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ORIGINAL RESEARCH PAPER

Importance of cortactin for efficient epithelial NF-κB activation by *Helicobacter pylori*, *Salmonella enterica* and *Pseudomonas aeruginosa*, but not *Campylobacter* spp.

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ABSTRACT

Transcription factors of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family control important signaling pathways in the regulation of the host innate immune system. Various bacterial pathogens in the human gastrointestinal tract induce NF-κB activity and provoke pro-inflammatory signaling events in infected epithelial cells. NF-κB activation requires the phosphorylation-dependent proteolysis of inhibitor of κB (IκB) molecules including the NF-κB precursors through ubiquitin-mediated proteolysis. The canonical NF-κB pathway merges on IκB kinases (IKKs), which are required for signal transduction. Using CRISPR-Cas9 technology, secreted embryonic alkaline phosphatase (SEAP) reporter assays and cytokine enzyme-linked immunosorbent assay (ELISA), we demonstrate that the actin-binding protein cortactin is involved in NF-κB activation and subsequent interleukin-8 (IL-8) production upon infection by *Helicobacter pylori*, *Salmonella enterica* and *Pseudomonas aeruginosa*. Our data indicate that cortactin is needed to efficiently activate the c-Sarcoma (Src) kinase, which can positively stimulate NF-κB during infection. In contrast, cortactin is not involved in activation of NF-κB and IL-8 expression upon infection with *Campylobacter* species *C. jejuni*, *C. coli* or *C. consisus*, suggesting that *Campylobacter* species pluralis (spp.) induce a different signaling pathway upstream of cortactin to trigger the innate immune response.

KEYWORDS

ADP-heptose, NF-κB, *Helicobacter pylori*, T4SS, cortactin, Src, interleukin-8, inflammation

INTRODUCTION

Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) is a family of conserved transcription factors representing major dimeric transcriptional regulators that are expressed in all differentiated mammalian cell types [1]. They inhabit important functions in a wide range of cellular processes, including an important conserved function in orchestrating immune responses and inflammation [2]. The NF-κB signaling network is composed of hetero- or homodimers of five protein monomers called p50, p52, RelA, RelB and c-Rel together with their inhibitor proteins, inhibitors of kappa B (IκBs) named e.g. IκBα, IκBβ and

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IκBε [3]. All five monomeric members share an amino-terminal Rel homology domain (RHD) comprising about 300 amino acids [2] that facilitates 15 potential dimerizations, DNA binding, interaction with IκBs as well as translocation in the nucleus [4].

In the cytoplasm of unstimulated cells, NF-κB dimers are inactive, as controlled by IκB inhibitors. Upon activation signals such as oxidative stress, viral and bacterial infections, pro-inflammatory cytokines or damage to DNA, IκBs are phosphorylated by the IκB kinases (IKK) complex. The phosphorylated IκBs are then ubiquitinated and degraded through the proteasome, resulting in the release of NF-κB dimers. Subsequently, released NF-κB dimers translocate to the nucleus to initiate a target transcriptional program by binding to their specific DNA sites [2]. Generally, NF-κB activity is regulated by two major routes, called the canonical and non-canonical NF-κB signal transduction cascades [5]. The canonical pathway is conveyed by activation of the NF-κB essential modulator (NEMO) within the IKK complex and is induced by recognition of signals generated from pro-inflammatory cytokine receptors, including the tumor necrosis factor receptor (TNFR) family, interleukin-1 receptor (IL-1R) members, antigen receptors and Toll-like microbial pattern recognition receptors (TLRs) [6, 7]. In contrast, the non-canonical pathway is regulated by stimulation of a NEMO-independent signaling module involving NF-κB-inducing kinase (NIK) and IKKα, and is stimulated by certain TNF family members, called CD40 ligand (CD40L), lymphotoxin-β (LTB), B cell activating factor (BAFF), and receptor activator of NF-κB ligand (RANKL) [6, 8]. The immediate reversible immune and inflammatory response takes place by stimulation of the canonical pathway, which is crucial for specific cytokine responses [2]. NF-κB activation finally results in the transcription and subsequent regulation of the target genes encoding a variety of pro-inflammatory chemokines and cytokines, such as IL-8, IL-6, IL-1β and TNF. One of the major pro-inflammatory reactions is the secretion of IL-8, overexpression of which is generally associated with cancer development [3, 9].

Most gastrointestinal infections are spread by ingestion of water or food contaminated by various pathogenic bacteria and cause high incidences of acute and chronic diseases worldwide [10]. For instance, bacteria such as *Campylobacter*, *Salmonella* and *Pseudomonas* species are the general agents of infection [10–12]. *Helicobacter pylori* are persistent, Gram-negative, micro-aerophilic bacteria inhabiting the gastric tissue of humans. The virulence properties of the bacteria depend on the existence of the numerous virulence factors that help to tightly attach, colonize and induce pathogenicity [13]. Many Gram-negative bacteria can activate NF-κB, and this pathway is well described for *H. pylori* [14]. It was reported that at least four *H. pylori* virulence factors are able to mediate NF-κB activation and IL-8 expression – the type IV secretion system (T4SS) encoded by the cytotoxin-associated gene (*cag*) pathogenicity island (PAI), its CagA effector protein [15], the lipopolysaccharide metabolite ADP-glycero-β-D-manno-heptose (ADP-Heptose) [16, 17], and γ-Glutamyl-transpeptidase (GGT) [18]. The major NF-κB inducer of *H. pylori*, ADP-Heptose, is presumably injected into the cytoplasm of the host cell, where it can bind to the alpha-protein kinase 1 (ALPK1), which induces its kinase activity to phosphorylate and self-oligomerize the TRAF-interacting protein with the forkhead-associated domain (TIFA) [16, 17]. Interaction of activated TIFA with the tumor necrosis factor receptor-associated factor 6 (TRAF6) forms the so-called TIFAsome. This complex enables binding of TGFβ-activated kinase 1 (TAK1) to stimulate the IKK complex resulting in the phosphorylation of specific N-terminal serine residues (Ser32/36) of IκBα [19]. This phosphorylation in return enables ubiquitination of IκBα through the recruitment of S-phase kinase-associated protein 1 (SKP1)-cullin 1 (CUL1)-F-box protein (SCF)/beta-transducin repeat-containing protein (βTRCP) ligase complex, and further degradation through the 26S proteasome, which releases the NF-κB heterodimer [2]. Thereafter, the release of RelA/p50 heterodimers results in their nuclear translocation, where they start the transcription of corresponding genes linked to cytokine expression [17, 19]. In addition, it has been demonstrated that in gastric epithelial cells infected with *H. pylori*, IKKβ tyrosine phosphorylation and binding to c-Sarcoma (Src) kinase is induced and that Src knockdown resulted in reduced IκBα and RelA phosphorylation [20].

We recently discovered that infection of gastric epithelial cells with *H. pylori* targets cortactin, coded by the gene *cttn*, to activate focal adhesion kinase (FAK), Src and Abl tyrosine kinases [21]. The protein cortactin contains multiple domains and is involved in regulating the actin cytoskeleton organization and cellular motility [22]. The domain structure of cortactin includes the amino-terminal domain (NTA), a central filamentous actin binding domain, a proline-rich area, and a Src-homology 3 (SH3) domain at the carboxy-terminus, which provides complex interactions of cortactin with other regulatory proteins [23]. Being a key factor in regulating cytoskeletal rearrangements, cortactin represents an attractive target to control host target cells by various microbial pathogens, including *H. pylori* [24, 25]. Cortactin's activity can be regulated through serine and tyrosine phosphorylation events by Src and Abl, by extracellular signal regulated kinases 1 and 2 (ERK1/2), or by p21-activated kinase 1 (PAK1) [26–30]. During *H. pylori* infection, cortactin was shown to undergo phosphorylation by ERK1/2 at Ser-405 and/or Ser-418, which finally results in FAK stimulation [21, 30–32]. In turn, FAK has been shown to interact with and stimulate Src and Abl kinase activities, which are important for CagA phosphorylation [21, 33]. Taking into account that (1) Src is potentially involved in NF-κB activation [34] and (2) cortactin functions upstream of the FAK/Src/Abl-axis pathway [21], we aimed to investigate if cortactin may contribute to NF-κB activation in *H. pylori* infected cells. We studied the role of cortactin in NF-κB activation and IL-8 release by utilizing AGS gastric epithelial wild-type (wt) cells and AGSΔ*cttn* cells with a complete knockout of the cortactin gene [21], which were infected with four different pathogens. Our results show cortactin-dependent, Src-mediated NF-κB activation upon

infection by the bacterial pathogens *H. pylori*, *Salmonella enterica* and *Pseudomonas aeruginosa*, but not by *Campylobacter* species pluralis (spp.), suggesting that the latter species trigger a cortactin-independent route.

MATERIALS AND METHODS

Cultivation of human AGS cells

The AGS gastric epithelial cell line established from adenocarcinoma (American Type Culture Collection #CRL-1739™) was grown in RPMI 1640 medium containing 10% FCS (fetal calf serum) from ThermoFisher Scientific (Waltham, MA, USA), 1% penicillin/streptomycin (Sigma-Aldrich, Steinheim, Germany) and 0.2% Normocin™ (InvivoGen, Toulouse, France) in an incubator set to 37°C and 5% carbon dioxide (CO_2). Cortactin knockout AGS cells (AGSΔ*cttn*) were described earlier and cultured like AGS wt, except for adding 2 µg mL^{-1} puromycin into the medium [35]. Twelve hours before infection of the cells with the below described bacterial pathogens, the medium was removed and fresh RPMI medium without antibiotics was added, followed by incubation of the cells at 37°C and 5% CO_2 [21].

Cultivation of bacteria for infection experiments

Table 1 presents all bacteria used in this survey. Type 1 *H. pylori* wt strain P12 [36] was grown from deep-frozen stocks in brain heart infusion (BHI) medium with 20% glycerol. The bacteria were cultivated on GC agar base plates containing 10% horse serum, 4 µg mL^{-1} amphotericin, along with 10 µg mL^{-1} vancomycin under microaerobic conditions produced in an AnaeroJar™ with 2.5 L capacity (Oxoid, Wesel, Germany) by using a CampyGen (Oxoid) gas kit [37]. The isogenic P12Δ*cagA*, P12Δ*cag*PAI, P12Δ*gmhA* and P12Δ*ggt* mutants (Table 1) were treated like the wt strains, with the exception that 8 µg mL^{-1} kanamycin, or 4 µg mL^{-1} chloramphenicol were additionally added to agar plates to select resistant colonies [38]. *Campylobacter jejuni* strain 81-176, *Campylobacter coli* strain 10-02932 and *Campylobacter concisus* strain CCUG 13144 were similarly cultivated from stocks as described before [39]. The bacteria were grown at 37°C for 48 h on *Campylobacter* blood-free selective agar base supplemented with *Campylobacter* growth supplement purchased from Oxoid (Wesel, Germany). *C. coli* and *C. jejuni* were cultivated in microaerobic atmosphere created in a 2.5 L AnaeroJar™ with a CampyGen sachet as described above, while *C. concisus* required the anaerobic gas generating kit BR0038B (Oxoid) [40]. *P. aeruginosa* strain PAO1 (ATCC 15692) and *S. enterica* Serovar Typhimurium strain STM (NCTC 12023) were routinely grown from −80°C stocks (LB medium with 20% glycerol) by using LB plates incubated at 37°C overnight. For infection, bacteria were resuspended in LB medium. The number of bacterial cells for infection were determined by measuring the optical density (OD_{600}) of bacterial cultures and adjusting the OD to 1.0, which corresponds to approximately 4×10^8 bacterial cells. For all infection experiments, a multiplicity of infection (MOI) of 50 was used.

Transient transfection assays

Transfection of AGSΔ*cttn* cells with peGFP-N1-Src wt, expressing constitutively active Src kinase [41], and pNF-κB-SEAP (secreted embryonic alkaline phosphatase) reporter plasmid (http://www.addgene.org) was carried out by using the transfection reagent Turbofect according to the protocol of the manufacturing company (ThermoFisher Scientific). AGSΔ*cttn* cells were seeded into 6-well plates with RPMI 1640 complete medium (with 10% FCS) and were grown until they reach approximately 70% confluency. From each plasmid 5 µg were used and mixed with 200 µL RPMI 1640 medium without supplements/antibiotics and 10 µL Turbofect followed by incubation at room temperature for 20 min. Subsequently, the mixture was carefully added to the cells and the cells were incubated at 37°C and 5% CO_2 for 24 h. Transfected cells were then subjected to infections with the different bacterial strains (Table 1).

ELISA immunoassay

For investigation of IL-8 levels released from AGS cells upon infection with *H. pylori*, the enzyme-linked immunosorbent assay (ELISA) was used [42]. Supernatants of infected AGS control and AGSΔ*cttn* knockout cells were subjected to ELISA measurements. The supernatants of uninfected AGS wt and AGSΔ*cttn* cells were used as negative controls. Concentrations of IL-8 were determined using colorimetric ELISA kit (Invitrogen, #88-8086) according to instructions of the manufacturer.

Table 1. Bacteria used in the present work

Species	Strain	Mutated gene	Antibiotic Resistance	Origin
Helicobacter pylori	P12	none	none	[36]
H. pylori	P12Δ*cag*PAI	HP0520-548*	kanamycin	[54]
H. pylori	P12Δ*gmhA*	HP0857*	chloramphenicol	[19]
H. pylori	P12Δ*cagA*	HP0548*	chloramphenicol	[55]
Salmonella enterica	STM	none	none	NCTC 12023
Pseudomonas aeruginosa	PAO1	none	none	ATCC 15692
Campylobacter jejuni	81-176	none	none	ATCC-BAA-2151
Campylobacter coli	10-02932	none	none	RKI 10-02932
Campylobacter concisus	CCUG 13144	none	none	ATCC 33237

*Gene nomenclature according to TIGR strain 26695 (accession number PRJNA57787).

SEAP reporter assay

The SEAP reporter assay was applied as recently described [43]. Briefly, the above reported plasmid was transfected into the cells for 48 h followed by infection (MOI 50) for 24 h in 6-well plates. To quantify the levels of SEAP, 20 µL of infected cell supernatant (or as negative control non-infected cell culture medium) were mixed with 180 µL QUANTI-Blue™ solution (Invivogen) in 96-well plates followed by incubation for 30 min at 37°C and optical density (OD_{620}) measurements with the Infinite F200 Pro microplate reader (Tecan, Grödig, Austria).

Protein gels and Western blotting

Protein separation by mass was done by using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). For further analysis, the proteins were blotted onto polyvinylidene fluoride (PVDF) membranes and were incubated with antibodies after blocking the membranes according to manufacturers' protocols with either 5% non-fat dry milk, 3% or 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 [44]. The utilized antibodies recognized specifically CagA (Austral Biologicals, San Ramon, CA, USA; #HPP-5003-9), CagY [42], GGT [45], cortactin (Merck-Millipore, Darmstadt, Germany; #05-180), GFP (Clontech, #632381), FlaA [46], GAPDH (Santa Cruz Biotechnology; #sc-47724) and a polyclonal anti-β-actin antiserum from rabbit that was raised against a peptide (GQKDSYVGDEAQSK) of β-actin (Biogenes GmbH, Berlin, Germany). For detection of either primary mouse (Invitrogen, Darmstadt, Germany; #31446) or rabbit (Invitrogen; #31460) antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies were used, and Western blot development was carried out as described before [47].

Statistics

All measurements were repeated at minimum three times. Analysis of all results was carried out by applying the one-way analysis of variance (ANOVA) followed by Tukey's test with the statistical software version 8.0 of GraphPad Prism. The P-values $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***) were considered as statistical significant.

Ethics statement

Ethics approval was not required for this research because it did not involve any animal or human subjects.

RESULTS

Cortactin is involved in pronounced NF-κB activation and IL-8 release upon infection with *H. pylori*

It is known that *H. pylori* triggers NF-κB activation as has been previously described by means of either ADP-Heptose metabolite, CagA and/or GGT, resulting in IL-8 secretion [3, 7, 8, 14–19]. To study whether cortactin deficiency might impact these cellular responses in *H. pylori* infection, we utilized gastric epithelial AGS wt cells and previously created AGSΔ*cttn* knockout cells [21]. The knockout of cortactin in the AGSΔ*cttn* cell line was verified by Western blot analysis against cortactin (Fig. 1A). For infection, we have chosen the well-described duodenal ulcer *H. pylori* strain P12, which has a functional T4SS [48, 49], and utilized isogenic knockouts in the *cag*PAI, *cagA*, *gmhA* and *ggt* genes (Table 1). P12Δ*cag*PAI and P12Δ*cagA* mutant bacteria are unable to deliver CagA, whereas P12Δ*gmhA*, and P12Δ*ggt* cannot express ADP-Heptose and GGT, respectively. Since the number of adherent AGS wt and AGSΔ*cttn* cells could affect the comparative analysis of NF-κB or IL-8, we confirmed that the confluency and number of cells among the two cell lines per well were similar (Fig. 1B). The correct expression or absence of the resulting proteins in isogenic *H. pylori* mutants were confirmed by Western blots with *H. pylori*-specific α-CagA, α-CagY and α-GGT antibodies with α-FlaA serving as a loading control (Fig. 1C).

Infection of AGS wt and AGSΔ*cttn* cells was performed with the aforementioned *H. pylori* isolates at an MOI of 50 for 8 h. The supernatants were harvested and IL-8 levels were determined using the ELISA immunoassay. AGS wt cells infected with P12 wt and P12Δ*ggt* led to an increase of secreted IL-8 levels by about 4-fold compared to the mock control (Fig. 2A). The isogenic *H. pylori* mutants P12Δ*cag*PAI and P12Δ*gmhA* were significantly less potent in IL-8 induction and revealed only background signals, while P12Δ*cagA* produced intermediate levels. Intriguingly, despite the same number of cells in both AGS wt and AGSΔ*cttn* infections, we found the IL-8 levels of AGSΔ*cttn* cells to be significantly lower than in AGS wt cells, in particular, when infected with P12 wt, P12Δ*cagA* and P12Δ*ggt* (Fig. 2B). Similar to infections of AGS wt, P12 wt and P12Δ*ggt* showed the highest IL-8 activation in AGSΔ*cttn* cells, with the remaining mutants being less potent in IL-8 induction (Fig. 2B).

To investigate if reduced IL-8 secretion is due to altered NF-κB activity, we transfected AGS wt and AGSΔ*cttn* cells with the NF-κB-SEAP reporter construct before infection. The findings indicate that the extent of NF-κB activation correlated with the levels of IL-8 release during infection with the *H. pylori* strains (Fig. 2A/B). Importantly, a significant reduction ($P \leq 0.001$) in NF-κB activity was observed in AGSΔ*cttn* cells in comparison to the parental AGS wt cells, implying that cortactin plays a role in triggering NF-κB activation upon infection with *H. pylori*.

Cortactin is involved in prominent NF-κB activation and IL-8 expression by *Salmonella, Pseudomonas*, but not *Campylobacter* spp.

As next, we asked whether cortactin requirement for effective NF-κB activation and IL-8 synthesis is a specific aspect of *H. pylori* infection, or whether this is also true for infections with other bacteria. To clarify this question, AGS wt and AGSΔ*cttn* cells were infected with the well-known pathogens *S. enterica, P. aeruginosa*, *C. jejuni*, *C. coli* or *C. concisus*. Infection with *S. enterica* and *P. aeruginosa* resulted in an increase in IL-8 production up to 2.7-fold,

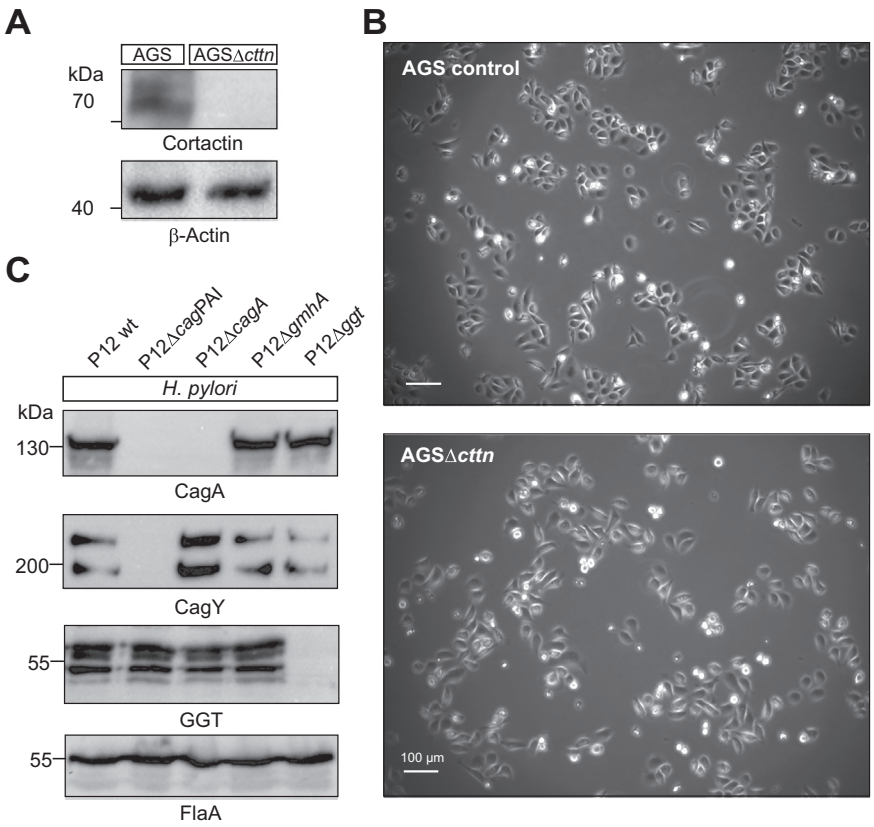

Fig. 1. Validation of various gene knockout mutations in gastric epithelial AGS cells and *H. pylori* isolates used in the current study. (A) Western blotting showing cortactin deletion in AGS cells. The β-actin staining provides the loading control. (B) Phase contrast microscopy of AGS wt control cells as well as AGSΔ*cttn* knockout cell line before infection. Scale bar is 100 µm. (C) Confirmed knockout of *cagA*, *cag*PAI, and *ggt* genes in *H. pylori* strain P12 by specific antibodies recognizing the indicated proteins, with the FlaA blot serving as the loading control. Antibodies against GmhA are not yet available, and therefore the *gmhA* gene knockout was confirmed by PCR (not shown)

while the levels of IL-8 secreted upon infection with *C. jejuni*, *C. coli* or *C. concisus* were only slightly upregulated compared with the uninfected control (Fig. 3A). Interestingly, *S. enterica* and *P. aeruginosa* induced only an increase of 1.7-fold and 1.8-fold in IL-8 expression by AGSΔ*cttn* cells, respectively (Fig. 3B), suggesting that the activation of NF-κB was cortactin-dependent. Stimulation of NF-κB activity followed a similar pattern with a 15-fold increase by *S. enterica* and a 14-fold increase by *P. aeruginosa* in AGS wt in comparison to the uninfected control, while in AGSΔ*cttn* cells the NF-κB activity reached only about 50% compared to AGS wt cells (Fig. 3). In contrast, no significant changes of NF-κB activation and IL-8 secretion levels between the two cell lines were obtained during infection with *C. jejuni*, *C. coli* and *C. concisus*, suggesting cortactin-independent NF-κB activation and release of IL-8 by these bacteria.

We recently reported that cortactin expression is essential for efficient activation of FAK, Src and Abl tyrosine kinases upon infection with *H. pylori* [21]. In addition, it was shown that tyrosine-phosphorylation of IKKβ by activated Src contributes to activation of NF-κB in response to *H. pylori* infection [20]. Therefore, we hypothesized that cortactin engagement via Src may be required for effective NF-κB activation and IL-8 production during infection with *H. pylori, S. enterica* and *P. aeruginosa*. To test this idea, AGSΔ*cttn* knockout cells were transfected either with GFP-tagged wt Src or constitutive active Src (Src Y527F) together with the NF-κB SEAP reporter construct prior to infection using identical conditions as described above. Similar expression levels of the Src constructs in each lane were verified by Western blot analysis using α-GFP antibodies (see Fig. 4, bottom). The results show that NF-κB activation and IL-8 secretion by AGSΔ*cttn* knockout cells were enhanced upon infection with *H. pylori, S. enterica* and *P. aeruginosa* by the expression of constitutively active Src, but not wt Src (Fig. 4A). On the contrary, the NF-κB activation and IL-8 secretion by *C. jejuni*, *C. coli* or *C. concisus* (Fig. 4B) were not changed by expression of constitutively active Src. These results let us suppose that cortactin expression is necessary for pronounced NF-κB stimulation through Src during infection with *H. pylori, S. enterica* and *P. aeruginosa*, but not *C. jejuni*, *C. coli* or *C. concisus.*

DISCUSSION

In mammalian cells, the NF-κB transcription factor is a crucial regulator of the host innate immune system [1–8]. Various gastrointestinal bacterial pathogens activate the NF-κB canonical signaling pathway. Upon stimulation signal,

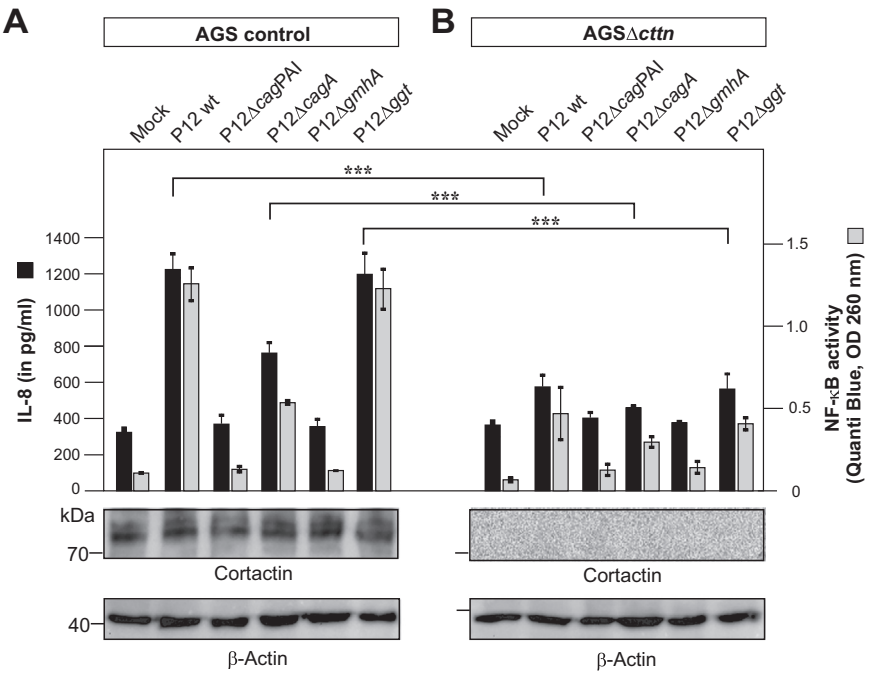

Fig. 2. Cortactin deficiency results in a significantly reduced pro-inflammatory response upon infection with *H. pylori*. (A) AGS wt cells as control and (B) AGSΔ*cttn* knockout cells were infected for 8 h with *H. pylori* P12 wt or its indicated mutants. The supernatants were analyzed by ELISA immunoassay to measure the amounts of secreted chemokine IL-8 (black bars). In addition, the AGS cell variants were subjected to transfection with the NF-κB-SEAP reporter plasmid prior to *H. pylori* infection for 24 h to monitor NK-κB activity (grey bars). The Western blots on the bottom confirm the expression or deficiency of cortactin in the cells with β-actin as loading control. Every experiment was performed in triplicate. Statistically significant differences were confirmed; *** $P \leq 0.001$

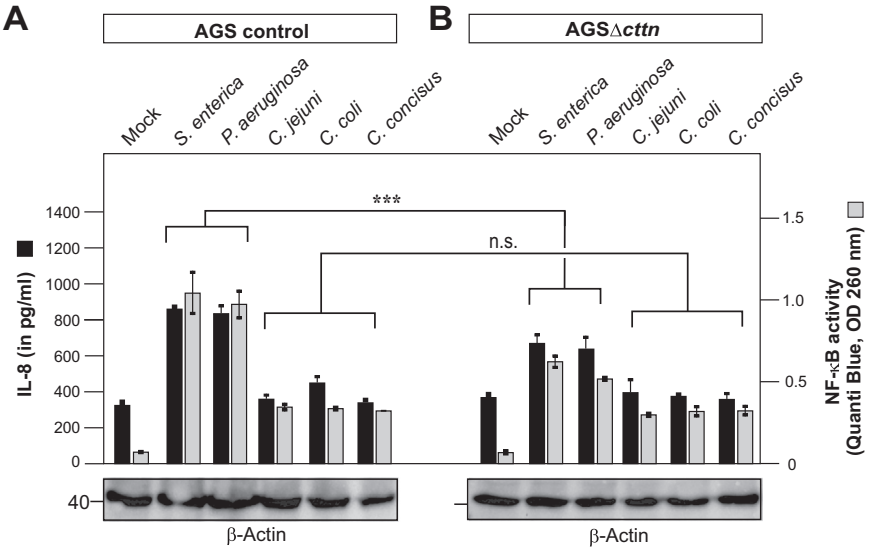

Fig. 3. Cortactin knockout is associated with significantly reduced pro-inflammatory responses upon infection with *S. enterica* and *P. aeruginosa*, but not with *C. jejuni*, *C. coli* or *C. concisus*. AGS wt control cells (A) and AGSΔ*cttn* knockout cells (B) were infected for 8 h with the indicated bacteria, and the supernatants were analyzed by the ELISA immunoassay to measure the amounts of the secreted chemokine IL-8 (black bars). In addition, the AGS cell variants were transfected with the NF-κB-linked SEAP reporter plasmid prior to *H. pylori* infection, and NK-κB activity was quantified (grey bars). The Western blots on the bottom show the expression or deficiency of cortactin in the cells with β-actin as loading control. Every experimental test was done in triplicate. Statistical significance is shown by *** ($P \leq 0.001$). n.s.; not significant

IκBs become degraded, followed by nuclear translocation of the activated NF-κB RelA/p50 heterodimer and transcription activation of numerous cytokine genes [2]. One of the predominant chemokines that are expressed in response to pathogens is IL-8, which belongs to the Glu-Leu-Arg (ELR) motif positive Cysteine-X-Cysteine (CXC) chemokine family

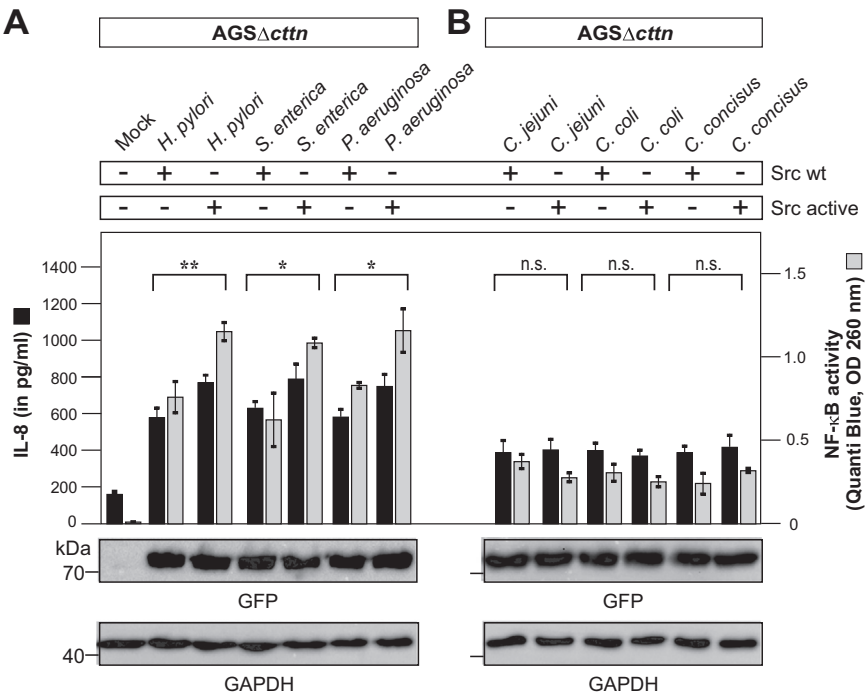

Fig. 4. Ectopic expression of constitutively active Src in AGSΔ*cttn* knockout cells rescues activation of NF-κB and IL-8 production upon infection with *H. pylori, S. enterica* and *P. aeruginosa*, but not with *C. jejuni*, *C. coli* or *C. concisus.* The AGSΔ*cttn* cell line was simultaneously transfected with two constructs, NF-κB-linked SEAP reporter plasmid and either wt Src kinase or constitutive active Src, respectively, followed by infection with the indicated strains. The supernatants were subjected to IL-8 ELISA (black bars) and an SEAP reporter assay (grey bars). The Western blots on the bottom show the expression of GFP-tagged Src constructs in the cells with GAPDH as loading control. Every experiment was performed in triplicate. * ($P \leq 0.05$) and ** ($P \leq 0.01$) indicate statistical significance. n.s.; not significant

and attracts several types of immune cells in response to bacterial entry [9, 50–52]. The IL-8 gene promoter carries various sites for NF-κB binding, which results in transcription of the IL-8 gene. Using the CRISPR-Cas9 knockout approach we show that cortactin, an actin-binding protein, represents a novel player in IL-8 regulation during infection of selected bacterial pathogens.

Cortactin represents a detailed described regulator of the host cell actin cytoskeleton [22–24]. Therefore, this protein is an attractive target for many pathogens during the infection process [24, 25]. Our group reported a signaling pathway in *H. pylori* infected cells, where this pathogen alters the phosphorylation status of cortactin by the help of the T4SS-injected virulence factor CagA [31, 32]. We produced a complete cortactin knockout in the stomach epithelial AGS cell line by CRISPR-Cas9 [21], which we used here to pinpoint a novel role of cortactin in NF-κB activation and secretion of IL-8. Our results showed a significant reduction of NF-κB activation and IL-8 release triggered by *H. pylori* infection of AGSΔ*cttn* cells compared to the AGS wt control. We have carefully checked in each of the infection experiments the similar AGS cell numbers and conditions, as confirmed by phase contrast microscopy and Western blotting. Thus, varying cell numbers cannot account for the observed effects. Furthermore, similar cortactin-dependent effects on NF-κB activation and IL-8 production were seen upon infection with other gastrointestinal pathogens such as *S. enterica* and *P. aeruginosa.* In contrast, these cortactin-dependent activities on NF-κB stimulation and IL-8 production were not observed during infection with *C. jejuni*, *C. concisus* or *C. coli*, further confirming that the above seen effects on NF-κB responses by *H. pylori, S. enterica* and *P. aeruginosa* are specific.

We have recently reported that cortactin deficiency in AGS cells results in diminished activation of the host kinase Src [21], which itself has been implicated to play a role in effective NF-κB activation by *H. pylori* [20]. Stimulation of NF-κB requires the central IKK-complex with IKKα and IKKβ as catalytic factors associated with the scaffold protein NEMO. Rieke and co-workers [20] showed that IKKβ is crucial for NF-κB activation by various stimuli such as *H. pylori* infection [53]. Interestingly, not only phosphorylation of serine residues, but also tyrosine phosphorylation, appeared to be essential for IKKβ activity. In fact, *H. pylori* infection transiently induced the formation of a Src and IKKβ complex [20]. Downregulation of Src expression by small interfering RNA or inhibition of Src kinase activity using specific inhibitors diminished phosphorylation of the downstream effectors IκBα and RelA [20]. Consequently, Src-mediated phosphorylation of IKKβ at tyrosine residues participated substantially to activation of NF-κB by *H. pylori*. Here we observed a similar defect in NF-κB activation during infection of cortactin-deficient AGSΔ*cttn* knockout cells. To prove the importance of cortactin in Src-mediated

stimulation of NF-κB, transfections of AGSΔ*cttn* cells were performed either with Src wt or constitutive active Src followed by infection, which confirmed the role of Src activation during infection with *H. pylori, S. enterica* and *P. aeruginosa*. In contrast, *C. jejuni*, *C. concisus* and *C. coli* triggered IL-8 synthesis and activation of NF-κB independent of Src and cortactin suggesting that these pathogens use other mechanisms which contribute to the induction of NF-κB activity and the response of the innate immune system. However, more studies will be needed to determine the particular signaling pathways upstream of cortactin.

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Conflict of interest: Nothing to declare.

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