Rapid Interaction of *Helicobacter pylori* With Microvilli of the Polar Human Gastric Epithelial Cell Line NCI-N87

ANNE-KATHRIN DIESING,[1]* CONSTANZE NOSSOL,[1] HEIDI FABER-ZUSCHRATTER,[1] WERNER ZUSCHRATTER,[2] LYDIA RENNER,[1] OLGA SOKOLOVA,[3] MICHAEL NAUMANN,[3] AND HERMANN-JOSEF ROTHKÖTTER[1]

[1]Institute of Anatomy, Otto-von-Guericke University Magdeburg, Leipziger Str. 44, 39120 Magdeburg, Germany
[2]Leibniz Institute for Neurobiology, Brennecke Str. 6, 39118 Magdeburg, Germany
[3]Institute of Experimental Internal Medicine, Otto-von-Guericke University Magdeburg, Leipziger Str. 44, 39120 Magdeburg, Germany

ABSTRACT

Infection with *Helicobacter pylori* results often in chronic gastritis, gastric ulcers or even gastric tumor development. Little is known about the initial interaction between gastric epithelial cells and *H. pylori*. The aim of the present study was to analyze the initial host contact to the bacteria. Monolayers of the human gastric epithelial cell line NCI-N87 grown on porous membranes were used and the apical side of the epithelium was exposed to the *H. pylori* wild-type strain P1 for 1 hr. Many epithelial cells were colonized by bacteria within the period of 60 min. Using scanning electron microscopy we detected that the bacteria were in close contact with the epithelia via microvilli. Further, transmission electron microscopy of the contact sites revealed no difference in the morphology of the microvilli in comparison to those not attached to the bacteria. The present study demonstrates the importance of microvilli on apical epithelial cells during the initial contact of the host by colonizing *H. pylori*. Anat Rec, 296:1800–1805, 2013. © 2013 The Authors. The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology published by Wiley Periodicals, Inc. on behalf of the American Association of Anatomists.

Key words: gastric epithelial cells; scanning electron microscopy; tight junction; trans epithelial electrical resistance

Infection of the gastric mucosa by *Helicobacter pylori* occurs in the childhood and about 50% of man carry the bacteria throughout their whole life. Only a part of infected persons develop clinical symptoms including gastritis, ulcers and cancer. Since the first identification of *H. pylori* in 1983 by Marshal and Warren, the interaction between the bacteria and gastric epithelium was in the focus of intensive research activities (Ruggiero, 2012). Whereas several signaling processes were described in detail (Naumann, 2005; Backert and Naumann, 2010), it is so far not clear, how the first physical contact between the epithelial surface and *H. pylori* is established. Ultra-structural analysis showed that after exposition of isolated primary gastric epithelial cells or the human gastric tumor derived AGS cell line (Barranco et al., 1983) with

H. pylori, the bacteria were closely located at the epithelial cell membrane, in part microvilli were attached to the bacteria (Smoot et al., 1993; Shaffer et al., 2011; Johnson et al., 2012). In biopsies from patients suffering from *H. pylori* infection the gastric cells develop protrusions directly in the neighborhood of the bacteria (Smoot et al., 1993). So far it is not known at what time point these morphological changes occur after the exposition of the epithelium with *H. pylori*. The results from *in vitro* experiments were often studied 4 h after bacterial exposition (Johnson et al., 2012), in biopsies from *H. pylori* infected patients the time point and the morphology of the initial contact of host epithelium and bacteria can not be determined. As recent studies demonstrate that isoenzymes of the protein kinase C are already phosphorylated beginning 30 min after *H. pylori* exposition *in vitro* (Sokolova et al., 2013), our study aimed at an early morphological characterization of the contact of bacteria with the epithelium 60 min after exposure.

The interaction of host and *H. pylori* is dependent on the presence of important proteins of the apical cell membrane and the tight junctions (Oliveira et al., 2009). Therefore the human gastric epithelial cell line NCI-N87 was used as it forms a tight monolayer and exhibits gastric epithelial characteristics like ZO-1 and E-cadherin expression (Chailler and Menard, 2005). The morphology of the host/bacterium interface was studied using scanning as well as transmission electron microscopy techniques.

MATERIAL AND METHODS

Cell Culture

The human NCI-N87 gastric cell line (American Type Culture Collection, Manassas, VA, ATCC CRL 5822) was cultured in RPMI 1640 supplemented with 10 % fetal calf serum (FCS, heat inactivated; both PAN-Biotech, Aidenbach, Germany) and incubated in a humid atmosphere at 37°C and 5% CO_2 (refereed as normal cell culture conditions).

To determine their generation time the cells were cultured in 96-well plates (culture area per well: 0.28 cm^2, volume 100 µL; NUNC, Wiesbaden, Germany), various seeding densities were used (1×10^4, 3×10^4, and 6×10^4 per 100 µL). Cells were allowed to adhere for 6 hr. At this time point all wells were washed with PBS. Cells of two wells of the 96-well-plate were harvested with Trypsin/EDTA (Sigma, Taufkirchen, Germany) for cell count. The cell number of 6 hr refers to the cell number at the beginning of the experiment (N_0). Fresh medium was applied to all other wells of the 96-well-plate. Further on, the medium was aspirated and the cells were washed and harvested with Trypsin/EDTA at the time points of 24, 48, and 72 hr (N) to calculate the cell number. In the experiment (n = 3) cell count was examined in duplicates for each time point. The generation time (tg) was calculated after the following equation:

$$tg = \frac{\log 2 \times dt}{\log N \times \log N_0}$$

For all other experiments the cells were seeded in a density of 2×10^5 cells mL^{-1} on porous 12-well inserts (area 1.12 cm^2, pore size 1 µm; ThinCerts[TM] Cell Culture
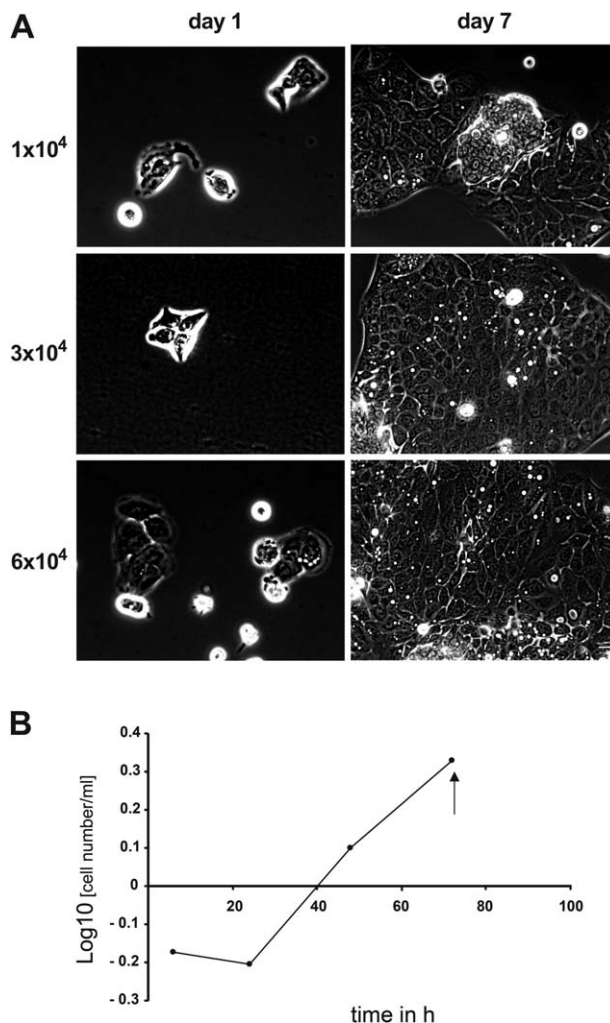

Fig. 1. (**A**) Development of NCI-N87 epithelial monolayers based on different cell numbers initially seeded (1×10^4, 3×10^4, 6×10^4). Some islands of dividing cells were observed after 24 hr and monolayers were established after 7 days of cultivation. (**B**) The cell proliferation analysis of NCI-N87 showed the relatively long population doubling time of 42 hr (arrow: calculation point for the time of generation; 72 hr).

Inserts, Greiner-bio one, Frickenhausen, Germany) and monolayers were grown for 7 days. Cell culture was regularly tested and found to be free of *Mycoplasma Spp.* (Venor® GeM Mycoplasma detection Kit; Minerva Biolabs, Berlin, Germany). One day before infection cells were washed with PBS and serum-free medium was added.

TEER Measurement

Measurement of transepithelial electrical resistance (TEER) was performed using a Millicell electrical resistance system (Millipore, Molsheim, France). Cells were deemed to be confluent at a TEER value of ≥ 0.90 kOhm $\times$ cm^2 (corresponding to a TEER value of 1 kOhm per 12-well insert), which was obtained after 7 days.

Bacteria Strains and Growth Conditions

H. pylori wild-type strain P1 was cultured on agar plates containing 10% horse serum under micro-
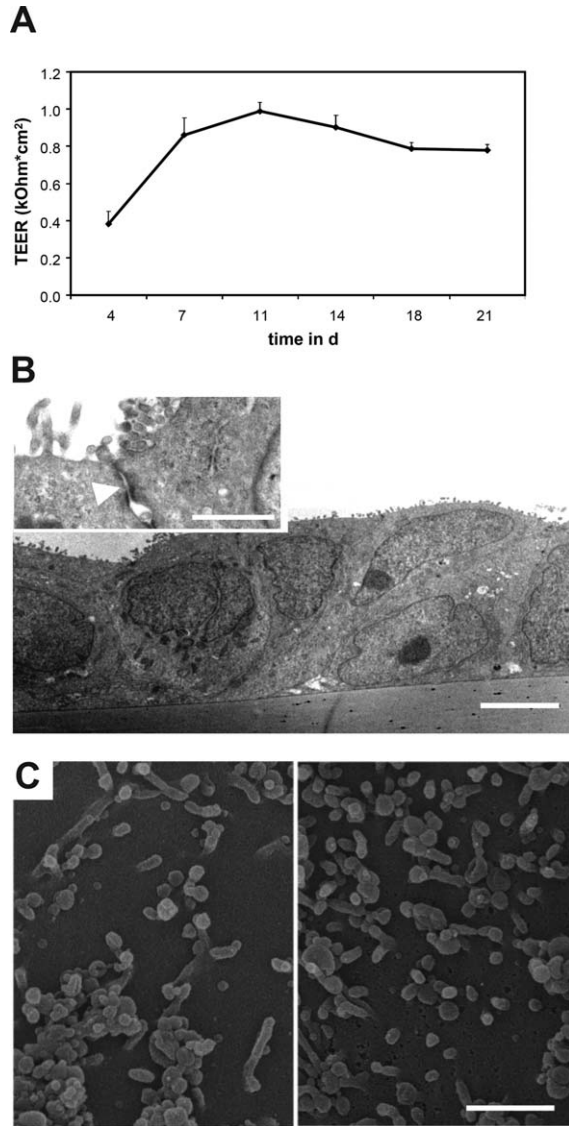
Fig. 2. (**A**) Trans epithelial electrical resistance (TEER) measurement was carried out on day 4, 7, 11, 14, 18, and 21. On day 7 the TEER was higher than 0.90 kOhm $\times$ cm^2. (**B**) Tight junction connected monolayers with many microvilli on day 7—bar 10 µm; insert: details of tight junctions (white arrow)—bar 500 nm (TEM). (**C**) Two areas of non-*H. pylori* exposed NCI-N87 cells—the length of microvilli and there density per area is variable—bar 500 nm (SEM).

pension of *H. pylori* cells in a ratio of 50:1 MOI for 1 hr. After this period membranes were detached from inserts and fixed for 30 min in 4% PFA. Membranes were than treated with fixation solution containing 0.5% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) over night. After fixation the membranes were washed in 0.1 M PB. Subsequently, samples were treated with 1% osmium tetroxide (Science Services, Munich, Germany) in 0.1 M PB for 1 hr. Samples were then rinsed in buffer, dehydrated and bloc-contrasted for 1 hr with 1% uranyl acetate in 70% ethanol. Finally, the membranes were infiltrated with Durcupan ACM (Fluka, Buchs, Switzerland) and flat embedded between plastic foils. The resin polymerized in an oven at 70°C for 2 days. Regions of interest were cut out and glued vertically onto a blank block of resin with a small groove. With an Ultracut S ultramicrotome (Reichert, Leica AG, Vienna, Austria) 1 µm semi-thin sections were cut and counterstained with toluidine blue for light microscopic measurements on slides. Ultrathin sections (50–70 nm) were collected on Formvar-coated slot grids of copper. They were further contrasted with 2% uranyl acetate and 0.1% lead citrate and examined in a LEO 906 E transmission electron microscope of Zeiss (Jena, Germany) equipped with a digital 1K Bio-Scan camera (Gatan, Pleasanton, USA).

aerophilic conditions at 37°C for 48 hr. Bacteria were harvested from agar plate in serum-free cell culture medium (RPMI) and added to the serum-starved NCI-N87 cells (grown on 12-well inserts) in a ratio of 50:1 multiplicity of infection (MOI), meaning 50 bacteria per cell. This MOI is in the order of magnitude used in earlier studies (Sokolova et al., 2013), and results in final bacterial numbers suitable for morphological evaluation.

Transmission Electron Microscopy (TEM)

Confluent, polar grown NCI-N87 cells on insert membranes at the age of 7 days were incubated with a sus-
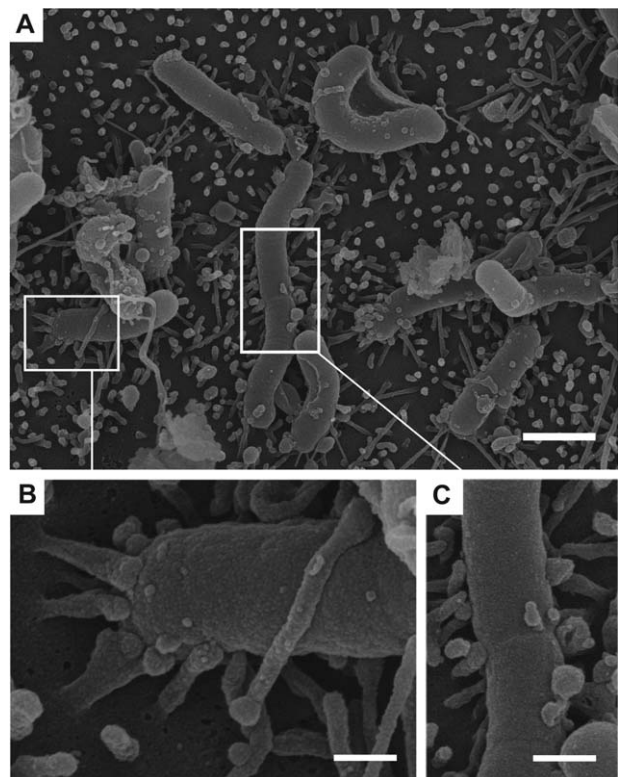

Fig. 3. Scanning electron microscopy of an epithelial monolayer of NCI-N87 cells, apical view, 1 hr after the exposition with bacteria (MOI 50:1). *H. pylori* are located on the epithelium, within this short period close attachments of microvilli/bacteria have been established. (**A**) overview—bar 1 µm, (**B**) budding of microvilli toward the bacteria cell wall—bar 200 nm, (**C**) some bacteria have few if any microvillus attachments—bar 250 nm.
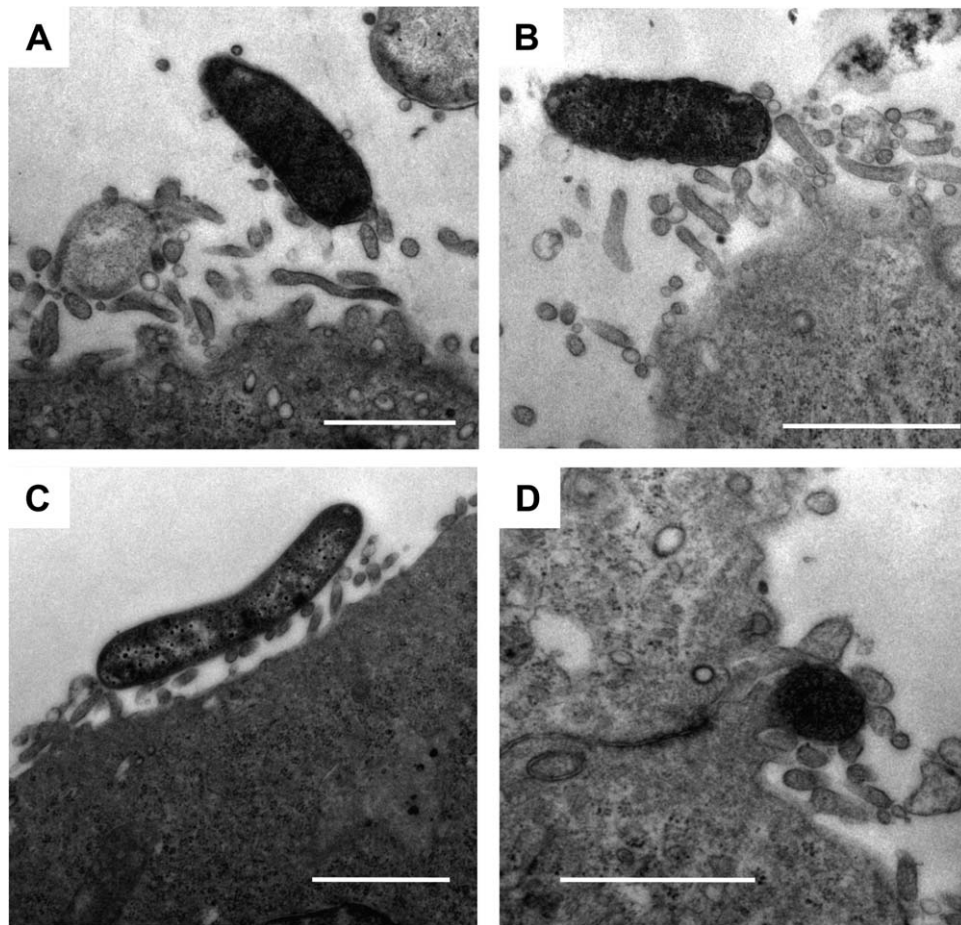
Fig. 4. Transmission electron microscopy of the same monolayer as shown in Fig. 3A–C: (**A**) Transmission electron micrographs of human gastric epithelial NCI-N87 cells exposed to *H. pylori* for 1 hr, bacteria are dark or pale contrasted–bar 1 μm. (**B**) *H. pylori* bacteria are closely associated to the microvilli of epithelial NCI-N87 cells, however, no specific cell contact structure is established–bar 1 μm. (**C**) Bacterium is located along the microvilli of one epithelial cell, compare to Fig. 3C–bar 1 μm. (**D**) Host cell microvilli of two neighboring cells surround bacteria after 1 hr of interaction–bar 1 μm.

Scanning Electron Micoscopy (SEM) of *H. pylori* in Contact WithNCI-N87 Cells

Procedure of incubation and fixation of NCI-N87 cells and bacteria was the same as described before in section TEM. Beside this, the fixed membranes were kept in 0.1 M Cacodyl-buffer until post-fixation with 1 % osmium tetroxide (Science Services, Munich, Germany) in H_2O. After three times washing in H_2O samples were dehydrated in a series of ethanol and then dried by an automated critical point dryer (Leica CDP300, Wetzlar, Germany) using liquid CO_2 substituting ethanol prior to evaporation at 31°C. SEM inspection of samples was performed by a Hitachi S-4800 SEM following Gold coating with a sputter coater (Bal-Tec, Pfäffikon, Switzerland).

RESULTS

Human gastric tumor NCI-N87 cells (1×10^4, 3×10^4, and 6×10^4) were seeded on 96-well-culture plates (culture area: per well 0.28 cm^2) and the growth of the cells until a confluent monolayer was studied for 7 days (Fig. 1A). At the time points of 24, 48, and 72 hr after seeding, the cell number was quantified. The cell number at the time point of 6 hr was used as starting cell number (N_0). Herein, only 33 % of the seeded NCI-N87 cells were adherent on the cell culture plates. The generation time (tg) was calculated in the log phase at the time point of 72 h (see section Material and Methods). Interestingly, the time for reduplication of the NCI-N87 cells was quite long and took 42 hr (Fig. 1B).

For electron microscopical studies the cells were grown on porous membrane inserts (12 well; culture area 1,12 cm^2) in a monolayer. The tightness of the monolayer was determined by measuring the transepithelial electrical resistance (TEER). The measurement was performed within a period of 21 days (Fig. 2A). The cells reached the state of complete tightness with a TEER value of 0.96 kOhm $\times$ cm^2 at around 7 days of cell culture. Therefore this time point was used for further experiments. NCI-N87 cells developed tight junction connected monolayers with a number of microvilli on the apical side after 7 days of cultivation (Fig. 2B; TEM). SEM showed at this time many microvilli of variable length on the surface of the cells, the density of microvilli per area was different (Fig. 2C).

H. pylori bacteria were added for 1 hr to the apical side of these NCI-N87 monolayers. After this short period a close interaction of the bacteria cell wall with epithelial microvilli was detected using scanning electron microscopy (Fig. 3A–C). The microvilli were in part thicker at the interacting site and some budding was observed. Comparable to non-exposed NCI-N87 monolayers (see Fig. 2C), here also a marked variation of microvillus length and density per area was observed. In addition, the host/bacteria interface was studied by transmission electron microscopy (Fig. 4). However, there were no specific ultrastructural differentiations of the microvilli, bacteria located in the area of two neighboring cells were often entrapped by several microvilli (Fig. 4A–D).

DISCUSSION

In the present study, the human gastric epithelial cell line NCI-N87 was used as polar monolayer in order to analyze the first contact of *H. pylori* at the cellular surface. This cell line has its origin from an adenocarcinome (Chailler and Menard, 2005). Based on the population doubling time of 42 hr (Fig. 1A) and using an adequate seeding density (see Fig. 1B) monolayers were obtained for the exposition with *H. pylori*. In contrast to other tumor-derived cells, NCI-N87 cells obviously have a replication time more comparable to epithelial cells (Chailler and Menard, 2005). NCI-N87 develop tight junctions and a brush border with numerous microvilli (Fig. 2B,C), B,C), a prerequisite for *H. pylori* exposition studies. Oliveira et al. have shown that structural proteins for tight-junctions such as ZO1 and E-cadherin and other apical cell membrane proteins (e.g. Ezrin) are essential for establishing a bacteria/host interaction comparable to the *in vivo* situation (Oliveira et al., 2009). After 1 hr of *H. pylori* exposition of the NCI-N87 cells bacteria were located at the tips of the microvilli, interestingly a certain budding of the microvillus tips had occured already, and neighboring cells had entrapped the bacteria.

The interaction of *H. pylori* with gastric epitheIia has been studied earlier - in cell lines, primary epithelial cells (Hemalatha et al., 1991; Corthesy-Theulaz et al., 1996; Heczko et al., 2000; Shaffer et al., 2011; Johnson et al., 2012) and human gastric biopsies (Smoot et al., 1993; Chun et al., 2002; Bimczok et al., 2010). In the human AGS tumor cell line, the interaction with bacteria was studied 4 hr after exposing the cells to *H. pylori* (Shaffer et al., 2011). However, AGS-cells develop few and very short microvilli and lack other structures of the apical cell membrane (Oliveira et al., 2009)—resulting in a different morphology in comparison to our experiments (Shaffer et al., 2011). Our TEM results are comparable to studies in human biopsies—but in patients the infection is longer lasting and time point of the initial contact is unknown (Chun et al., 2002). Thus, NCI-N87 cells represent a suitable cell model to study the pathology of *H. pylori* infection, especially as comparable morphology was observed as in human biopsies, and as they provide the apical cell environment necessary for effective colonization by *H. pylori* (Chailler and Menard, 2005; Oliveira et al., 2009).

The entrapping of *H. pylori* by microvilli of neighboring cells may reflect an important first sign for the initiation of the immune response against *H. pylori*. Recently it has been reported that gastric dendritic cells obtained from patients suffering from *H. pylori* infection induce $CD4^+$ Th1 cells (Bimczok et al., 2010). Thus, stimulation of dendritic cells by *H. pylori* may lead to the permeation of proinflammatory cytokines within the inflamed tissue in chronic *H. pylori* infections (Drakes et al., 2006). The tight junction associated protein tricellulin is expressed between neighboring cells and enables stomach dendritic cells to interact with the epithelial surface (Mariano et al., 2011). Thus, our observed entrapment of *H. pylori* by neighboring cells is possibly a prerequisite of the interaction between the bacteria and dendritic cells.

Future experiments are necessary to explain whether the early contact between microvilli and *H. pylori* is sufficient to induce NF-κB activation in gastric epithelial cells (Sokolova et al., 2013). Further, it has to be clarified whether the initial physical microvillus/*H. pylori* interaction already results in *H. pylori*-induced maturation of dendritic cells (Guiney et al., 2003; Kranzer et al., 2004) and in the recently described association of CagA with c-Met, E-cadherin, and p120-catenin that regulates the invasion of *H. pylori* (Oliveira et al., 2009).

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