Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in *Helicobacter pylori* infection

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SUMMARY

Toll-like receptors (TLRs) expressed by mucosal epithelium play an essential role in the defense against microbes by recognizing conserved bacterial molecules. For the first time TLR4, TLR5 and TLR9 have been microanatomically localized in patients with noninflamed gastric mucosa and *Helicobacter pylori* gastritis by immunohistochemistry. Because polarized expression of TLRs in apical and basolateral epithelial compartments is thought to modulate mucosal immunity, subcellular TLR distribution by gastric epithelium was investigated using confocal microscopy. TLR4, TLR5 and TLR9 were expressed by gastric epithelium in antrum and corpus of all patients with *H. pylori* gastritis (*n* = 14) and with noninflamed gastric mucosa (*n* = 5). TLR4 was expressed at the apical and the basolateral pole of the gastric epithelium as well in noninflamed gastric mucosa as in *H. pylori* gastritis. TLR5 and TLR9 expression in the noninflamed gastric mucosa was identical to that of TLR4 with localization at the apical and the basolateral epithelial pole. However, in *H. pylori* gastritis TLR5 and TLR9 expression on the gastric epithelium changed to an exclusive basolateral localization without detectable expression at the apical pole. In the human stomach, the gastric epithelium expressed TLR4, TLR5 and TLR9, which gives it the possibility to interact with *H. pylori*. Furthermore, gastric epithelial TLR4 expression is highly polarized in an apical and a basolateral compartment, whereas TLR5 and TLR9 polarization seems to be a process dynamically influenced by *H. pylori* infection. This polarized and dynamically regulated gastric epithelial expression of TLRs supports a sentinel role for these receptors in the mucosal immunity to *H. pylori*.

Keywords toll-like receptor TLR *Helicobacter pylori* gastric mucosa *Helicobacter pylori* gastritis

INTRODUCTION

Infection of the gastric mucosa by *Helicobacter pylori* is worldwide one of the most common human bacterial infections. *H. pylori* causes chronic active gastritis and gastroduodenal ulceration [1–4]. During *H. pylori* infection the bacterium colonizes the mucus layer overlying the gastric surface epithelium and causes inflammation of the underlying mucosa, which is called chronic active gastritis.

Toll-like receptors (TLRs) are recently found to play an essential role in the first line of host defense by recognition of microbial components. These receptors recognize conserved molecular patterns that are expressed by infectious agents. By this way TLRs mediate the production of proinflammatory cytokines and chemokines resulting in inflammation. Recently, the ligands for most receptors of the TLR family have been identified. TLR2 recognizes and signals bacterial lipoprotein and peptidoglycans from Gram-positive bacteria. TLR3 is considered to recognize double-stranded RNA. Lipopolysaccharides (LPS) from Gram-negative bacteria signal through TLR4. TLR5 is identified to recognize bacterial flagellin and represents therefore a pattern recognition receptor for motile pathogens. Cellular response to the CpG motive of bacterial DNA is mediated by TLR9 [5–9].

First *in vitro* data suggest that TLRs expressed on gastric epithelial cells interact with *H. pylori*. It has been demonstrated that TLR5 expressed on human gastric epithelial cell lines is required for *H. pylori* induced NF-kappaB activation and chemokine expression [10]. TLR4 expression by gastric epithelium is discussed controversely and the precise function of TLR4 is unclear [11,12]. TLR9 has not been investigated in the stomach so far.

In the gut epithelium polarized expression of TLRs in an apical and a basolateral epithelial compartment is thought to be a central principle of mucosal immunity, which discriminates between pathogenic microbes invading the epithelium and nonpathogenic microbes facing the luminal surface [13–15].

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TLR expression has not previously been analysed *in vivo* in the human stomach. Therefore, in this study TLR4, TLR5 and TLR9 were microanatomically localized in patients with noninflammed gastric mucosa and in patients with *H. pylori* infection. Furthermore, to elucidate a possible role of epithelial polarization in the human stomach during *H. pylori* infection the subcellular TLR distribution in gastric epithelial cells was investigated in detail by confocal microscopy.

MATERIALS AND METHODS

Patients and selection of gastric tissue
Surgical tissue specimens from gastric antrum and corpus mucosa of 5 patients (4 with tumour of the pancreas, 1 with oesophageal carcinoma) without *H. pylori* gastritis and of 14 patients with chronic active *H. pylori* gastritis were investigated in this study by immunohistochemistry and confocal microscopy. In the patients without *H. pylori* gastritis no inflammatory cells were detectable in the antrum and in the corpus mucosa and *H. pylori* colonization was excluded in both compartments by a modified Giemsa stain. Chronic active *H. pylori* gastritis showed a moderate to severe chronic inflammatory infiltrate of T- and B-lymphocytes, plasma cells and monocytes and a mild to severe activity with neutrophils in the lamina propria, within the gastric epithelium and in the foveolar lumen. *H. pylori* colonization was mild to severe as determined by a modified Giemsa-stain.

This study, in which the human tissue was collected from gastrectomy specimens used for routine histopathological diagnosis, has been permitted by a local ethical commission.

Immunhistochemistry
For immunohistological analyses tissue specimens were fixed in 10% formalin buffered at pH 7·0 for 24 h and paraffin-embedded. The following antibodies were used at the dilutions indicated: polyclonal rabbit anti-TLR4 (1 : 500, Zymed, San Francisco, USA), polyclonal rabbit anti-TLR5 (1 : 1000, Zymed), monoclonal mouse anti-TLR9 (1 : 200, Biocarta, Hamburg, Germany). For immunohistochemical staining tissue sections were treated with microwave. Non-specific binding sites were blocked with buffered casein solution (Power Block Universal Blocking Reagent, Bio Genex, San Ramon, USA) for 10min at RT. Sections were incubated with the first-step antibody at 4°C overnight. As second stage reagent a Biotin/Streptavidin-peroxidase detection system (Super Sensitive Multilink HRP Detection System, Bio Genex) was used according to the instruction of the manufacture with 3,3′-diaminobenzidine tetra-hydrochloride (DAB) solution as substrate. To exclude a nonspecific staining in each case a negative control was performed. For this purpose polyclonal TLR4 and TLR5 antibodies were blocked with the peptide used for antibody production (Zymed) at 37°C for 30 min. For TLR9 the first monoclonal antibody was replaced by an isotype control antibody. In both procedures no staining of the gastric tissue was detectable.

Immunofluorescence and confocal microscopy
For confocal microscopy the formalin fixed and paraffin-embedded sections were stained with the TLR4, TLR5 and TLR9 antibodies as described above, followed by incubation with Cy3 conjugated donkey-antimouse IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Nuclei and *H. pylori* were stained with DAPI (Sigma, Hannover, Germany) and pseudocoloured in blue and green, respectively. Stained tissue sections were observed in a confocal microscope (TCS SP2, Leica, Heidelberg, Germany). To separate DAPI signal from nuclei and bacteria region of interest (ROI) scanning mode was used.

As negative control polyclonal TLR4 and TLR5 antibodies were blocked with the peptide used for antibody production (Zymed, San Francisco, USA) at 37°C for 30 min. For TLR9 the first monoclonal antibody was replaced by an isotype control antibody. In both procedures no staining of the gastric tissue was detectable.

RESULTS

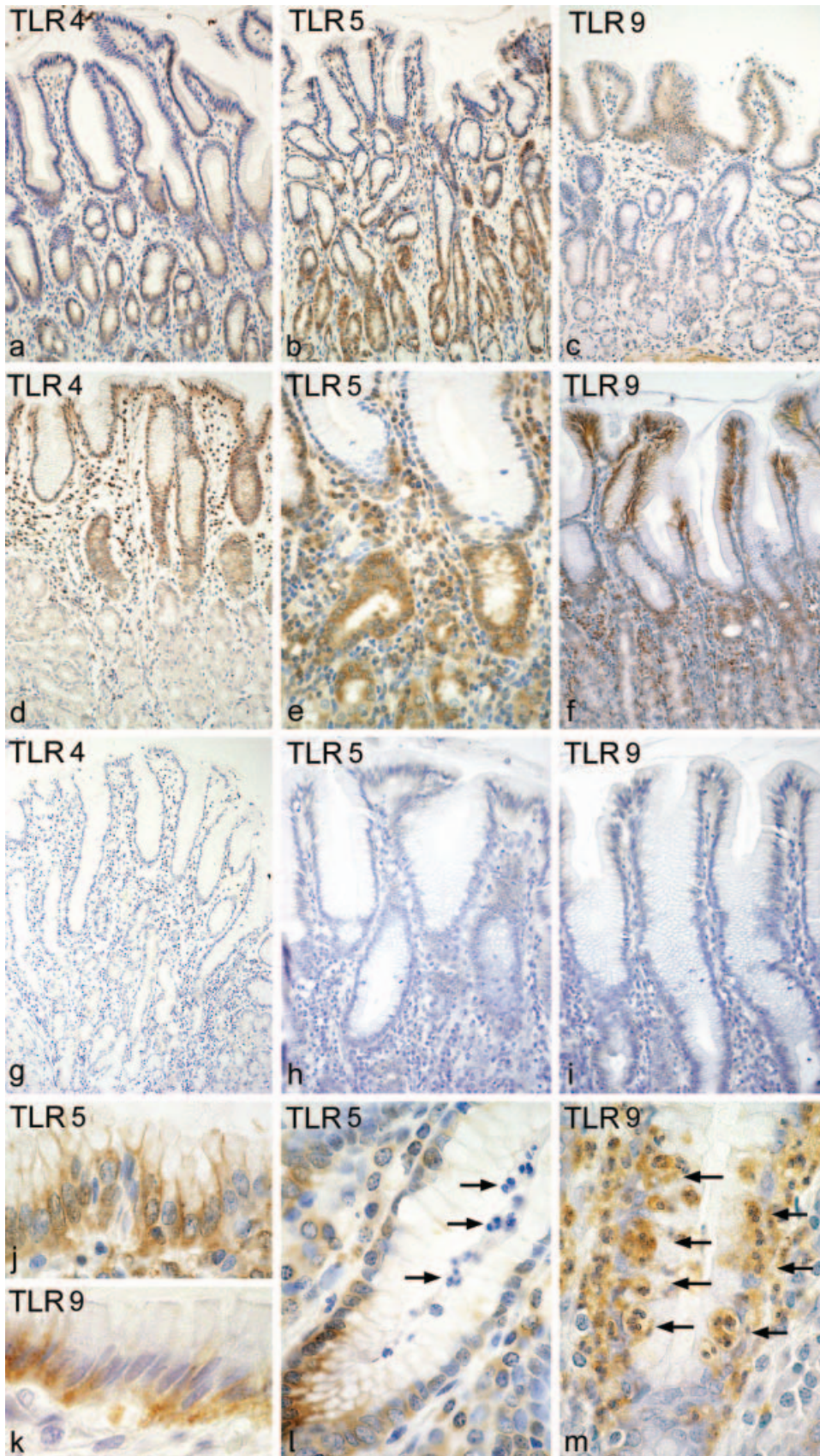*Toll-like receptor expression in noninflamed gastric mucosa and chronic active* H. pylori *gastritis*
TLR4, TLR5 and TLR9 were expressed by gastric epithelium in antrum and corpus of all patients as well in noninflamed gastric mucosa (*n* = 5; Fig. 1a–c) as in chronic active *H. pylori* gastritis (*n* = 14; Figs 1d–f,j,k). TLR4 and TLR5 expression was accentuated in the glandular neck region and extended to the luminal surface (Figs 1a,b,d,e). In contrast to TLR4 and TLR5, TLR9 expression was predominantly localized at the gastric surface epithelium with nearly no detectable expression in the glandular neck region (Figs 1c,f).

TLR4 and TLR9 expression by gastric epithelium tended to be stronger in *H. pylori* gastritis (Figs 1d,f) than in the noninflamed gastric mucosa (Figs 1a,c). TLR5, however, showed a strong expression by gastric epithelium as well in the noninflamed mucosa as in chronic active *H. pylori* gastritis (Figs 1b,e).

In chronic active *H. pylori* gastritis some neutrophils localized in the lamina propria, within the gastric epithelium and in the foveolar lumen expressed TLR4 and TLR9 (Fig. 1m). TLR5, however, was not detected on neutrophils (Fig. 1l). TLR4 and TLR5 were additionally expressed by mononuclear cells representing plasmacells and macrophages (Figs 1d,e). TLR9 expression by mononuclear cells was not detectable.

Subcellular distribution of Toll-like receptors by gastric epithelium in noninflamed gastric mucosa and chronic active *H. pylori* gastritis.

Fig. 1. Toll-like receptor expression in noninflamed gastric mucosa and chronic active H. pylori gastritis determined by immunohistochemistry Gastric epithelial cells as well in noninflamed gastric mucosa (a–c) as in chronic active *H. pylori* gastritis (d–f,j,k) express TLR4, TLR5 and TLR9. Epithelial TLR4 and TLR5 expression is accentuated in the glandular neck region (a,b,d,e). TLR9 expression was predominantly localized at the gastric surface epithelium (c,f). TLR4 and TLR9 expression by gastric epithelium tends to be stronger in *H. pylori* gastritis (d,f) than in the noninflamed gastric mucosa (a,c). TLR5 shows a strong expression by gastric epithelium as well in the noninflamed mucosa (b) as in chronic active *H. pylori* gastritis (e). Fig j and k show TLR5 and TLR9 expression by gastric epithelium in *H. pylori* gastritis in more detail. Negative controls for TLR4, TLR5 and TLR9 show no staining (g–i) TLR4 and TLR5 stain mononuclear cells in the lamina propria of *H. pylori* gastritis (d,e). Many neutrophils in chronic active *H. pylori* gastritis express TLR4 and some neutrophils express TLR9 (arrows, m). TLR5, however, is not found on neutrophils (arrows; l). Magnification: a–d, f × 20; e × 40; g–k × 100.

Confocal microscopy confirmed TLR4, TLR5 and TLR9 expression by gastric epithelium as well in noninflamed gastric mucosa (Figs 2a,c,d,g,h) as in chronic active *H. pylori* gastritis (Figs 2b,e,f,i,j) in all patients.

TLR4 was expressed at the apical and the basolateral pole of the gastric surface epithelium. This expression pattern was identical in the noninflamed gastric mucosa and in *H. pylori* gastritis (Figs 2a,b). In *H. pylori* gastritis the bacterium was directly attached to the TLR4 receptor expressed at the apical epithelial pole (Fig. 2b), which was confirmed by vertical confocal scanning sections (Fig. 2b inset). This suggests that TLR4 may not only *in vitro*, but also *in vivo* function as a cellular binding receptor for *H. pylori* [12].

On subcellular level TLR5 (Figs 2c,d) and TLR9 (Figs 2g,h) were expressed in the noninflamed gastric mucosa of all patients as well at the apical pole as at the basolateral pole of the gastric epithelium.

In contrast, in chronic active H. pylori gastritis TLR5 (Figs 2e,f) and TLR9 (Figs 2i,j) expression of the gastric epithelium was exclusively localized at the basolateral pole of the epithelial cells without detectable expression at the apical pole.

Our data demonstrate that TLR expression by gastric epithelium is highly polarized. Moreover, TLR5 and TLR9 polarization was found to differ between noninflamed gastric mucosa and *H. pylori* gastritis and seems therefore to be a dynamical process during *H. pylori* infection.

DISCUSSION

In *H. pylori* infection the bacterium colonizes the mucus layer overlying the gastric surface epithelium and causes inflammation and immune response of the underlying mucosa, which is called chronic active gastritis. There is evidence that gastric epithelial cells themselves play an active role in the innate immunity against *H. pylori* particulary by secreting chemokines. But so far the receptors, which trigger this epithelial driven inflammatory response, are not clearly defined and localized in the human stomach. Mucosal epithelium has been reported to express several TLRs, suggesting that these receptors play a role in gastrointestinal epithelial innate immune response.

In our study for the first time TLR4, TLR5 and TLR9 were localized *in vivo* in the human stomach. TLR4, TLR5 and TLR9 were expressed by gastric epithelial cells not only in chronic active *H. pylori* gastritis, but also constitutively in noninflamed gastric mucosa. TLR4 expression by gastric epithelium is discussed controversely, because TLR4 is expressed on gastric epithelial cell lines, but primary gastric epithelial cells were not found to express TLR4 [11,12]. We have identified for the first time TLR4 expression *in vivo* in the human stomach and can demonstrate a clear TLR4 protein expression of gastric epithelial cells as well in

noninflamed gastric mucosa as in chronic active gastritis. The precise function of TLR4 on gastric epithelium, however, remains unclear, as TLR4 seems not to be involved in NF-kappaB activation of the gastric epithelium [11,12]. In a very recent study in the gastric mucosa a nonconventional role of TLR4 beyond innate immunity is discussed [12] in analogy to the intestinal epithelium [16].

TLR5 expressed on gastric epithelial cell lines was found to recognize the *H. pylori* flagellin, which results in NK-kappaB activation with subsequent chemokine expression by epithelial cells [10]. In respect to these *in vitro* data, TLR5 expression by gastric epithelium *in vivo* implies that TLR5 is involved in the epithelial driven inflammatory response to *H. pylori*. Indeed, in the human stomach *H. pylori* induces gastric epithelial cells to produce cytokines and chemokines, predominantly IL-8 and Groa, which coordinate neutrophil trafficking from the mucosal vessel into the gastric epithelium to the site of infection [17].

TLR9 expression is not investigated in the human stomach so far. For the first time we could demonstrate TLR9 expression by gastric epithelium. This is in line with very recent *in vitro* data demonstrating that colonic epithelial cell lines express TLR9 mRNA. Because these cell lines respond to *E.coli* DNA via TLR9 by increased IL-8 production [18], further studies have to investigate whether *H. pylori* DNA induces a similar response of the gastric epithelial with IL-8 production and following neutrophil recruitment.

A crucial question in mucosal immunity is how to discriminate between pathogenic and nonpathogenic microbes. Now there is first evidence that compartimentalization of TLRs may play an important role in this process. In the gut the polarized expression of TLRs in an apical and and a basolateral epithelial compartment is thought to be a central principle for the discrimination between pathogenic bacteria invading the epithelium and nonpathogenic microbes facing the luminal surface [13,14,19]. In the stomach epithelium we could verify this principle of a highly polarized TLR expression for the investigated TLRs.

TLR4 was expressed at the apical and the basolateral membrane compartment of the gastric epithelium. The pattern of TLR4 compartimentalization did not change *in vivo* in the human stomach during *H. pylori* infection. Having in mind that TLR4 is a binding receptor for *H. pylori in vitro* [12] and that *H. pylori* was directly attached to apical expressed TLR4 in patients with gastritis, it seems probably that TLR4 may also *in vivo* function as a bacterial binding receptor.

In the noninflamed gastric mucosa TLR5 and TLR9 showed an identical subcellular expression by gastric epithelium as found for TLR4 with TLR expression at the apical and the basolateral epithelial pole. In this study for the first time we could demonstrate that TLR9 was not only expressed in the cytoplasma, but also on the cell surface. To exclude an unspecific cell surface

Fig. 2. Subcellular distribution of Toll-like receptors in gastric epithelium in noninflamed gastric mucosa and chronic active H. pylori gastritis determined by confocal microscopy Fluorescence microscopy shows TLR4 expression by gastric epithelium at the apical and at the basolateral epithelial pole. This expression pattern was identical in the (a) noninflamed gastric mucosa and (b) in *H. pylori* gastritis. In *H. pylori* infection the bacterium was directly attached to TLR4 expressed at the apical epithelial pole (b), which was confirmed by vertical confocal scanning sections (b inset). TLR5 (c, overview; d, detail) and TLR9 (g overview; h, detail) expression in the noninflamed gastric mucosa is identical to TLR4 with expression at the apical and at the basolateral pole of the gastric epithelium. However, in chronic active *H. pylori* gastritis gastric epithelium with directly overlying *H. pylori* expressed TLR5 (e, overview; f, detail) and TLR9 (i, overview; j, detail) exclusively at the basolateral pole of the gastric epithelial cells. No TLR5 and TLR9 expression at the apical pole was detectable, so that the apical cell borders are no more visible. TLR expression shows a red fluorescence. Nuclei and *H. pylori* are stained with DAPI and are pseudocoulored in blue and green, respectively.
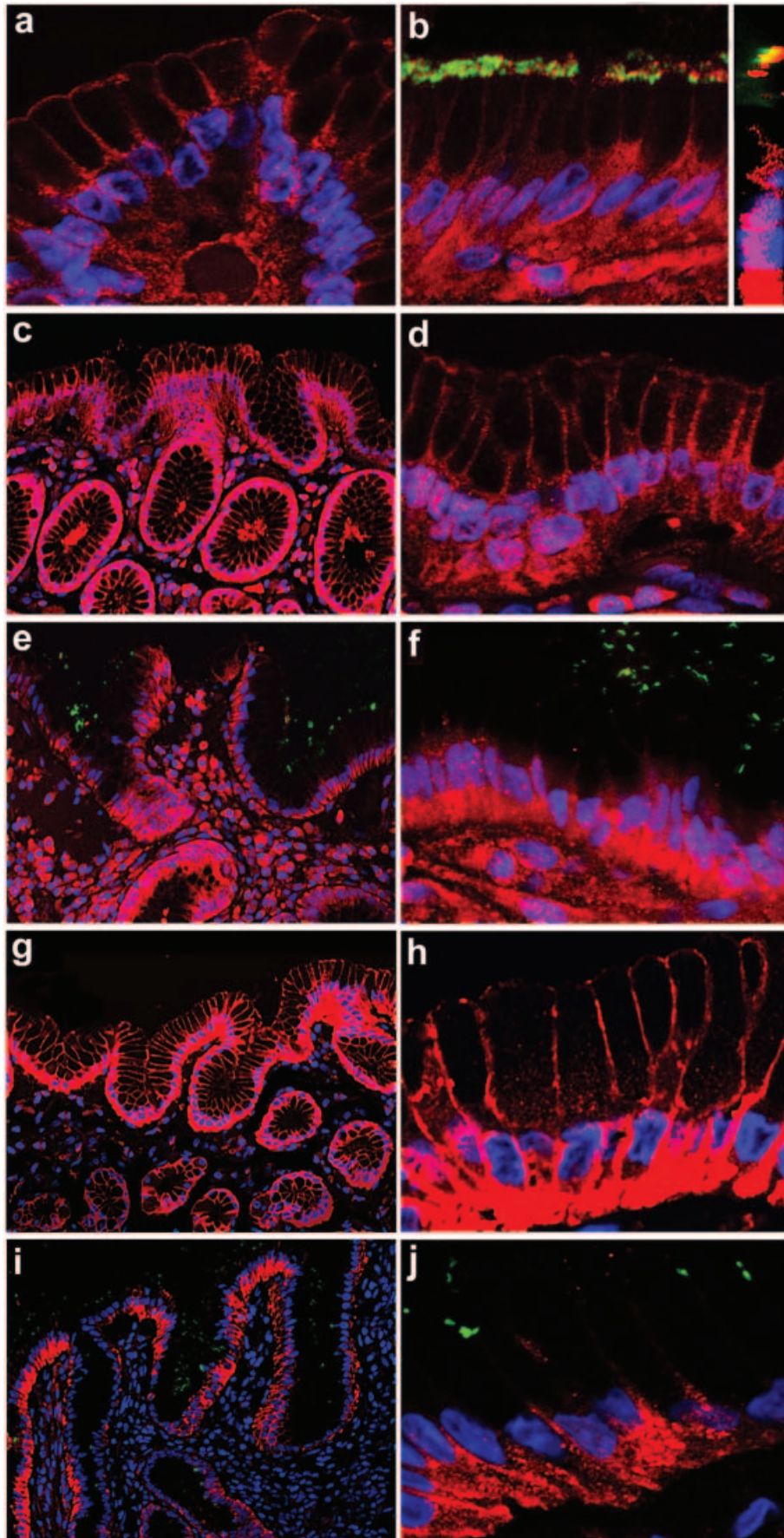
staining, we tested the human B cell line TK6 with the same TLR9 antibody. Here we found an exclusive intracellular localization of TLR9, which is in line with literature (data not shown).

However, in patients with chronic active *H. pylori* gastritis TLR5 and TLR9 expression by gastric epithelium changed to an exclusive basolateral localization.

Because in the intestinum basolaterally expressed TLR5 will only activate proinflammatory gene expression by microbes that have invaded or translocated their flagellin across the epithelium [13,14], further studies have to investigate whether translocation of *H. pylori* ligands to the basolateral pole of the gastric epithelium is necessary for mediating inflammation of the underlying gastric mucosa.

For the first time we were able to demonstrate *in vivo* for TLR5 and TLR9 that subcellular TLR distribution in an apical and a basolateral epithelial compartment is not a static, but a dynamic process, which seems to be regulated by *H. pylori* infection. A similar process of dynamic regulation of TLR polarization is described *in vitro* for intestinal epithelium, which shows a TLR4-trafficking from apical membrane to cytoplasmic compartments in response to the TLR4 ligand LPS [20]. In analogy the dynamic regulation of TLR5 and TLR9 receptor polarization may be influenced by *H. pylori* flagellin or DNA, the specific ligands for TLR5 and TLR9.

In vivo TLR expression by gastric epithelium confirm in vitro data that TLRs are involved in the innate immune response to *H. pylori*. Moreover, the principle of epithelial TLR compartimentalization seems to be not only valid in the gut, but also in the human stomach. In conclusion the polarized and dynamically regulated gastric epithelial expression of TLRs supports a sentinel role for these receptors in gastric mucosal immunity.

ACKNOWLEDGEMENTS

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