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 6.0×10^7 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 (1.5×10^4 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

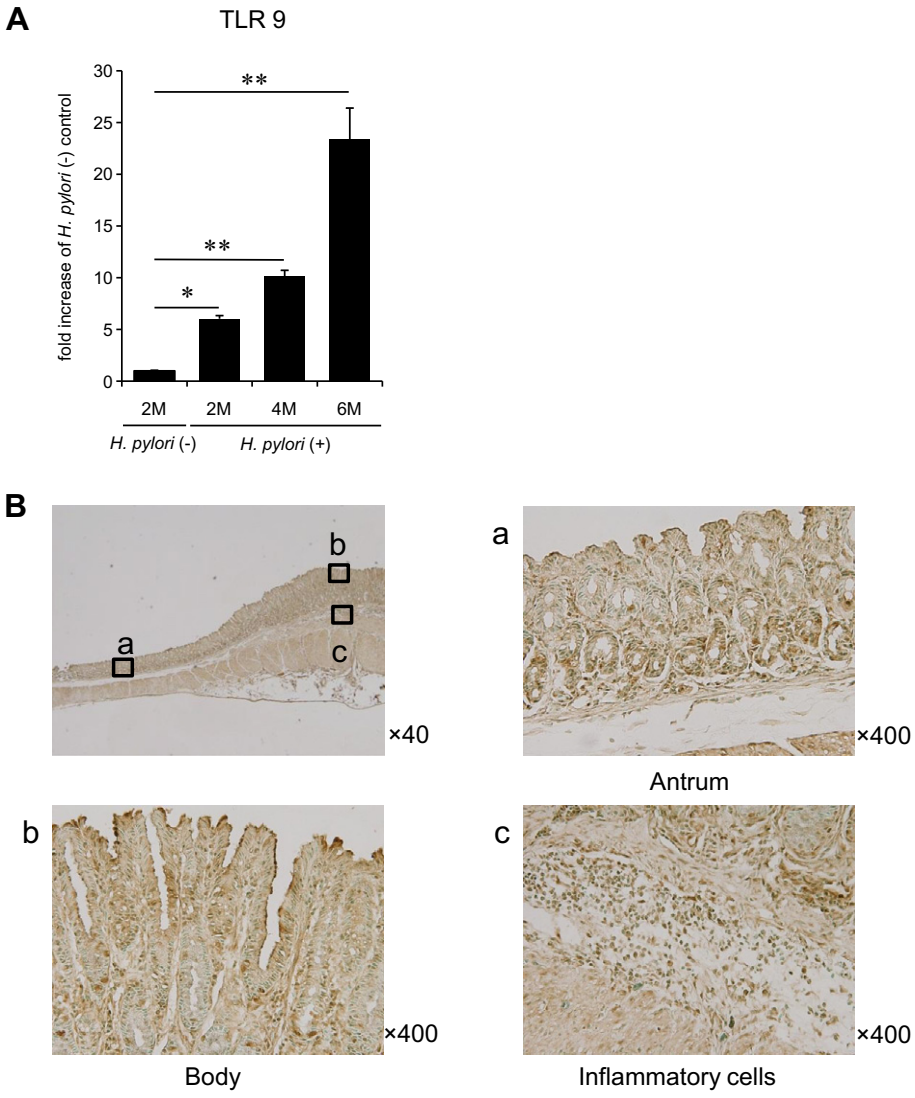

Fig. 1. Time-dependent changes and immunohistochemical analysis of the expression of toll-like receptor (TLR) 9 in *Helicobacter pylori (H. pylori)*-induced gastritis. (A) Expression of TLR9 mRNA after *H. pylori* infection. Expression level of TLR9 mRNA was determined by real-time quantitative reverse transcriptase-polymerase chain reaction. mRNA levels are expressed as ratios to the mean value for *H. pylori*-uninfected mice. Each column represents the mean ± standard error of mean. $n = 6$. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. *H. pylori*-uninfected mice. (B) Immunohistochemical staining for TLR9 in *H. pylori*-induced gastritis. (a) Gastric epithelial cells in the antrum. (b) Gastric epithelial cells in the body. (c) Inflammatory cells infiltrating to the lamina propria and submucosa. The abbreviations 2M, 4M, and 6M represent 2 months, 4 months, and 6 months, respectively, after *H. pylori* infection.

anti-mouse TLR9/CD289 antibody (IMGENEX Corp.); rat monoclonal anti-mouse F4/80 (a marker of macrophages) antibody (Serotec Ltd., Oxford, UK); rat monoclonal anti-mouse DCs antibody (BD Biosciences, San Jose, CA); rat monoclonal anti-mouse CD3 antibody (R&D Systems, Inc., Minneapolis, MN); and rat monoclonal anti-mouse platelet/endothelial cell adhesion molecule (PECAM)-1 antibody (Santa Cruz Biotechnology, Inc.) were diluted in antibody diluent using background reducing components solution (Dako) and specimens were incubated overnight at 4 °C. The specimens were washed in PBS and then the primary antibodies were reacted with donkey anti-rabbit IgG labeled with Alexa Fluor 488 (Life Technologies Corp.) or donkey anti-rat IgG labeled with Alexa Fluor 594 (Life Technologies Corp.). The specimens were examined using a confocal microscope equipped with argon and argon–krypton laser sources.

2.9. Statistical analysis

Values are expressed as means ± standard error of mean (SEM). One-way analysis of variance was used to test for significance of differences among treatment group means, and results were analyzed by Fisher's protected least significant difference test. Differences with p values less than 0.05 were considered significant.

3. Results

3.1. Time-dependent changes and immunohistochemical analysis of the expression of TLR9 in H. pylori-induced gastritis

The expression level of TLR9 increased significantly in a time-dependent manner by 6.0-, 10.1-, and 23.4-fold at 2, 4, and 6 months, respectively, after inoculation with *H. pylori* compared to that in *H. pylori*-uninfected mice (Fig. 1A).

The gastric epithelial cells in both the antrum (a) and the body (b) showed immunoreactivity for TLR9, and the immunoreactivity was also observed in inflammatory cells infiltrating to the lamina propria and submucosa (c) (Fig. 1B).

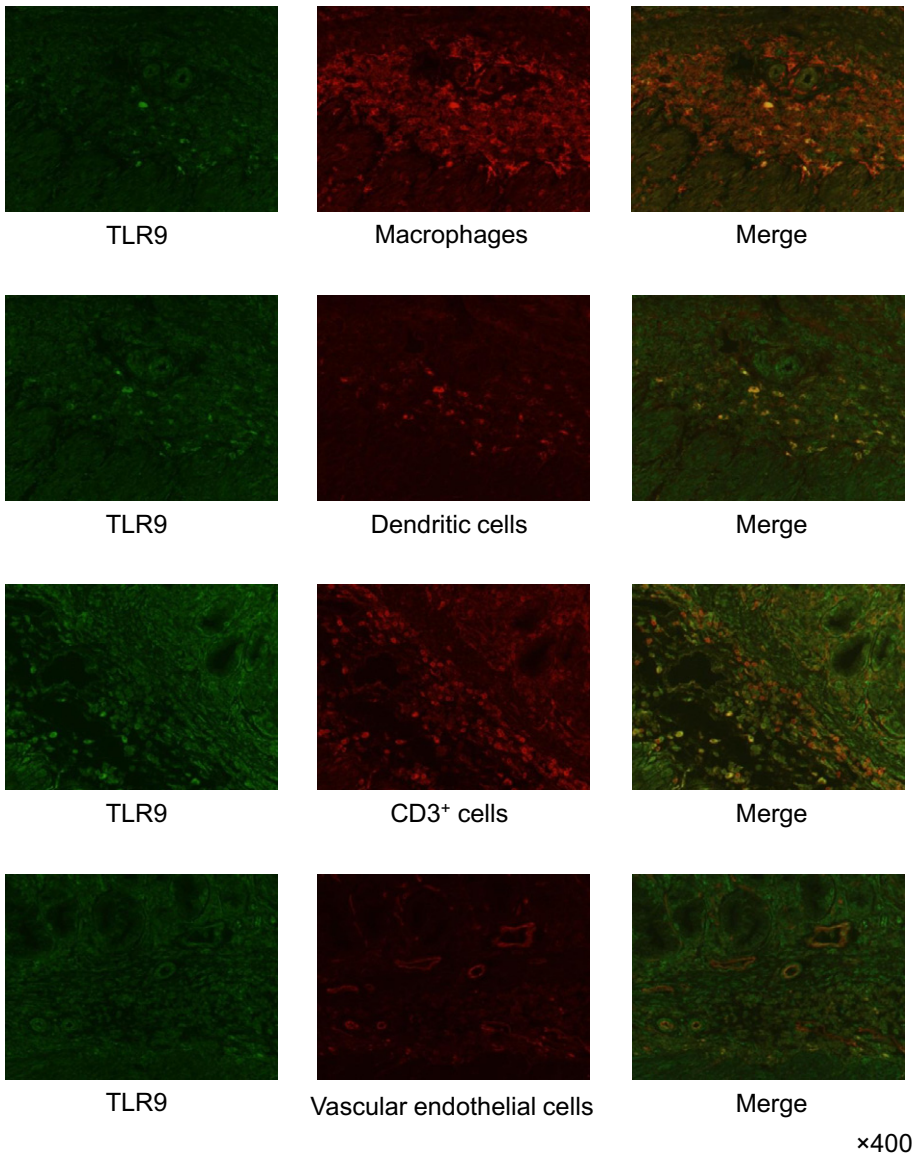

Fig. 2. Double-immunofluorescence examination for toll-like receptor 9 in macrophages, dendritic cells, $CD3^+$ cells, and vascular endothelial cells in the gastric mucosa infected with *Helicobacter pylori* (original magnification: 400×).

3.2. Double-immunofluorescence examination for TLR9 in macrophages, DCs, $CD3^+$ cells, and vascular endothelial cells

Double-immunofluorescence examination revealed that inflammatory cells expressing TLR9 proteins infiltrating to the lamina propria and submucosa included macrophages, DCs, and $CD3^+$ cells. In addition, TLR9 proteins were expressed on vascular endothelial cells (Fig. 2).

3.3. Effects of genetic disruption of TLR9 on H. pylori-induced gastritis

All mice inoculated with *H. pylori* were successfully infected as confirmed by bacterial culture. About 10^5 viable *H. pylori* per gram of tissue were present in the stomach of mice. The wild-type mice and TLR9 KO mice showed similar mean levels of viable *H. pylori* 2, 4, and 6 months after inoculation with *H. pylori* (Fig. 3A).

There was no significant difference in MPO activities, expression level of TNF-α and IFN-γ in the gastric tissue between wild-type and TLR9 KO mice without *H. pylori* infection.

MPO activities in the gastric tissue of TLR9 KO mice increased by 2.7- and 2.5-fold, compared to that in *H. pylori*-infected wild-type mice at 2 and 4 months after inoculation with *H. pylori*, respectively. MPO activities in the gastric tissue of wild-type and TLR9 KO mice were similar at 6 months after inoculation with *H. pylori* (Fig. 3B).

Infection with *H. pylori* increased the expression levels of TNF-α and IFN-γ (Th1-type cytokines) mRNA in the gastric tissue. After 2 months of inoculation, mRNA levels of TNF-α and IFN-γ in the gastric tissue of TLR9 KO mice increased by 5.3- and 6.7-fold, respectively, compared to those in *H. pylori*-infected wild-type mice. In addition, the expression levels of TNF-α and IFN-γ mRNA in the gastric tissue of TLR9 KO mice increased by 2.3- and 3.5-fold, respectively, compared to those in *H. pylori*-infected wild-type mice at 4 months after inoculation with *H. pylori*. These inflammatory parameters were similar in the wild-type and TLR9 KO mice at 6 months after inoculation with *H. pylori* (Fig. 3C and D).

The expression level of IL-4 (Th2-type cytokine) did not differ between the wild-type and TLR9 KO mice with or without *H. pylori* infection throughout the experiment (Fig. 3E).

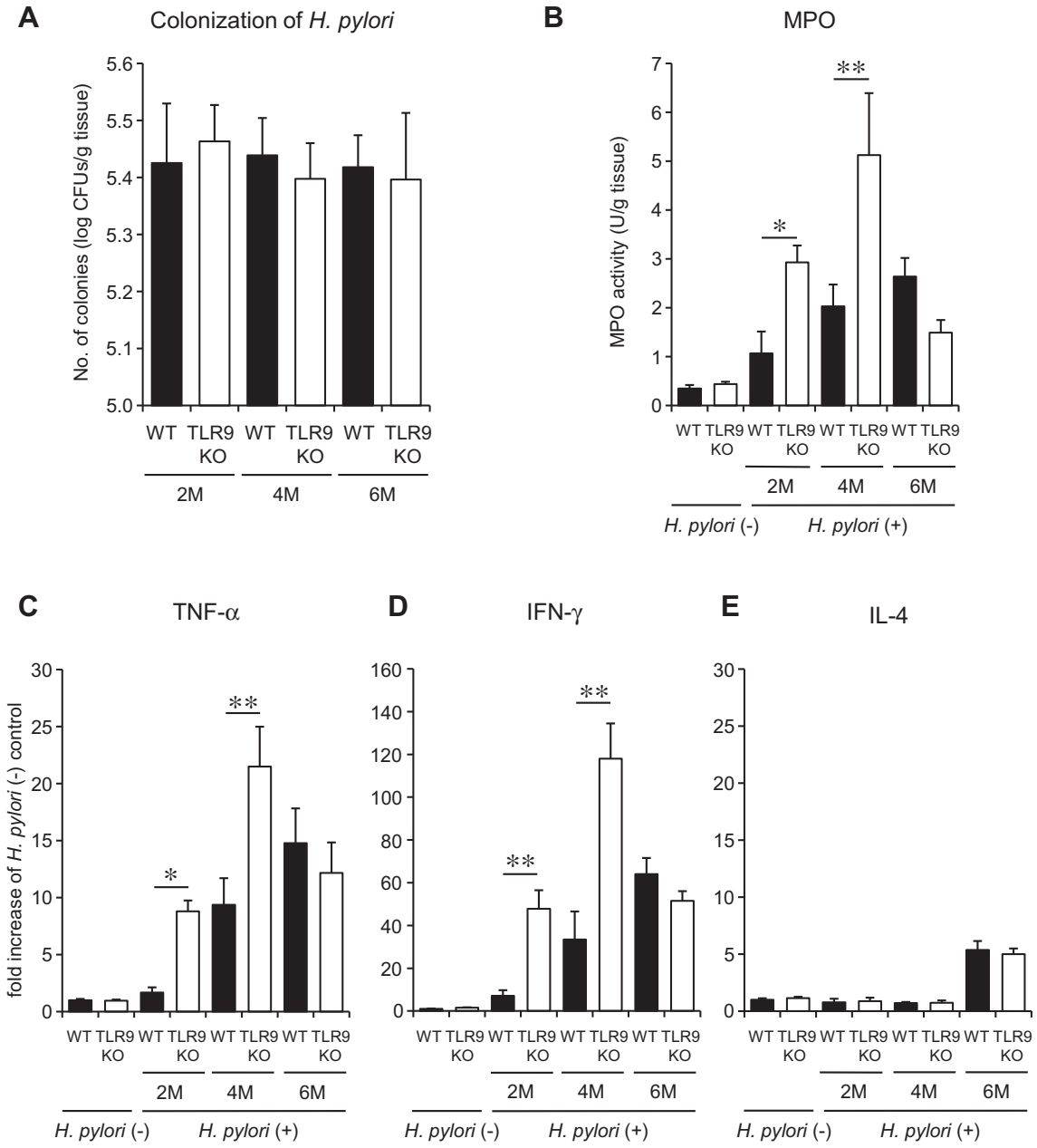

Fig. 3. Effects of genetic disruption of toll-like receptor (TLR) 9 on *Helicobacter pylori* (*H. pylori*)-induced gastritis. (A) Colonization of *H. pylori* in the stomach. Each column represents the mean ± standard error of mean (SEM). $n = 5$ or 6. Results are expressed as log colony-forming units per gram of tissue. (B) Myeloperoxidase (MPO) activities. Each column represents the mean ± SEM. $n = 5$ or 6. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. *H. pylori*-infected wild-type mice. Results are expressed as units per gram of tissue. (C–E) Expression of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-4 mRNAs. Expression levels of TNF-α, IFN-γ, and IL-4 mRNA were determined by real-time quantitative reverse transcriptase-polymerase chain reaction. mRNA levels are expressed as ratios to the mean value for *H. pylori*-uninfected wild-type mice. Each column represents the mean ± SEM. $n = 5$ or 6. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. *H. pylori*-infected wild-type mice. WT, wild-type; 2M, 4M, and 6M, 2 months, 4 months, and 6 months after *H. pylori* infection.

3.4. Effects of genetic disruption of TLR9 on the expression of type-I IFN and administration of IFN-α on H. pylori-induced gastritis

There was no significant difference in expression level of IFN-α and IFN-β in the gastric tissue between wild-type and TLR9 KO mice without *H. pylori* infection.

The expression levels of IFN-α and IFN-β mRNA in TLR9 KO mice tended to decrease by 0.53-fold ($p = 0.18$ vs. *H. pylori*-infected wild-type mice) and 0.42-fold ($p = 0.17$ vs. *H. pylori*-infected wild-type mice), respectively, 2 months after inoculation with *H. pylori* and by 0.63-fold ($p = 0.10$ vs. *H. pylori*-infected wild-type mice) and 0.75-fold ($p = 0.21$ vs. *H. pylori*-infected wild-type mice), respectively, 4 months after inoculation compared to those in *H. pylori*-infected wild-type mice. These parameters were similar in wild-type and TLR9 KO mice after 6 months (Fig. 4A and B).

Administration of recombinant IFN-α tended to decrease MPO activity by 0.22-fold ($p = 0.066$ vs. *H. pylori*-infected wild-type mice), but did not reduce the expression levels of TNF-α and IFN-γ mRNA in *H. pylori*-infected wild-type mice. On the other hand, administration of recombinant IFN-α significantly reduced MPO activity by 0.15-fold, and the expression levels of TNF-α and IFN-γ mRNA by 0.49- and 0.53-fold, respectively, in *H. pylori*-infected TLR9 KO mice (Fig. 4C–E).

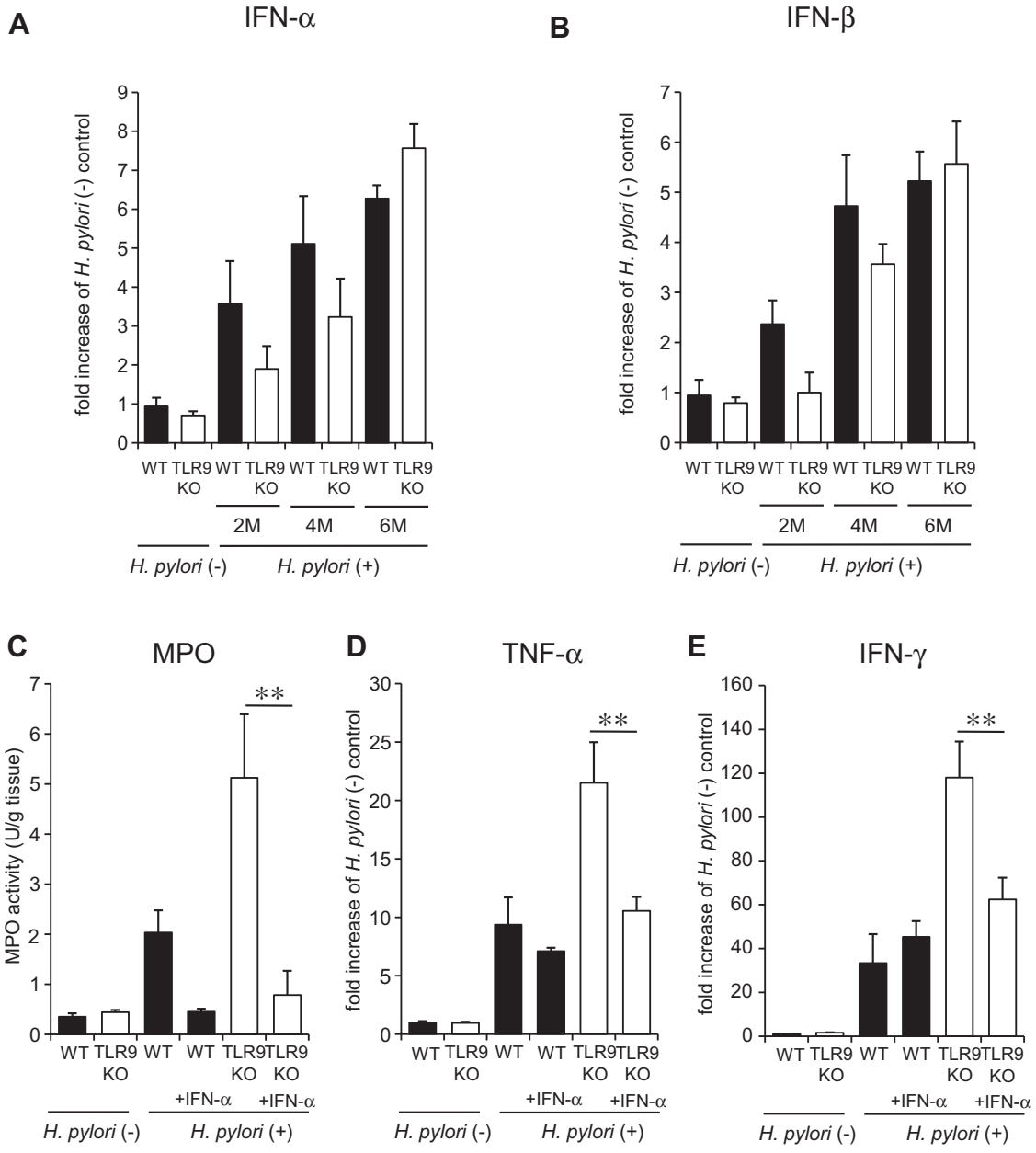

Fig. 4. Effects of genetic disruption of toll-like receptor (TLR) 9 and administration of interferon (IFN)-α on *Helicobacter pylori (H. pylori)*-induced gastritis. (A, B) Expression of IFN-α and IFN-β mRNAs. Expression levels of IFN-α and IFN-β mRNA were determined by real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). mRNA levels are expressed as ratios to the mean value for *H. pylori*-uninfected wild-type mice. Each column represents the mean ± standard error of mean (SEM). $n = 5$ or 6. (C) Effects of administration of IFN-α on myeloperoxidase (MPO) activities. Each column represents the mean ± SEM. $n = 5$ or 6. $^{**}p < 0.01$ vs. *H. pylori*-infected TLR9KO mice. Results are expressed as units per gram of tissue. (D, E) Effects of administration of IFN-α on the expression of tumor necrosis factor (TNF)-α and IFN-γ mRNAs. Expression levels of TNF-α and IFN-γ mRNA were determined by real-time quantitative RT-PCR. mRNA levels are expressed as ratios to the mean value for *H. pylori*-uninfected wild-type mice. Each column represents the mean ± SEM. $n = 5$ or 6. $^{**}p < 0.01$ vs. *H. pylori*-infected TLR9 KO mice. WT, wild-type; 2M, 4M, and 6M, 2 months, 4 months, and 6 months after *H. pylori* infection.

4. Discussion

In the present study, we showed that TLR9 was overexpressed in the gastric mucosa by *H. pylori* infection. In contrast to our initial hypothesis that TLR9 contributes to enhancing the proinflammatory response in *H. pylori*-induced gastritis, genetic disruption of TLR9 resulted in an increase in *H. pylori*-induced gastric mucosal inflammation characterized by neutrophil infiltration and upregulation of TNF-α and IFN-γ in the early phase of *H. pylori* infection. *H. pylori* infection-induced increase in the expression levels of IFN-α and IFN-β was lower in TLR9 KO mice compared to that in wild-type mice, and administration of IFN-α attenuated neutrophil infiltration and the overexpression of TNF-α and IFN-γ in TLR9 KO mice, which can account for the significance of TLR9 signaling pathway as having IFN-α-mediated anti-inflammatory properties in *H. pylori*-induced gastritis.

We found that the gastric tissue of TLR9 KO mice had higher neutrophil infiltration and Th1 cytokine levels than the gastric tissue of *H. pylori*-infected wild-type mice and thus showed that TLR9 has anti-inflammatory actions on *H. pylori*-induced gastritis. A majority of evidence suggests that the main role of TLR9 signaling in innate immunity is exerting proinflammatory action through

Th1 responses [3,4]. However, the anti-inflammatory action of TLR9 has recently been implicated in several types of inflammatory diseases. Rachmilewitz et al. showed anti-inflammatory effects of CpG-oligodeoxynucleotide (CpG-ODN), a synthetic analog of TLR9 containing CpG motifs, on dextran sulfate sodium (DSS)-induced colitis [16]. In addition, they showed that treatment with irradiated probiotic bacteria containing unmethylated CpG-DNA prevented DSS-induced colitis [17]. This preventive effect of irradiated probiotic bacteria was abolished in TLR9 KO mice, which suggested that the anti-inflammatory effect of irradiated probiotic bacteria is dependent on TLR9 in DSS-induced colitis. Anti-inflammatory effect of TLR9 signaling is also reported in different experimental animal models of inflammatory diseases such as pneumonia induced by *Streptococcus pneumoniae* [18]. The results of these previous studies and our study indicate that the role of TLR9 signaling in inflammation may vary in a disease-specific manner.

TLR9 has the characteristic ability of inducing type-I IFN production. Activation of multiple signaling pathways, including myeloid differentiation factor (MyD88)-IRF7 [19,20], MyD88-IRF1 [21], and MyD88-TNF receptor-associated factor (TRAF) 6-NF-κB essential modulator (NEMO)-TRAF family member-associated NF-κB activator (TANK)-IRF3 [22–24] was involved in TLR9-triggered production of type-I IFN. Type-I IFN was initially described as a potent antiviral protein, but recent studies showed that type-I IFN is a multifunctional immunomodulatory cytokine with profound effects on the signal transduction cascade, including regulation of Th1 response [5]. Katakura et al. reported that CpG-ODN prevented the severity of DSS-induced colitis in both wild-type mice and RAG1 KO mice lacking T cells and B cells, but it did not affect the severity of colitis in SCID mice lacking type-I IFN [25]. These results indicate that induction of type-I IFN is responsible for the protective effect of CpG-ODN via TLR9 in an experimental colitis model [26]. Consistent with these results, our results suggest that anti-inflammatory action of TLR9 is elicited by the inhibitory effect of IFN-α on transcription of Th1 response, because administration of IFN-α significantly decreased neutrophil infiltration and the expression levels of TNF-α and IFN-γ mRNA in *H. pylori*-infected TLR9 KO mice 4 months after inoculation with *H. pylori*. The reason that administration of IFN-α did not decrease TNF-α and IFN-γ levels in *H. pylori*-infected wild-type mice in this study might be that IFN-α was already present abundantly in wild-type mice, and the level of IFN-α reached the quantity that was adequate to exert suppressive effects on TNF-α and IFN-γ expression. However, the possibility also remains that some unknown molecules except IFN-α act as anti-inflammatory suppressors in the settings of the present study.

We demonstrated that TLR9 signaling exerted anti-inflammatory effects on the early phase of *H. pylori*-infection through the reduction of Th1 response. However, why genetic disruption of TLR9 did not induce significant increase in neutrophil infiltration and TNF-α and IFN-γ mRNA levels 6 months after inoculation with *H. pylori* remains unknown. The mean levels of viable *H. pylori* in wild-type mice were similar to those in TLR9 KO mice at 2, 4, and 6 months after inoculation with *H. pylori*; therefore, the possibility that deficiency of TLR9 affected the viability of *H. pylori* was considered to be low. *H. pylori*-induced gastritis is recognized as long-term progressive disease, and it develops in a multistep process from superficial gastritis, atrophic gastritis to intestinal metaplasia. After the early phase of *H. pylori* infection, the anti-inflammatory effects of TLR9 via type-I IFN might be interfered by a variety of other proinflammatory responses, resulting in reduced effect of TLR9 via type-I IFN.

In conclusion, TLR9 signaling plays important roles in inhibition of *H. pylori*-induced gastritis in the early phase through the suppression of Th1 differentiation via induction of IFN-α.

Financial support

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology in Japan.

Acknowledgment

We thank Ms. Emi Suzuki-Yoshioka for the technical assistance.

References

[1] S. Akira, H. Hemmi, Recognition of pathogen-associated molecular patterns by TLR family, Immunol. Lett. 85 (2003) 85–95.
[2] H. Hemmi, O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, S. Akira, A Toll-like receptor recognizes bacterial DNA, Nature 408 (2000) 740–745.
[3] B. Beutler, Innate immunity: an overview, Mol. Immunol. 40 (2004) 845–859.
[4] K. Takeda, T. Kaisho, S. Akira, Toll-like receptors, Annu. Rev. Immunol. 21 (2003) 335–376.
[5] C.A. Biron, Interferons alpha and beta as immune regulators – a new look, Immunity 14 (2001) 661–664.
[6] T. Tanigawa, T. Watanabe, M. Hamaguchi, E. Sasaki, K. Tominaga, Y. Fujiwara, N. Oshitani, T. Matsumoto, K. Higuchi, T. Arakawa, Anti-inflammatory effect of two isoforms of COX in *H. pylori*-induced gastritis in mice: possible involvement of PGE2, Am. J. Physiol. Gastrointest. Liver Physiol. 286 (2004) G148–G156.
[7] K. Otani, T. Watanabe, T. Tanigawa, H. Okazaki, H. Yamagami, K. Watanabe, K. Tominaga, Y. Fujiwara, N. Oshitani, T. Arakawa, Anti-inflammatory effects of IL-17A on *Helicobacter pylori*-induced gastritis, Biochem. Biophys. Res. Commun. 382 (2009) 252–258.
[8] M. Yamato, T. Watanabe, K. Higuchi, K. Taira, T. Tanigawa, M. Shiba, K. Tominaga, Y. Fujiwara, N. Oshitani, K. Takeuchi, T. Arakawa, Anti-inflammatory effects of pravastatin on *Helicobacter pylori*-induced gastritis in mice, Dig. Dis. Sci. 52 (2007) 2833–2839.
[9] T.R. Mosmann, R.L. Coffman, TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties, Annu. Rev. Immunol. 7 (1989) 145–173.
[10] L.E. Smythies, K.B. Waites, J.R. Lindsey, P.R. Harris, P. Ghiara, P.D. Smith, *Helicobacter pylori*-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-gamma, gene-deficient mice, J. Immunol. 165 (2000) 1022–1029.
[11] K.A. Eaton, M. Mefford, T. Thevenot, The role of T cell subsets and cytokines in the pathogenesis of *Helicobacter pylori* gastritis in mice, J. Immunol. 166 (2001) 7456–7461.
[12] B. Schmausser, M. Andrulis, S. Endrich, S.K. Lee, C. Josenhans, H.K. Muller-Hermelink, M. Eck, Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in *Helicobacter pylori* infection, Clin. Exp. Immunol. 136 (2004) 521–526.
[13] A. Lee, J. O'Rourke, M.C. De Ungria, B. Robertson, G. Daskalopoulos, M.F. Dixon, A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain, Gastroenterology 112 (1997) 1386–1397.
[14] J.E. Krawisz, P. Sharon, W.F. Stenson, Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models, Gastroenterology 87 (1984) 1344–1350.
[15] P.P. Bradley, D.A. Priebat, R.D. Christensen, G. Rothstein, Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker, J. Invest. Dermatol. 78 (1982) 206–209.
[16] D. Rachmilewitz, F. Karmeli, K. Takabayashi, T. Hayashi, L. Leider-Trejo, J. Lee, L.M. Leoni, E. Raz, Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis, Gastroenterology 122 (2002) 1428–1441.
[17] D. Rachmilewitz, K. Katakura, F. Karmeli, T. Hayashi, C. Reinus, B. Rudensky, S. Akira, K. Takeda, J. Lee, K. Takabayashi, E. Raz, Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis, Gastroenterology 126 (2004) 520–528.
[18] B. Albiger, S. Dahlberg, A. Sandgren, F. Wartha, K. Beiter, H. Katsuragi, S. Akira, S. Normark, B. Henriques-Normark, Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection, Cell. Microbiol. 9 (2007) 633–644.
[19] T. Kawai, S. Sato, K.J. Ishii, C. Coban, H. Hemmi, M. Yamamoto, K. Terai, M. Matsuda, J. Inoue, S. Uematsu, O. Takeuchi, S. Akira, Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6, Nat. Immunol. 5 (2004) 1061–1068.
[20] K. Honda, H. Yanai, T. Mizutani, H. Negishi, N. Shimada, N. Suzuki, Y. Ohba, A. Takaoka, W.C. Yeh, T. Taniguchi, Role of a transductional–transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling, Proc. Natl. Acad. Sci. USA 101 (2004) 15416–15421.
[21] H. Negishi, Y. Fujita, H. Yanai, S. Sakaguchi, X. Ouyang, M. Shinohara, H. Takayanagi, Y. Ohba, T. Taniguchi, K. Honda, Evidence for licensing of IFN-gamma-induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program, Proc. Natl. Acad. Sci. USA 103 (2006) 15136–15141.

[22] L.A. O'Neill, A.G. Bowie, The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling, Nat. Rev. Immunol. 7 (2007) 353–364.
[23] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (2006) 783–801.
[24] G.M. Barton, R. Medzhitov, Linking Toll-like receptors to IFN-alpha/beta expression, Nat. Immunol. 4 (2003) 432–433.
[25] K. Katakura, J. Lee, D. Rachmilewitz, G. Li, L. Eckmann, E. Raz, Toll-like receptor 9-induced type I IFN protects mice from experimental colitis, J. Clin. Invest. 115 (2005) 695–702.
[26] J. Lee, D. Rachmilewitz, E. Raz, Homeostatic effects of TLR9 signaling in experimental colitis, Ann. N. Y. Acad. Sci. 1072 (2006) 351–355.