Biochemical and Biophysical Research Communications 426 (2012) 342–349

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Toll-like receptor 9 signaling has anti-inflammatory effects on the early phase of *Helicobacter pylori*-induced gastritis

Koji Otani, Tetsuya Tanigawa *, Toshio Watanabe, Yuji Nadatani, Mitsue Sogawa, Hirokazu Yamagami, Masatsugu Shiba, Kenji Watanabe, Kazunari Tominaga, Yasuhiro Fujiwara, Tetsuo Arakawa

*Department of Gastroenterology, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan*

ARTICLE INFO

*Article history:*
Received 5 August 2012
Available online 24 August 2012

*Keywords:*
Tumor necrosis factor-α
Interferon-α
Interferon-β
Interferon-γ

ABSTRACT

*Helicobacter pylori* (*H. pylori*)-induced immune responses in the gastric mucosa are skewed toward T helper (Th) 1 phenotype, which is characterized by predominant production of tumor necrosis factor (TNF)-α and interferon (IFN)-γ by helper T cells. Toll-like receptors (TLRs) play an essential role in mucosal defense against microbes through the recognition of bacterial molecules. Among the members of the TLR family, TLR9 recognizes bacterial unmethylated CpG DNA sites, and signal transduction of TLR9 induces production of a variety of cytokines, including type-I IFN (IFN-α/β). We investigated the expression and role of TLR9 in *H. pylori*-induced gastritis in mice. Expression of TLR9 mRNA in the gastric tissue increased after infection with *H. pylori*. TLR9 was mainly expressed in the macrophages, dendritic cells, and $CD3^+$ cells in the gastric mucosa. Neutrophil infiltration and the expression levels of TNF-α and IFN-γ mRNA were higher in TLR9 knockout (KO) mice than in wild-type mice at 2 and 4 months after *H. pylori* inoculation. These differences in inflammatory parameters between *H. pylori*-infected wild-type and TLR9 KO mice disappeared 6 months after *H. pylori* inoculation. Expression of interleukin-4 mRNA, typical Th2 cytokine, in the gastric tissue did not differ between *H. pylori*-infected wild-type and TLR9 KO mice. Expression level of IFN-α/β mRNA in the TLR9 KO mice was lower than that in wild-type mice by 4 months after inoculation. Administration of IFN-α reduced *H. pylori* infection-induced increase in neutrophil infiltration and the expression levels of TNF-α and IFN-γ mRNA in TLR9 KO mice. Our findings suggest that TLR9 signaling plays important roles in the suppression of *H. pylori*-induced gastritis in the early phase via downregulation of Th1-type cytokines modulated by IFN-α.



## 1. Introduction

Innate immunity in the gastrointestinal tract involves a well-coordinated mechanism for defense against microbial pathogens and maintenance of the integrity of the gastrointestinal mucosa. In response to the pathogenic bacterial products and components, different inflammatory signal transduction pathways are induced that activate innate immune responses in the gastrointestinal mucosa. Toll-like receptors (TLRs) play a pivotal role in innate immune responses against pathogenic microbes through the recognition of pathogen-associated molecular patterns [1]. TLR signaling triggers transcriptional activation of inflammatory cytokines, chemokines, and costimulatory molecules. To date, 11 TLR genes have been identified in humans and 13 in mice. Among the subsets of TLRs, TLR9 identifies unmethylated CpG-DNA sites in bacterial DNA [2]. Accumulating evidence indicates that TLR9 mediates innate immune responses by activating proinflammatory cytokines to eliminate microbial pathogens. The signaling pathways downstream of TLR9 are nuclear factor-κ B (NF-κB), mitogen-activated protein kinase (MAPK), and interferon regulatory factor (IRF)-5, which are responsible for the induction of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ [3,4]. In addition, recent studies have shown that TLR9 signaling pathway induces type-I IFN (IFN-α/β) which modulate inflammatory responses [5].

*Helicobacter pylori* (*H. pylori*) is a major cause of chronic gastritis characterized by upregulation of proinflammatory cytokines and enzymes and infiltration of inflammatory cells into the gastric mucosa [6–8]. Generally, the regulation of immune responses is conventionally explained by the balance of T helper (Th) 1 and Th2

*Abbreviations:* TLRs, toll-like receptors; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; IRF, interferon regulatory factor; TNF, tumor necrosis factor; IFN, interferon; Th, T helper; IL, interleukin; KO, knockout; SS, Sydney strain; CFUs, colony-forming units; PBS, phosphate-buffered saline; MPO, myeloperoxidase; BCA, bicinchoninic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; DCs, dendritic cells; PECAM, platelet/endothelial cell adhesion molecule; SEM, standard error of mean; CpG-ODN, CpG-oligodeoxynucleotide; DSS, dextran sulfate sodium; MyD88, myeloid differentiation factor 88; TRAF, TNF receptor-associated factor; NEMO, NF-κB essential modulator; TANK, TRAF family member-associated NF-κB activator.

* Corresponding author. Fax: +81 6 6645 3813.
*E-mail address:* ttanigawa@med.osaka-cu.ac.jp (T. Tanigawa).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.080

cells; Th1 cells secrete TNF-α and IFN-γ whereas Th2 cells secrete interleukin (IL)-4 [9]. *H. pylori*-induced gastritis is assumed to be a disease mediated by immunologically competent cells of Th1 phenotype through the secretion of TNF-α and IFN-γ [10,11].

It is reported that TLR9 expression tended to be stronger in the gastric epithelium in *H. pylori*-induced gastritis than in the noninflamed gastric mucosa [12]. This result indicates that TLR9 signaling plays some role in the immune response of gastric mucosa to *H. pylori*. However, the mechanism of TLR9 signaling involved in the immune response to *H. pylori* in the stomach remains to be elucidated. Thus, we examined the expression and role of TLR9 in *H. pylori*-induced gastritis in mice.

## 2. Materials and methods

### 2.1. Animals

Specific pathogen-free C57BL/6J mice (4 weeks old; weight, 10–15 g) were obtained from Charles River Japan Inc. (Atsugi, Japan). TLR9 knockout (KO) mice, which were backcrossed eight times on the C57BL/6J background, originally generated by Dr. S. Akira (Osaka University, Osaka, Japan), were obtained from Oriental Bioservice Inc. (Kyoto, Japan).

For animal experiments, all mice were housed in polycarbonate cages with paper-chip bedding in an air-conditioned biohazard room with a 12-h light/12-h dark cycle. All animals had free access to food and water. All experimental procedures were approved by the Animal Care Committee of Osaka City University Graduate School of Medicine.

### 2.2. H. pylori preparation and inoculation of mice

We used the Sydney strain (SS)-1 of *H. pylori*, which readily colonizes the stomach and induces gastritis in C57BL/6J mice [13]. *H. pylori* broth culture was prepared as previously described [7]. Mice were orogastrically inoculated with 0.3 mL of the broth culture of *H. pylori* as $6.0 \times 10^7$ colony-forming units (CFUs)/animal using a feeding needle after being fasted for 18 h on three successive occasions within a 7-day period. Animals of the uninfected groups were administered broth medium alone.

### 2.3. Experimental design

Wild-type and TLR9 KO mice were inoculated with *H. pylori* (SS-1 strain). After 2, 4, and 6 months of inoculation with *H. pylori*, the mice were sacrificed. In another experiment, wild-type and TLR9 KO mice were intraperitoneally injected with recombinant mouse IFN-α ($1.5 \times 10^4$ units/day; Pestka Biomedical Laboratories, Inc., Piscataway, NJ) once a day for 3 days after 4 months of inoculation with *H. pylori*; then, the mice were sacrificed and the stomach was obtained. The stomach was incised along the greater curvature and rinsed gently in phosphate-buffered saline (PBS), and the forestomach was removed and discarded. The glandular stomach was longitudinally incised into four fragments. The first fragment was placed in a culture medium tube for transportation; the second was subjected to measurement of myeloperoxidase (MPO) activity, a marker of infiltration of neutrophils into the gastric mucosa; the third was placed in 0.5 mL RNAlater (Life Technologies Corp., Carlsbad, CA) for RNA extraction; and the fourth was fixed in periodate-lysine-paraformaldehyde for histological and immunohistochemical examination.

### 2.4. Determination of H. pylori colonization

Specimens were weighed and homogenized with 0.3 mL PBS (pH 7.6) and further diluted, and 0.1 mL aliquots were inoculated onto *Helicobacter*-selective agar plates (Plate *Helicobacter* Agar; Nissui Pharmaceutical, Tokyo, Japan) and incubated at 37 °C for 7 days under microaerophilic conditions. The number of colonies was counted, and viable *H. pylori* was expressed as log CFUs per gram of tissue.

### 2.5. Measurement of myeloperoxidase (MPO) activity

MPO activity of gastric tissue, a marker of neutrophil infiltration [14], was assayed by the method of Bradley et al. [15]. Briefly, the specimens were homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Wako Pure Chemical Industries, Osaka, Japan). Each suspension was then centrifuged, and MPO in the resulting supernatant was assayed using a spectrophotometer (Beckman Instruments, Fullerton, CA). One unit of MPO activity was defined as the amount required to degrade 1 μmol of peroxide per min at 25 °C. MPO activities were expressed as units per gram of tissue.

### 2.6. Real-time quantitative reverse transcriptase-polymerase chain reaction analysis

Total RNA was isolated from the gastric tissue using an Isogen Kit (Nippon Gene Co., Ltd., Tokyo, Japan). The RNA was resuspended in RNase-free Tris–HCl EDTA, buffer and the concentration was measured by absorbance at a wavelength of 260 nm. Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were performed as previously described [7]. The probes used for analysis were as follows: TLR9 (Mm00446193_m1), IFN-α (Mm03030145_gH), IFN-β (Mm00439552_s1), IFN-γ (Mm01168134_m1), TNF-α (Mm00443258_m1), and IL-4 (Mm00445260_m1). The mRNA levels of these cytokines in the gastric tissue were standardized to glyceraldehyde-3-phosphate dehydrogenase mRNA and were expressed as ratios to the mean value for normal gastric tissue.

### 2.7. Histological and immunohistochemical analysis

Tissue samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek Japan, Tokyo, Japan). Serially-cut 5-μm-thick cryostat sections were mounted on silanized slide (Dako, Tokyo, Japan). Hematoxylin and eosin staining was performed for morphological observations. Immunohistochemical staining was performed using ImmunoCruz Staining System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Endogenous peroxidase was inactivated by immersing the specimens in 0.3% hydrogen peroxide ($H_2O_2$), sodium azide, and isotonic PBS for 10 min. Specimens were incubated in serum block solution for 30 min. Rabbit polyclonal anti-mouse TLR9/CD289 (IMGENEX Corp., San Diego, CA) was diluted in serum block solution and the specimens were incubated overnight at 4 °C. After being washed in PBS, the specimens were incubated with biotinylated secondary antibody for 30 min. After washing in PBS, specimens were incubated with horseradish peroxidase–streptavidin complex for 30 min. Finally, the specimens were treated with 0.03% 3,3′-diaminobenzidine-4HCl (Wako Pure Chemical Industries) for visualization of immunoreactivity and counterstained with methyl green (Dako).

### 2.8. Immunofluorescence histochemistry

To evaluate the colocalization of TLR9 with macrophages, dendritic cells (DCs), $CD3^+$ cells, and endothelial cells, double labeling by immunofluorescence was performed using confocal laser scanning microscopy. Serial 5-μm-thick cryostat sections were mounted on silanized slides (Dako). Specimens were incubated in protein block serum-free solution (Dako) for 30 min. Rabbit polyclonal