylated CagL and analysed by flow cytometry. Unstained cells are shown in black, cells treated only with NeutrAvidin™-PE in grey and cells incubated with CagL and NeutrAvidin™-PE are shown in red. A representative histogram is shown. Three independent experiments were performed.

two $\alpha_V\beta_6$ expression patterns were observed repeatedly. In half of the measurements no shift in fluorescence was observed, for the other half a bimodal distribution was obtained with one maximum indicating a cell population with a very low expression level of $\alpha_V\beta_6$ and a smaller cell population with a slightly higher $\alpha_V\beta_6$ expression. The low or absent $\alpha_V\beta_6$ expression is in agreement with the failure of A-549 cells to adhere to CagL. The small population of A-549 cells with weak $\alpha_V\beta_6$ expression would fit to the small fluorescence shift in flow cytometry with labelled CagL. Collectively, the results of the flow cytometry experiments point towards $\alpha_V\beta_6$ as a necessary and sufficient cell receptor for CagL.

CagL binds integrin $\alpha_V\beta_6$ specifically and with high affinity *in vitro*

The flow cytometry experiments confirmed our suggestion that integrin $\alpha_V\beta_6$ could act as primary cellular receptor for CagL [22]. Previously, we had not been able to show direct binding of recombinant CagL to any purified integrin ectodomain [22]. Instead we had to resort to a competitive assay, in which soluble CagL at increasing concentrations competed with the soluble integrin ligands fibronectin or LAP-1 used at a fixed concentration for binding to various immobilized integrin ectodomains. This assay revealed competition of CagL for binding to the ectodomains of $\alpha_V\beta_6$ and

Table 1. Summary of traits examined in this and previous studies for eight human cell lines. Adhesion to CagL was tested in [18] for MKN-45 and in [22] for the other cell lines. (+) stands for weak binding of CagL to A-549 cells or for absent or weak expression of $\alpha_V\beta_6$ in these cells

Cell line		Binding of CagL	Expression of		Adhesion to CagLwt
			$\alpha_V\beta_6$	$\alpha_5\beta_1$	
HT-29	Colorectal adenocarcinoma	+	+	–	+
23132/87	Gastric adenocarcinoma	+	+	Low	+
MKN-45	Gastric adenocarcinoma	+	+	+	+
SW480β_6	Colorectal carcinoma; β6 transfected	+	+	+	+
SW480 mock	Colorectal carcinoma; control transfected	–	–	+	–
A-549	Lung carcinoma	(+)	(+)	+	–
WM-115	Melanoma	–	–	+	–
293T (HEK)	Embryonic kidney	–	–	+	–

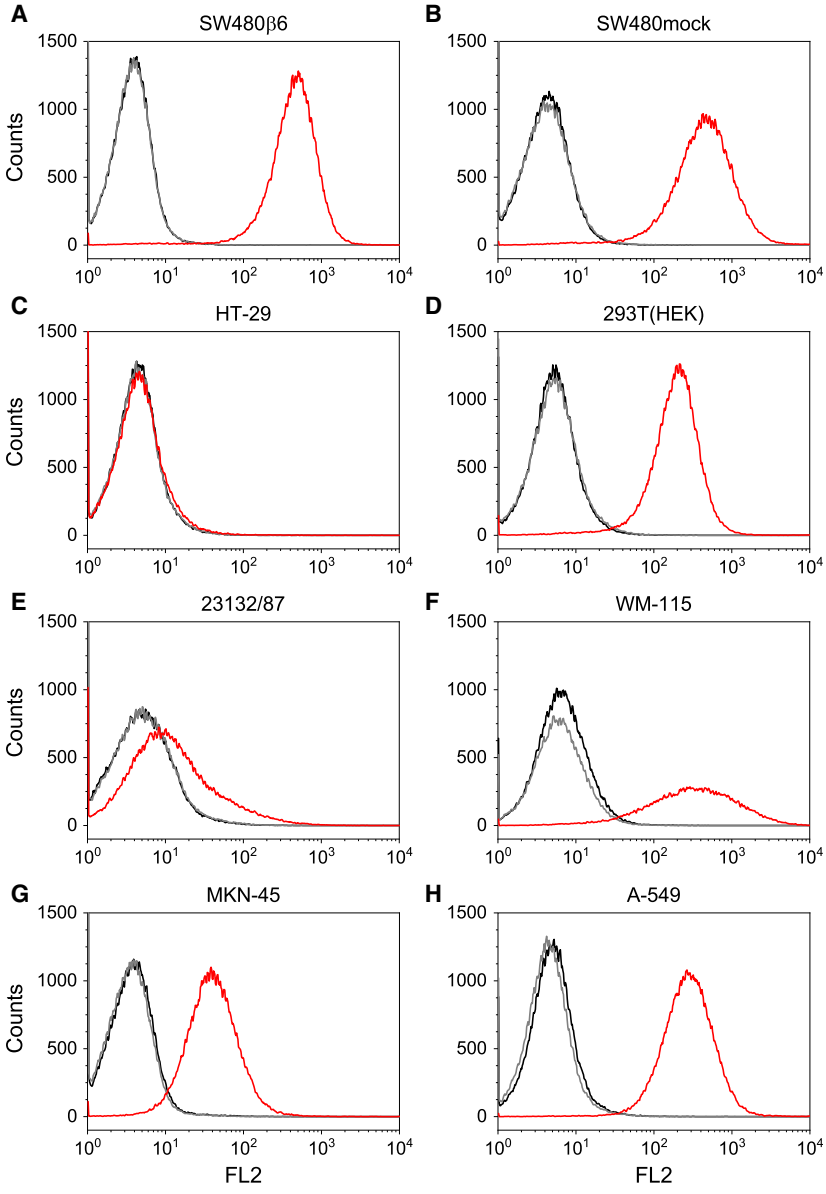

Fig. 2. Integrin $\alpha_5\beta_1$ expression on eight human cell lines. (A–H) Cells were analysed regarding their integrin $\alpha_5\beta_1$ expression by flow cytometry. The unstained cells are shown in black, the control antibody treated cells in grey and the anti-CD49e (α_5 specific) antibody treated cells in red. A representative histogram is shown. Three independent experiments were performed.

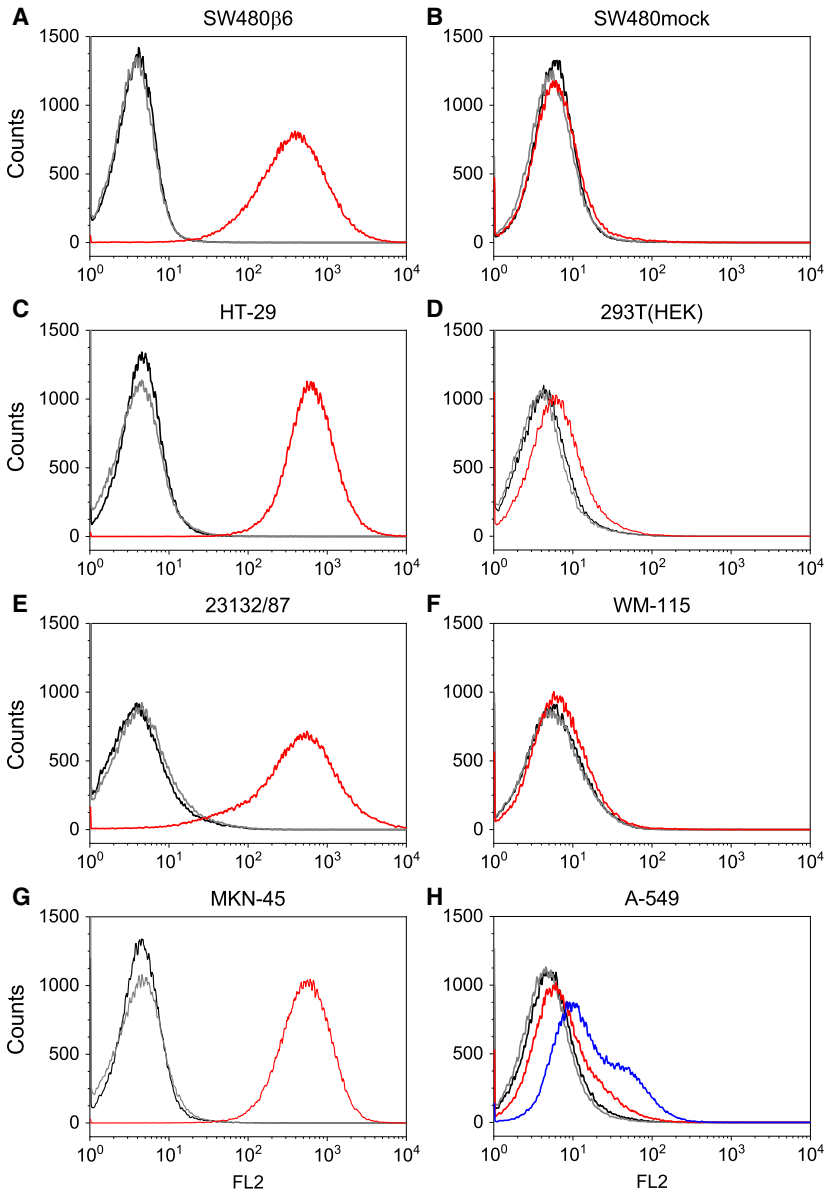

Fig. 3. Integrin $\alpha_V\beta_6$ expression on eight human cell lines. (A–H) Cells were analysed regarding their integrin $\alpha_V\beta_6$ expression by flow cytometry. The unstained cells are shown in black, the control antibody treated cells in grey and the 3G9 ($\alpha_V\beta_6$ specific) treated cells in red. For cell line A-549 (H), two expression patterns were repetitively observed whereof the second one is shown in blue. A representative histogram is shown. Three independent experiments were performed.

$\alpha_V\beta_8$ and to a much smaller extent to $\alpha_V\beta_3$ [22]. We had found no competition for binding to $\alpha_5\beta_1$, $\alpha_V\beta_1$ and $\alpha_V\beta_5$. The disadvantage of these experiments was that they do not deliver affinities as quantitative result, but rather the half maximal inhibitory concentration (IC_{50}). These IC_{50} values do not only depend on the affinity of CagL for the integrin, but also on the affinity of the competed integrin ligands fibronectin or LAP-1 for the immobilized integrin.

To obtain comparable quantitative measures for the interaction of CagL with various integrins, we developed a solid phase binding assay that directly measures binding of CagL to integrins. We immobilized the ectodomains of six RGD-dependent integrins ($\alpha_5\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$ and $\alpha_V\beta_8$) and incubated them with biotinylated CagL. Bound CagL was detected with horse radish peroxidase (HRP)-coupled NeutrAvidin™ in a colorimetric assay. We obtained sigmoidal binding curves, which, however, showed no clear upper plateau except for $\alpha_V\beta_6$ (Fig. 4A). Nevertheless, we fitted the data with the Langmuir equation to obtain estimates of the half maximal effective concentrations (EC_{50} values). For $\alpha_V\beta_6$, the EC_{50} was in the low, single digit nanomolar range, whereas $\alpha_V\beta_1$, $\alpha_V\beta_3$ and $\alpha_V\beta_8$ showed a roughly 100-fold lower and $\alpha_5\beta_1$ and $\alpha_V\beta_5$ an about 1000-fold lower apparent affinity (Table 2). By and large, these results are consistent with our previous results from competitive

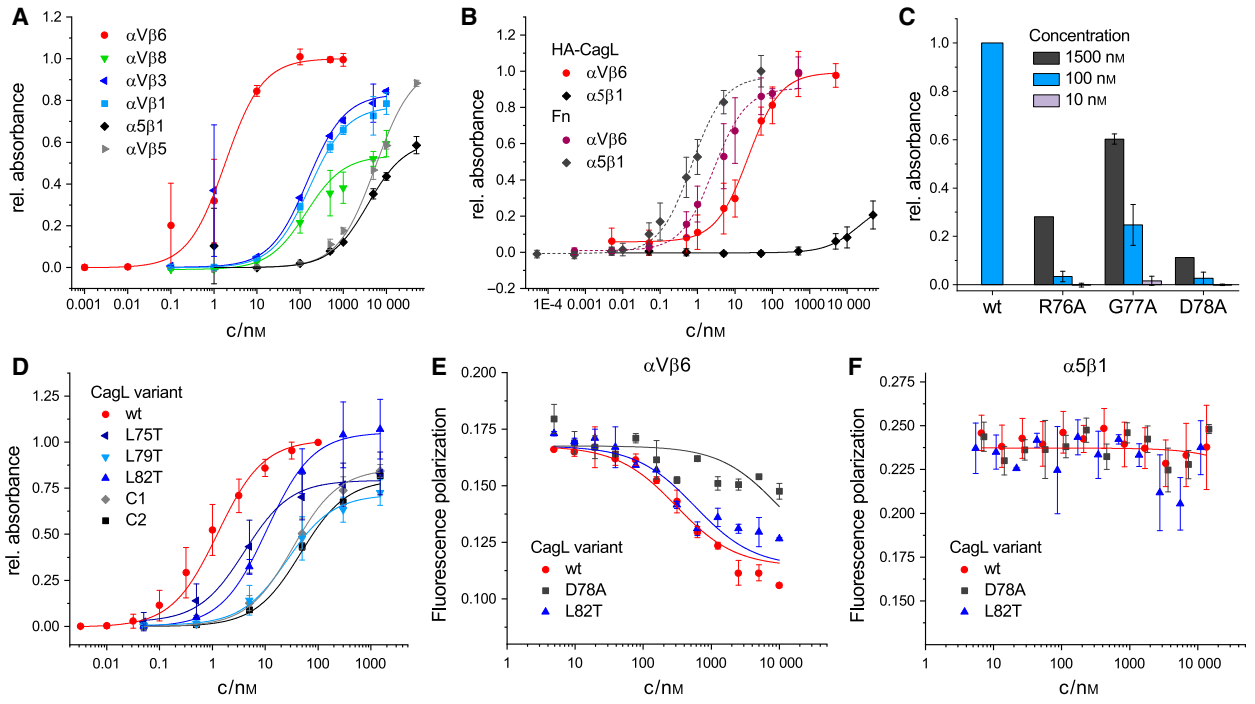

Fig. 4. CagL specifically binds integrin $\alpha_V\beta_6$ in an RGDLXXL-dependent manner. (A) Immobilized integrin ectodomains ($\alpha_5\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$ and $\alpha_V\beta_8$) were incubated with a dilution series of biotinylated CagL. Binding was detected with NeutrAvidin™-HRP in an ELISA. Data from three independent experiments are illustrated as mean with standard deviation. (B) Binding of HA-tagged CagL (solid lines) and fibronectin (dotted lines) to immobilized ectodomains of integrins $\alpha_5\beta_1$ and $\alpha_V\beta_6$ is shown as the mean and standard deviation of three independent experiments. (C, D) The binding of biotinylated CagL variants to immobilized ectodomains of integrin $\alpha_V\beta_6$ was detected with NeutrAvidin™-HRP in an ELISA. The diagram is representative for one of three measurements. Error bars represent the standard deviation of three technical replicates. (E, F) Competitive fluorescence polarization experiments of CagL variants with integrin $\alpha_V\beta_6$ and $\alpha_5\beta_1$ were performed using FITC-labelled pro-TGF-β3 peptide and FITC-labelled cycloRGD peptide as fluorescence probes, respectively. $\alpha_V\beta_6$ headpiece or $\alpha_5\beta_1$ ectodomain were pre-equilibrated with various concentrations of CagL indicated on the *x*-axis for 1 h before mixing with FITC-labelled peptide. Values and error bars represent mean and standard deviation of two ($\alpha_V\beta_6$) or three ($\alpha_5\beta_1$) experiments.

binding studies [22]. However, the low apparent affinity of CagL for $\alpha_5\beta_1$ is at odds with previous SPR studies that yielded K_d values of 90 nM [9] or 183 nM and 39 nM for the clasped and unclasped ectodomain preparations of $\alpha_5\beta_1$, respectively [38]. Therefore, we checked whether the immobilized $\alpha_5\beta_1$ is functional in our assay using a fibronectin titration as positive control. The $\alpha_5\beta_1$ ectodomain bound fibronectin with higher affinity compared to $\alpha_V\beta_6$ (Fig. 4B). To test whether biotinylation of CagL impeded binding to $\alpha_5\beta_1$ we titrated N-terminally HA-tagged CagL against $\alpha_5\beta_1$ and $\alpha_V\beta_6$ (Fig. 4B). HA-tagged CagL bound $\alpha_V\beta_6$ with high affinity, but not $\alpha_5\beta_1$. Both $\alpha_V\beta_6$ (EC_{50} = 22.7 nM) and $\alpha_5\beta_1$ had lower affinity for HA-tagged CagL than for biotinylated CagL. This may be due to differences in the detection procedures. Biotinylated CagL was detected in a single step with tetravalent NeutrAvidin™-HRP, while HA-tagged CagL was detected in a two-step procedure with a primary anti-HA antibody and a HRP-coupled secondary antibody.

To independently confirm our results, we used competitive fluorescence polarization (FP) experiments for $\alpha_V\beta_6$ and $\alpha_5\beta_1$ (Fig. 4E,F). This approach is well-established and very reliable [39]. Measurements are performed in solution and do not require labelling of CagL or integrins. Integrins were pre-equilibrated with increasing concentrations of CagL before adding the fluorescent probes, a FITC-labelled pro-TGF-β3 peptide for $\alpha_V\beta_6$ [32] or a FITC-labelled cyclic RGD peptide for $\alpha_5\beta_1$. [39]. The measurements confirmed high affinity binding of CagL to $\alpha_V\beta_6$ whereas no binding was observed to $\alpha_5\beta_1$. The K_d of CagL and $\alpha_V\beta_6$ was calculated to be 40.3 nM (23.3–69.7 nM; 95% confidence interval) using equations given by Rossi and Taylor [40] and the known K_d of the FITC-labelled probe for $\alpha_V\beta_6$ [32]. Thus, although the absolute K_d value from competitive FP and the EC_{50} value from ELISA differ, the two methods agree qualitatively and show consistently that binding of CagL to $\alpha_5\beta_1$ is several orders of magnitude weaker than binding to $\alpha_V\beta_6$.

Table 2. Affinity of biotinylated wildtype CagL for ectodomains of six RGD-binding integrins *in vitro*. The half maximum effective concentrations were calculated from at least three independent experiments with three measurements each

Integrin	EC_{50} (nM)
$\alpha_5\beta_1$	5762 ± 808.7
$\alpha_V\beta_1$	138.0 ± 31.27
$\alpha_V\beta_3$	166.5 ± 8.567
$\alpha_V\beta_5$	4025 ± 404.7
$\alpha_V\beta_6$	1.825 ± 0.183
$\alpha_V\beta_8$	149.6 ± 4.813

CagL binding to integrin $\alpha_V\beta_6$ *in vitro* is RGD-dependent

CagL was identified as potential integrin ligand of the *cag*T4SS, because it is the only protein encoded by the *cag*PAI with an RGD motif [9]. Many cellular effects elicited by CagL, including adhesion of SW480β_6 cells, depend on its RGD motif [9,18,19,22,25,26]. Therefore, we tested RGD variants with single amino acid substitutions for their ability to bind to the $\alpha_V\beta_6$ ectodomains by ELISA (Fig. 4C). Binding of $CagL^{R76A}$ and $CagL^{D78A}$ (i.e. the AGD and RGA variants of CagL, respectively) was almost abolished at a concentration of 100 nM and no longer detectable at 10 nM, a concentration well above the EC_{50} of wildtype CagL. Binding of $CagL^{G77A}$ (RAD) was also reduced, but to a lesser extent than for the other two variants. Binding of $CagL^{D78A}$ to $\alpha_V\beta_6$ was also tested by competitive FP (Fig. 4E). These experiments showed strongly reduced binding, but could not be evaluated quantitatively. Thus, binding of CagL to $\alpha_V\beta_6$ was strictly dependent on its RGD motif.

The CagL LXXL motif contributes to integrin $\alpha_V\beta_6$ binding *in vitro*

In CagL, the sequence LALL is located immediately C-terminal of the RGD motif. The consensus RGDLXXL motif with conserved residues at positions D + 1 (Leu/Met) and D + 4 (Leu/Ile) is typical for ligands of integrin $\alpha_V\beta_6$ [41–44]. The two conserved Leu residues are important for binding to $\alpha_V\beta_6$ [32] and the LXXL sequence adopts helical conformation to position both Leu side chains on the same side [45]. Structures of $\alpha_V\beta_6$ in complex with an RGDLXXL-containing peptide from pro-TGF-β3 [32] or with pro-TGF-β1, an RGDLXXI-containing protein ligand [46], showed that both Leu/Ile side chains occupy hydrophobic pockets of the β_6 subunit that are not present in β_1 and in most other integrin β subunits. In CagL the side chains of Leu79 and Leu82 from the RGDLXXL motif point toward the hydrophobic core of CagL, but still mutation to Thr had impeded cell adhesion, suggesting that they could be accessible for receptor binding [22]. Moreover, the CagL RGD motif itself is unusually located in an α-helix. Comparison of multiple CagL structures had revealed flexibility N-terminal to the glycine of the RGD motif [18]. Therefore, we had suggested that CagL might partially unfold to expose the RGDLXXL motif. Two CagL variants with stabilizing inter-helix disulphide bridges N-terminal (CagL C1) or C-terminal (CagL C2) of the RGDLXXL motif reduced or abrogated cell adhesion, corroborating the suspected unfolding [18,22].

To test whether the flexibility around the RGD motif or the side chains of Leu79 and Leu82 from the LXXL motif are important for the interaction of CagL with $\alpha_V\beta_6$, we measured binding of CagL variants to $\alpha_V\beta_6$ (Fig. 4D). ELISAs revealed about 9-fold reduced EC_{50} value for $CagL^{L82T}$ and about 20-fold reduced EC_{50} for $CagL^{L79T}$ (Table 3) These values are comparable to the effect that deletion of leucine from the LXXL motif of pro-TGF-β3 peptides has on affinity for $\alpha_V\beta_6$ [32]. Mutation of the control Leu75 outside the LXXL motif reduced the EC_{50} only about 4-fold. Competitive FP measurements with $CagL^{L82T}$ also showed lower affinity for $\alpha_V\beta_6$ (Fig. 4E). With a K_d of 86.5 nM (50.3–148.8 nM; 95% confidence interval) the difference to wildtype CagL was only about 2-fold and thus smaller than in ELISAs. The disulphide-stabilized C1 and C2 variants of CagL also showed reduced apparent affinity for $\alpha_V\beta_6$ (Fig. 4D) with EC_{50} values of 43.4 and 46.6 nM, respectively (Table 3).

In conclusion, fixing helix α2 that contains the RGD motif to the neighbouring helix α5 reduced affinity for $\alpha_V\beta_6$. Likewise, mutation of two Leu residues reduced affinity for $\alpha_V\beta_6$ although their side chains are buried in the hydrophobic core of the protein and, therefore, are not accessible for interaction with a receptor. Partial unfolding of CagL may thus be necessary to allow high affinity binding of $\alpha_V\beta_6$.

Table 3. Affinity of biotinylated CagL variants for the immobilized $\alpha_V\beta_6$ ectodomain. The half maximum effective concentrations were calculated from at least three independent experiments with three measurements each

Variant	EC_{50} (nM)
wt	1.257 ± 0.176
L75T	4.077 ± 1.038
L79T	26.11 ± 5.386
L82T	11.32 ± 1.170
C1	43.37 ± 6.568
C2	46.64 ± 6.495

CagA phosphorylation and IL-8 induction is enhanced in β_6 expressing cells

To determine whether $\alpha_V\beta_6$ expression is required for *cag*T4SS function, we analysed CagA translocation into cells and IL-8 secretion by cells infected with different *H. pylori* strains for 8 h. We compared the effects in SW480β_6 cells with those in SW480 cells as $\alpha_V\beta_6$ negative control. CagA translocation was quantified with the anti-pan-phosphotyrosine antibody PY99, as CagA tyrosine phosphorylation only occurs in the host cell cytoplasm. Tyrosine phosphorylation of CagA was detected for SW480β_6 as well as for SW480 cells (Fig. 5A,B). The relative amount of phosphorylated CagA was significantly enhanced 2- to 3-fold in the β_6 transfected cells depending on the *H. pylori* strain. In addition, the amount of IL-8 secreted into the cell culture supernatant was determined by ELISA. Again, both SW480β_6 and SW480 cells showed increased IL-8 secretion upon infection with *H. pylori* (Fig. 6). Once more, it was dependent on the *H. pylori* strain, whether $\alpha_V\beta_6$ influenced IL-8 secretion. Compared to SW480 cells, SW480β_6 cells showed slightly reduced IL-8 secretion upon infection with *H. pylori* G27 wildtype. In contrast, IL-8 secretion was higher in $\alpha_V\beta_6$ expressing cells upon infection with three other *H. pylori* strains. This increased IL-8 secretion in SW480β_6 cells was particularly significant for the infection with *H. pylori* wildtype strains P12 and NCTC11637. These results demonstrate that $\alpha_V\beta_6$ is not absolutely necessary for CagA translocation and IL-8 induction but its presence strongly enhances the extent of these phenomena suggesting a potential complementarity or redundancy of host cell receptors.

A function-blocking antibody against integrin $\alpha_V\beta_6$ inhibits CagA translocation

The gastric epithelial cell line AGS is arguably the most common model system to study *H. pylori* infection *in vitro*. AGS cells express integrin $\alpha_V\beta_6$ in addition to $\alpha_5\beta_1$ and $\alpha_V\beta_5$, but show no expression of $\alpha_V\beta_3$ and $\alpha_V\beta_8$ [47,48]. To investigate whether integrin $\alpha_V\beta_6$

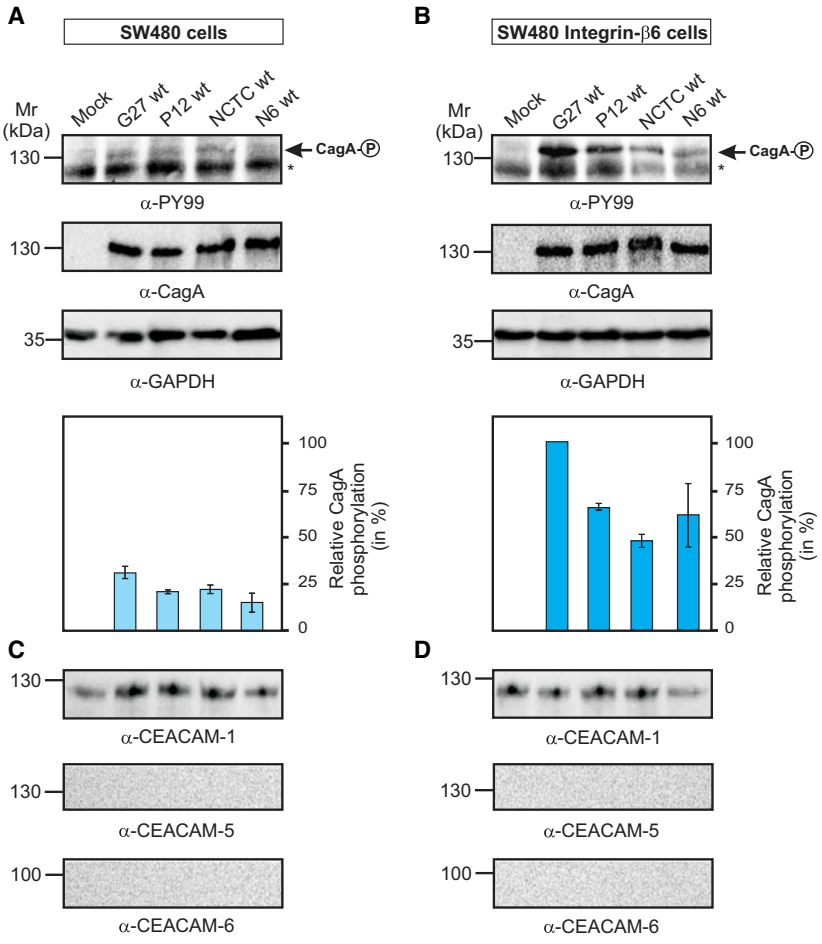

Fig. 5. Translocation and phosphorylation of *Helicobacter pylori* CagA is enhanced in SW480 cells expressing integrin $\alpha_V\beta_6$. (A) SW480 cells and (B) SW480β_6 cells expressing integrin $\alpha_V\beta_6$ were infected with the indicated *H. pylori* strains for 8 h. Phosphorylation of CagA was investigated using the anti-pan-phosphotyrosine antibody PY99. The relative CagA phosphorylation levels were quantified by densitometric measurement of band intensities. The anti-CagA and anti-GAPDH blots served as loading controls. Three independent experiments were performed. Error bars represent standard deviations. (C) and (D) Expression of three CEACAMs was analysed by western blots with monoclonal, monospecific antibodies.

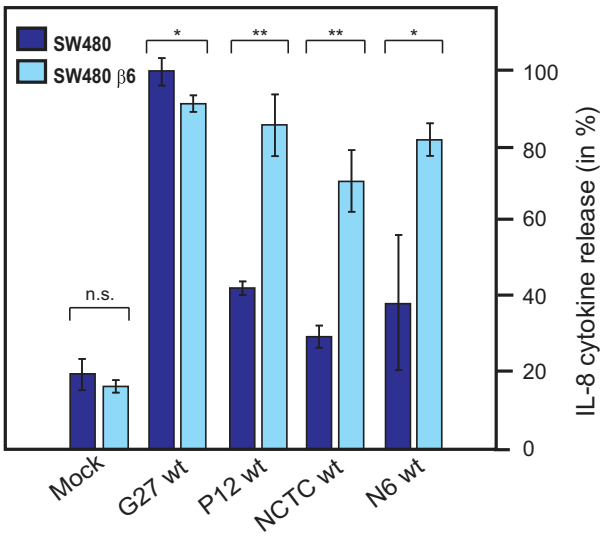

Fig. 6. Differential regulation of IL-8 cytokine production in *Helicobacter pylori* infected SW480 cell lines. SW480 cells (left) and SW480β_6 cells expressing integrin $\alpha_V\beta_6$ (right) were infected with the indicated *H. pylori* strains for 8 h. The amount of IL-8 secreted into the cell culture supernatant was detected by ELISA. Three independent experiments were performed. Statistical evaluation was performed by using Student's *t* test with SIGMAPLOT statistical software (version 13.0). $*P < 0.05$; $**P < 0.005$; $P \geq 0.05$ was considered not significant (n.s.). Error bars represent standard deviations.

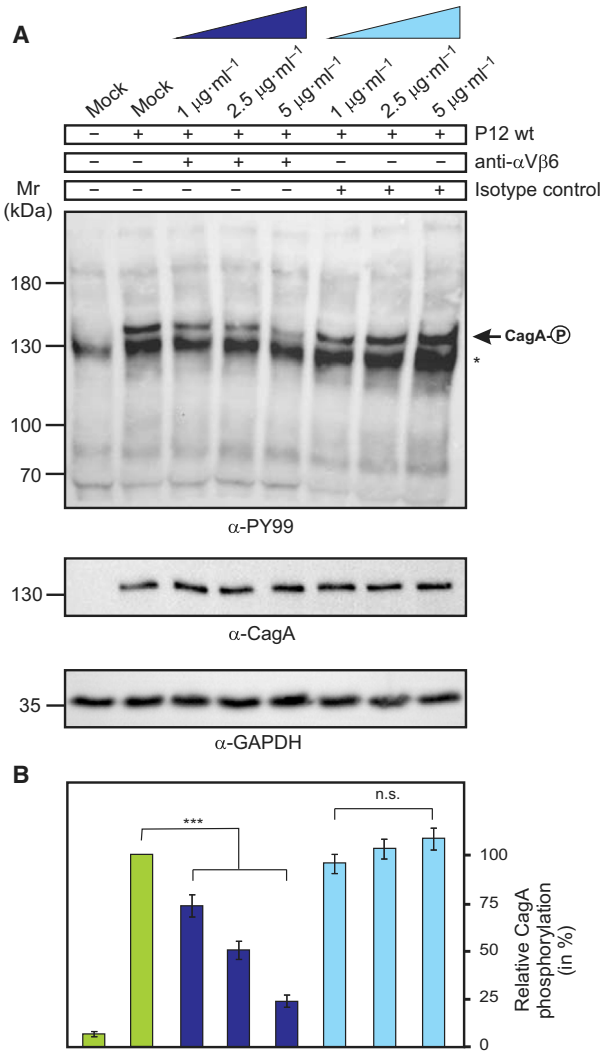

Fig. 7. Dose-dependent inhibition of CagA translocation and phosphorylation in *Helicobacter pylori* infected AGS cells by the function-blocking anti-$\alpha_V\beta_6$ antibody 3G9. (A) AGS cells were infected with *H. pylori* for 3 h in the absence or with various concentrations of anti-$\alpha_V\beta_6$ antibody 3G9 or the isotype-matched control antibody 1E6. Phosphorylation of CagA was investigated using the anti-pan-phosphotyrosine antibody PY99. The anti-CagA and anti-GAPDH blots served as loading controls. (B) The relative CagA phosphorylation levels were quantified by densitometric measurement of band intensities. Three independent experiments were performed. Statistical evaluation was done by using Student's *t* test with SIGMAPLOT statistical software (version 13.0). $***P < 0.001$; $P \geq 0.05$ was considered not significant (n.s.). Error bars represent standard deviations.

may function as host cell receptor for the *cag*T4SS in AGS cells, we analysed the $\alpha_V\beta_6$ dependency of the CagA translocation using the anti-$\alpha_V\beta_6$ function-blocking antibody 3G9 [37]. AGS cells were infected with *H. pylori* P12 wildtype for 3 h in the absence or presence of the anti-$\alpha_V\beta_6$ antibody. CagA phosphorylation was detected using the anti-pan-phosphotyrosine antibody PY99. The relative phosphorylation was set to 100% for infected cells in the absence of the anti-$\alpha_V\beta_6$ antibody 3G9. The extent of CagA phosphorylation decreased with increasing concentration of the antibody 3G9 but clearly remained detectable, even at the highest antibody concentration (Fig. 7). The isotype-matched control antibody 1E6 had no effect. Hence, integrin $\alpha_V\beta_6$ does not appear to be necessary for but rather to enhance *cag*T4SS function in AGS cells, hinting to the presence of further *cag*T4SS host cell receptors in this widely used cell line.

Discussion

The integrin β1 subunit, and integrin $\alpha_5\beta_1$ in particular, were identified as essential host cell receptor for the *cag*T4SS of *H. pylori* [9,27]. CagL was the first *cag*T4SS protein described as $\alpha_5\beta_1$ ligand [9]. Meanwhile, however, the picture has become more complex. Several other *cag*T4SS proteins were found to directly interact with integrin $\alpha_5\beta_1$ [27,38,49]. Likewise, additional CagL receptors were found, namely integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ [20,24]. Here we show that

$\alpha_V\beta_6$ bound CagL with the highest affinity among six RGD-binding integrins. We also show that expression of integrin $\alpha_V\beta_6$ on host cells infected with *H. pylori* enhanced CagA translocation into and stimulated IL-8 secretion by these cells.

This study systematically evaluates binding of recombinant CagL to various integrins under identical assay conditions *in vitro*. Therefore, it provides results that allow a reliable ranking of affinities. The high affinity that we found for CagL binding to $\alpha_V\beta_6$ is consistent with the presence of the RGDLXXL motif, which typically confers specificity for $\alpha_V\beta_6$ to RGD-containing integrin ligands [32,50]. The results from ELISAs with recombinant integrin ectodomains are consistent with data from flow cytometry that assays binding of recombinant CagL to intact integrins on the surface of living cells. The lower affinity that we found for $\alpha_5\beta_1$ is at odds with published data. Right now, we have no clear explanation for the big difference between the EC_{50} value that we measured and published K_d values for CagL and integrin $\alpha_5\beta_1$ [9,38]. Different protein constructs and preparations for integrin and CagL, different assays and different buffer conditions represent potential causes. Therefore, we verified the functionality of $\alpha_5\beta_1$ in our experiments with binding to fibronectin as positive control. Experiments with HA-tagged CagL confirmed that the lack of binding to $\alpha_5\beta_1$ was not due to CagL biotinylation. We double-checked CagL binding to $\alpha_5\beta_1$ and $\alpha_V\beta_6$ with competitive FP as alternative method. Fluorescence polarization confirmed that CagL binds with substantially higher affinity to $\alpha_V\beta_6$ than to $\alpha_5\beta_1$. The measured differences in affinity for $\alpha_V\beta_6$ between the two assays employed in this work may again be due to different buffer conditions and the different integrin constructs used. Our FP experiments were carried out with the physiologic cations Ca^{2+} and Mg^{2+}, while our ELISAs additionally contained the integrin activator Mn^{2+}. Moreover, different constructs of $\alpha_V\beta_6$ were used: for FP measurements, we used the $\alpha_V\beta_6$ headpiece that was recently crystallized with a pro-TGF-β peptide [32], while the complete $\alpha_V\beta_6$ ectodomain was used for ELISAs.

The integrin β_1 subunit and integrin $\alpha_5\beta_1$ in particular were consistently found to be important for CagA translocation [9,27] and for induction of IL-8 secretion [25,28]. This requirement for $\alpha_5\beta_1$ does not contradict our finding that CagL binding to $\alpha_5\beta_1$ is weak *in vitro*, because three other *cag*PAI proteins, CagA, CagI and CagY, were also found to interact with the integrin β_1 subunit in yeast two hybrid assays [27,49] or to directly bind purified integrin $\alpha_5\beta_1$ or its ectodomain in pull-down and SPR studies [27,38]. In another recent *in vitro* study it was shown that *H. pylori* binding to immobilized $\alpha_5\beta_1$ integrin under shear flow was dependent on CagY, while CagI or CagL were not required [51]. It is still under debate which of these factors bind to integrin $\alpha_5\beta_1$ upon infection and CagA delivery may require the concerted interaction of various *cag*PAI proteins with $\alpha_5\beta_1$. Also, our analysis of $\alpha_5\beta_1$ expression does not contradict an important role of $\alpha_5\beta_1$ for *cag*T4SS function upon infection. For the two gastric cell lines 23132/87 and MKN-45 CagA injection and IL-8 induction by *H. pylori* had previously been shown [52] and we found $\alpha_5\beta_1$ on the surface of both lines, although expression in 23132/87 was low.

At first sight, our results appear to be at odds with reports that cellular effects in response to purified CagL are mediated by integrin $\alpha_5\beta_1$. Closer inspection reveals that this may not be the case. Our data show that the affinity of CagL for $\alpha_5\beta_1$ is several orders of magnitude lower than that for $\alpha_V\beta_6$, however, they do not completely rule out an interaction of CagL with integrin $\alpha_5\beta_1$ because of the very high affinity for $\alpha_V\beta_6$. In several cellular assays, low affinity interactions may become physiologically relevant due to the avidity effect when multiple integrins of one cell interact with multiple CagL molecules immobilized on a surface. Such a scenario could hold for the binding of CagL-coated latex beads to AGS cells that was shown to be $\alpha_5\beta_1$-dependent [9]. Avidity effects cannot be invoked to explain how soluble, purified CagL induces IL-8 secretion in an $\alpha_5\beta_1$ integrin-dependent manner [25]. However, IL-8 secretion in these experiments was stimulated with soluble CagL at concentrations between 4 and 8 μM and reached baseline levels at roughly 0.5 μM. This appears consistent with our ELISAs, which showed CagL binding to $\alpha_5\beta_1$ at CagL concentrations above 1 μM with an EC_{50} of about 6 μM.

Our infection experiments using SW480 and AGS cells showed that expression of integrin $\alpha_V\beta_6$ can significantly promote CagA translocation into cells, while blocking $\alpha_V\beta_6$ function reduced CagA translocation. These finding strongly suggest that the binding of CagL to integrin $\alpha_V\beta_6$ is functionally important during *H. pylori* infections. Recent studies revealed that host cell receptors other than integrins $\alpha_5\beta_1$ and $\alpha_V\beta_6$ also promote CagA translocation. The outer membrane adhesin HopQ, which is encoded outside the *cag*PAI, can facilitate CagA injection into cells expressing specific carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) as HopQ receptors [53–55]. However, neither CEACAMs nor $\alpha_V\beta_6$ appear to be

absolutely required for *cag*T4SS functionality. The HopQ-CEACAM interaction is dispensable in Kato III cells, as a Δ*hopQ* mutant could inject CagA into these cells almost as efficiently as wildtype *H. pylori* [55]. Likewise, the CagL-$\alpha_V\beta_6$ interaction is dispensable for *cag*T4SS function in HEK cells expressing high levels of specific CEACAMs. *H. pylori* cannot translocate CagA into HEK cells that do not express $\alpha_V\beta_6$ or CEACAMs [56]. However, transfection of only CEACAM1 or CEACAM5 renders HEK cells susceptible to CagA injection [54,55]. Very recently CagA delivery into the gastroduodenal cell line AZ-521 was shown to require both integrin β_1 and CEACAM1 or CEACAM5 [57]. After we had finished the manuscript, another paper reported that all integrins are dispensable for CagA translocation, while at least one HopQ-binding CEACAM member was required [48]. Therefore, we checked for CEACAMs on SW480 cells and found expression of CEACAM1, while CEACAM5 and CEACAM6 were absent (Fig. 5C,D). The expression of CEACAM1 combined with the susceptibility for CagA translocation in SW480 cells is consistent with the finding that CEACAMs play a role in CagA translocation [48]. Zhao *et al.* found no reduction of CagA translocation into host cells upon deletion of the integrin α_V subunit. A potential difference to our experiments with a function-blocking anti-$\alpha_V\beta_6$ antibody could be that genetic knockout of integrin subunits can result in upregulation of CEACAMs. At the protein level, Zhao *et al.* [48] found about 4.5-fold increased expression of the HopQ receptor CEACAM1 in integrin-deficient Kato III cells. As a result, wildtype *H. pylori* showed increased adhesion to integrin-deficient Kato III cells. From their experiments with AGS cells Zhao *et al.* [48] concluded 'that in AGS cells (an) other receptor(s) distinct from CEACAMs seem(s) to support the process of CagA translocation'. Thus, multiple, and partly redundant, host cell receptors for various *H. pylori* surface proteins may synergistically support *cag*T4SS function and CagA injection in a yet unknown fashion. We are not aware of any other T4SS, for which such an elaborate interplay with host cell receptors was found. High expression levels of one receptor, as often achieved upon transfection, may be sufficient for *cag*T4SS function, rendering expression of other receptors unnecessary. Due to its high affinity for the T4SS adhesin CagL, integrin $\alpha_V\beta_6$ may be one of several important factors determining whether cells are susceptible to CagA delivery by *H. pylori*. Therefore, future experiments should address expression of multiple host cell receptors involved in *cag*T4SS function, including not only integrin $\alpha_5\beta_1$ and CEACAMs, but also integrin $\alpha_V\beta_6$.

For a long time it remained enigmatic how *H. pylori* could interact with integrin $\alpha_5\beta_1$. In polarized cells, $\alpha_5\beta_1$ is expressed at basolateral surfaces, whereas *H. pylori* approach gastric epithelial cells from the apical side. The same problem probably holds for binding of *H. pylori* CagL to $\alpha_V\beta_6$. There is little data in the literature regarding the subcellular distribution of $\alpha_V\beta_6$ on polarized cells and we found no such data for stomach epithelium. The scarce data indicates mostly basolateral localization with circumferential expression in only few tissues [58]. However, basolateral expression of $\alpha_V\beta_6$ may not be a conundrum any more. First, CEACAMs that also support *cag*T4SS function and CagA translocation localize to the apical side of polarized cells. Second, *H. pylori* secrete the protease HtrA that cleaves proteins in adherens junctions and tight junctions to facilitate paracellular migration of *H. pylori* giving it access to the basolateral side [59].

The panel of cell lines that we analysed contained two gastric epithelial lines, 23132/87 and MKN-45, and we found $\alpha_V\beta_6$ expression in both. AGS cells, another human gastric adenocarcinoma line, also express integrin $\alpha_V\beta_6$ [47,48]. Integrin $\alpha_V\beta_6$ is restricted to epithelial cells. Its expression is very low under normal conditions, but upregulated upon injury and infection. High expression levels of $\alpha_V\beta_6$ are also found in many cancers including gastric cancer [60,61]. However, β_6 mRNA was also detected in normal stomach epithelium of rhesus monkeys [62]. At the protein level, normal human surface epithelial and mucous neck cells of pyloric gastric mucosa stained strongly for anti-β_6 [63]. Intriguingly, the $\alpha_V\beta_6$ expression pattern suggests a potential link to *H. pylori* infections. CEACAM expression in the stomach is upregulated during gastritis, which might support interaction of *H. pylori* with epithelial cells and thereby enhance CagA delivery [54,55]. It was suggested that this might increase pathogenicity [54]. Similarly $\alpha_V\beta_6$ expression might be upregulated due to inflammation caused by *H. pylori* infection and in turn facilitate enhanced CagA injection.

Taken together, our results suggest that future studies regarding the function of CagL and of the *cag*T4SS should consider a potential contribution of integrin $\alpha_V\beta_6$, as $\alpha_V\beta_6$ appears to be a specific, high-affinity receptor for CagL. Expression of integrin $\alpha_V\beta_6$ in normal stomach epithelium and several gastric epithelial cell lines, its upregulation upon infection, and the association of integrin $\alpha_V\beta_6$ with cancer further warrant a closer look at integrin $\alpha_V\beta_6$ as putative primary CagL receptor in the human stomach epithelium.

Materials and methods

Expression and purification of CagL variants

An expression construct for HA-tagged CagL was generated using an existing pETM-11 expression vector for N-terminally His_6-tagged CagL [18]. The fragment encoding CagL amino acids 21–237 was amplified by PCR with an oligonucleotide including the sequence coding for the HA-tag (YPYDVPDYA). CagL variants were expressed and isolated as described before [18]. Shortly, N-terminally tagged CagL was expressed insolubly in *Escherichia coli* BL21 CodonPlus(DE3)-RIL (Agilent Technologies, Santa Clara, CA, USA) at 37 °C for 3.5 h and lysed mechanically with a Cell Homogenizer. The inclusion bodies were isolated at 5000 ***g*** in a cooled centrifuge, washed twice with PBS and dissolved in 6 M Urea in PBS. The unfolded CagL was bound to Ni-NTA (Cube Biotech, Monheim am Rhein, Germany), washed with 6 M Urea in PBS, supplemented successively with 1 M NaCl, 12.5% glycerol, 10 mM dithiothreitol (DTT) or 25 mM imidazole. CagL was refolded using seven steps of a 1.5-fold dilution series of 6 M Urea in PBS with refolding buffer (containing 50 mM Tris, 20 mM NaCl, 0.8 mM KCl, 1 mM EDTA, 2 mM reduced glutathione and 0.2 mM oxidized glutathione at a final pH 8.2 at 4 °C). The matrix was washed with refolding buffer and CagL was eluted with 250 mM imidazole in PBS. The fusion protein was cleaved with tobacco etch virus (TEV) protease during dialysis against PBS overnight at 4 °C. Cleaved CagL was further isolated by negative affinity chromatography using Ni-NTA, followed by gel filtration (HiLoad 16/60 Superdex75 pg; GE Healthcare, Chalfont St. Giles, UK) using Tris buffered saline (TBS) or PBS as running buffer. Monomeric CagL fractions were pooled, concentrated and stored at −20 °C.

Biotinylation of CagL variants

If required, the purified CagL variants were dialyzed twice against 100-fold excess of PBS. Sulfo-NHS-LC-Biotin (G Biosciences, St. Louis, MO, USA) was used in a 10-fold excess as amine reactive biotinylation agent dissolved in water directly before use. The biotinylation was performed for 2 h on ice and stopped with one reaction volume of 1 M Tris pH 8. Afterwards the protein was purified with a desalting column using PBS as buffer. Proteins were aliquoted and stored at −20 °C.

ELISA

To measure CagL binding to integrins, each well of a transparent F96 MaxiSorp Nunc-Immuno Plate (Thermo Scientific, Waltham, MA, USA) was coated overnight with 50 µL of 5 $\mu g \cdot mL^{-1}$ recombinant integrin ectodomains ($\alpha_5\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$ and $\alpha_V\beta_8$; R&D Systems, Minneapolis, MN, USA) in TBS+ (25 mM Tris pH 7.5, 27 mM KCl, 137 mM NaCl, 0.5 mM $MgCl_2/CaCl_2/MnCl_2$). Wells were emptied and blocked with 200 µL TBS+ supplemented with 0.1% Tween20 (TBST+) for 2 h. After washing three times with 200 µL TBST+, 50 µL of the CagL protein solution were applied and incubated for 1 h, followed by three washing steps (200 µL) with TBST+. 50 µL of NeutrAvidin™-HRP conjugate (Thermo Scientific) in TBST+ were applied for the biotinylated CagL variants. HA-CagL was treated with 50 µL monoclonal anti-HA antibody HA.11 clone 16B12 diluted 1 : 2000 in TBST+. Fibronectin was treated with 50 µL anti-human fibronectin antibody 1 : 500 in TBST+ (Clone P1H11; R&D systems) for 45 min. Both primary antibodies were incubated with goat anti-mouse HRP conjugate (Jackson ImmunoResearch, West Grove, PA, USA) after repeated washing. The bound protein was detected with 3,3′,5,5′-tetramethylbenzidine (TMB, Applichem, Darmstadt, Germany). Resulting absorbances were measured in a Tecan Infinite 200 microplate reader (Tecan Group, Männedorf, Switzerland) at 450 nm and normalized to the CagL wildtype binding to $\alpha_V\beta_6$ after background subtraction. All incubation steps were performed at 4 °C on a tumbling shaker.

Fluorescence polarization for competitive binding

Protein expression and purification of integrin $\alpha_V\beta_6$ headpiece and $\alpha_5\beta_1$ unclasped ectodomain and FP competitive binding affinity measurements of integrins to CagL were preformed similar as described [32,39]. Briefly, each sample (10 µL) contained 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 nM FITC-probe, 100 nM integrin, and indicated concentration of CagL in 20 mM Tris buffer (pH 7.4). $\alpha_V\beta_6$ and $\alpha_5\beta_1$ were pre-equilibrated with CagL for 1 h before mixing with fluorescence probe. The mixture was allowed to equilibrate for 1 h before recording FP on a Synergy NEO HTS multi-mode microplate reader (Biotek, Winooski, VT, USA). For wildtype CagL and $CagL^{L82T}$ a K_d value for the $\alpha_V\beta_6$ headpiece was derived from the data using the following method. The total CagL concentration resulting in half-maximal displacement of the FITC-labelled probe was determined by fitting a three-parameter dose-response curve in the program GRAPHPAD PRISM (GraphPad Software, San Diego, CA, USA). This value corresponds to the I_{50} value in equation (18) given by Rossi and Taylor [40]. K_d was calculated from I_{50} with equations (17) and (18) from [40].

Cell culture and *H. pylori* bacteria

Cells were cultivated in 5% (v/v) CO_2 atmosphere at 37 °C using media supplemented with 10% FCS, 2 mM L-glutamine, 100 $U \cdot mL^{-1}$ penicillin and 100 $U \cdot mL^{-1}$ streptomycin (Lonza Group, Basel, Switzerland). For HT-29 (V. Orian-Rousseau, Karlsruhe Institute of Technology,

Karlsruhe, Germany) DMEM/Ham's F-12 (GE Healthcare, Chalfont St. Giles, UK) was used. MKN-45 (DSMZ, Braunschweig, Germany) and 23132/87 (DSMZ, Braunschweig, Germany) cells were kept in RPMI 1640 (Lonza Group, Basel, Switzerland). A-549 (M. Heilemann, Goethe-University Frankfurt, Germany), 293T (HEK) (V. Orian-Rousseau, Karlsruhe Institute of Technology, Karlsruhe, Germany), WM-115 (N. Sewald, Bielefeld University, Bielefeld, Germany) and SW480 mock and SW480β_6 (D. Sheppard, University of California, San Francisco, CA, USA) cells were grown in DMEM High Modified (GE Healthcare, Chalfont St. Giles, UK). For infection experiments SW480 was maintained in DMEM and human gastric epithelial AGS cells (ATCC CRL 1739) were grown in RPMI. Medium was exchanged every 2–4 days and cells were split before confluence using trypsin/EDTA (Lonza, Basel, Switzerland) depending on their rate of growth. *H. pylori* wildtype strains P12, G27, NCTC11637 and N6 were grown on GC agar plates supplemented with 10% horse serum, 1% vitamin mix and vancomycin (10 $\mu g \cdot mL^{-1}$) at 37 °C under microaerophilic conditions using CampyGen gas packs (Oxoid Limited, Hampshire, UK) [64].

Flow cytometry analysis of the integrin expression

Cells were detached with 5 mM EDTA in PBS by incubating at 37 °C for 10–30 min depending on cell line characteristics, centrifuged for 5 min at 200 ***g*** and resuspended in PBS at a density of 5×10^6 cells·mL^{-1}. To hinder unspecific antibody binding Fc-receptor blocking reagent was used. Therefore 2 mL cell suspension was pelleted, resuspended in 80 µL PBS and 20 µL FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated for 10 min at 4 °C on a rotation wheel. The samples were pelleted and resuspended in 1 mL PBS supplemented with primary antibodies (2 $\mu g \cdot mL^{-1}$) for the specifically stained samples or not supplemented for the unstained and control samples. After incubation for 1 h at 4 °C on a rotation wheel, samples were washed twice with 1 mL PBS and resuspended in 1 mL PBS for unstained samples, in 1 mL PBS with 2 $\mu g \cdot mL^{-1}$ isotype-control antibodies for control stained samples and in 1 mL PBS with 2 $\mu g \cdot mL^{-1}$ secondary antibodies for specifically stained samples. The samples were incubated at 4 °C for 1 h on a rotation wheel and washed two times with 1 mL PBS. Finally all samples were filtrated (Ø 30 µm), vortexed and analysed with a CyFlow®space flow cytometer (Partec, Münster, Germany). For the analysis of the $\alpha_V\beta_6$ expression, 3G9 [37] was used as primary antibody, whereas the α_5 subunit of $\alpha_5\beta_1$ was marked with anti-CD49e (JBS5; Thermo Scientific). Goat anti-mouse IgG-PE (Santa Cruz Biotechnology, Dallas, TX, USA) served as secondary antibody.

CagL binding probed by flow cytometry

Cells were harvested from flasks at approximately 90% confluence with 5 mM EDTA in PBS. Cells were washed in 1 mL PBS once and then resuspended at 1.2–3.0 $\times 10^6$ cells·mL^{-1} in TBS+. Cells were washed in TBS+ and then incubated with 20 µL of Fc-receptor blocking reagent (Miltenyi Biotec) in 200 µL TBS+. Unlabelled reference and control samples were then incubated with TBS+ for 45 min while CagL samples were treated with 1 µM biotinylated CagL wildtype. After washing two times with TBS+, the control and the CagL samples were incubated with NeutrAvidin™-PE (Thermo Scientific) whereas the unlabelled reference was once again incubated in TBS+ for 45 min. All samples were finally washed two times in TBS+ and after resuspending and vortexing analysed on a FACSCalibur (BD biosciences, Franklin Lakes, NJ, USA) using the software BD CELLQUEST™ Pro version 4.0.2.

Infection assays

For infection experiments, AGS, SW480 mock or SW480β_6 cells were cultured on six-well plates until reaching confluency of ~ 70%. Then, cells were washed twice with Dulbecco's phosphate buffered saline and fresh antibiotics-free medium was added to the cells. For infection, *H. pylori* were suspended in BHI medium, and the number of bacteria was determined through optical density measurement at OD_{600}. The cells were infected at a multiplicity of infection (MOI) of 100 [65].

SDS/PAGE and western blotting

To prepare whole cell extracts for western blotting, infected cells were harvested by adding hot Laemmli buffer to the culture plates. Protein lysates were separated by SDS/PAGE and blotted onto PVDF membranes (Immobilon-P; Millipore, Darmstadt, Germany) [66]. Membranes were blocked in TBST with 3% BSA or 5% skim milk for 1 h at room temperature or overnight at 4 °C. Then, the membranes were probed with rabbit polyclonal anti-CagA antibody (Austral Biologicals, San Ramon, CA, USA) or for detection of phosphorylated CagA with anti-pan-phosphotyrosine antibody PY99 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), according to the instructions of the manufacturer. Incubation with anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) served as a loading control. CEACAMs were detected as described [57] with monoclonal antibodies specific for CEACAM1 (clone 18/20), CEACAM5 (clone 3E10) and CEACAM6 (clone 1H7) [54]. Immunodetection was performed using HRP–conjugated secondary antibodies and Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ, USA) as described. After documentation on a Bio-Rad ChemiDoc imaging system, the band intensities

were quantified using IMAGE LAB software (BioRad, Hercules, CA, USA).

Quantification of secreted IL-8

SW480 and SW480β_6 cells were infected with different *H. pylori* strains at a multiplicity of infection (MOI) of 50 and the co-culture supernatants were collected after 8 h of infection. The amount of IL-8 secreted into the cell culture supernatant was detected by ELISA using the OptEIA human IL-8 kit II (BD Biosciences, Franklin Lakes, NJ, USA) [56].

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MB purified CagL and performed ELISAs. MB and JS performed flow cytometry experiments. NT performed *H. pylori* infection experiments. XD and JL performed FP experiments. HHN conceived the study. TAS and SB contributed reagents. MB and HHN wrote the paper. All authors read and approved of the final version.

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