Overexpression of Tim-3 reduces *Helicobacter pylori*-associated inflammation through TLR4/NFκB signaling *in vitro*

FUCAI WANG1,2*, ZHIRONG MAO1*, DONGSHENG LIU1*, JING YU1, YOUHUA WANG1, WEN YE1,2, DONGJIA LIN2, NANJIN ZHOU3 and YONG XIE1

1Department of Gastroenterology, The First Affiliated Hospital of Nanchang University, Gastroenterology Institute of Jiangxi, Key Laboratory of Digestive Diseases of Jiangxi; 2Department of Immunology, Medical College of Nanchang University; 3Institute of Immunology and Biological Therapy, Jiangxi Academy of Medical Sciences, Nanchang, Jiangxi 330006, P.R. China

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**Abstract.** The present study aimed to investigate the interaction between T-cell immunoglobulin and mucin-domain-containing molecule-3 (Tim-3) and Toll-like receptor 4 (TLR4)/nuclear factor κB (NF-κB) signaling in *Helicobacter pylori*-infected RAW264.7 macrophage cells. RAW264.7 cells were co-cultured with *H. pylori* SS1 at different bacteria/cell ratios, and subsequently the mRNA expression of Tim-3, TLR4, and myeloid differentiation factor 88 (MyD88) was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Furthermore, the effect of Tim-3 overexpression was examined by transfection of RAW264.7 with pLVX-IRES-ZsGreen-Tim-3 and co-culturing with *H. pylori*. mRNA and protein expression levels were then analyzed for Tim-3, TLR4, and phosphorylated (p-) NF-κB by RT-qPCR and western blot analysis respectively. The concentrations of pro-inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), interferon-γ (IFN-γ) and interleukin 10 (IL-10)] released in the culture supernatants were measured by ELISA. *H. pylori* stimulation resulted in a significant increase of Tim-3, TLR4, and MyD88 mRNA expression in RAW264.7 cells. *H. pylori* stimulation upregulated Tim-3 expression even in the Tim-3-overexpressing RAW264.7 cells compared with unstimulated cells. TLR4, MyD88, and pNF-κB protein expression and pro-inflammatory cytokines (TNF-α, IL-6, and IFN-γ) release levels were increased in the control RAW264.7 cells following *H. pylori* infection, but not in the Tim-3-overexpressing RAW264.7 cells. By contrast, IL-10 levels were decreased following *H. pylori* infection in both control and Tim-3-overexpressing RAW264.7 cells. Overexpression of Tim-3 reduced *H. pylori*-associated inflammation in RAW264.7 macrophages, by downregulating expression of proteins in the TLR4 pathway and release of pro-inflammatory cytokines. These findings suggest that Tim-3 serves a crucial role in the negative regulation of *H. pylori*-associated inflammation and may be a novel therapeutic target for *H. pylori* infection.

**Introduction**

Chronic infection by *Helicobacter pylori* is achieved through the colonization of an almost exclusive niche and through evading detection by the host's cellular immune defense mechanisms (1). *H. pylori* is the only microorganism known to colonize the human stomach and to inhabit gastric mucosal cells. To achieve this colonization, *H. pylori* must escape detection by both innate and adaptive immune responses (2). Macrophages, phagocytic cells that are part of the innate immune system, are located in various tissues. A great number of cytokines assist macrophages in their role as custodians of the innate immune system as they mediate the transition from innate to adaptive immunity (3). Therefore, macrophages may serve an important role in *H. pylori* infection.

Toll-like receptor (TLR) 4, a pattern recognition receptor, recognizes pathogen-associated molecular patterns. It activates macrophages to secrete cytokines, which initiate and regulate the immune response (4). T-cell immunoglobulin and mucin-domain-containing molecule 3 (Tim-3) is an important member of the TIM family, a recently discovered family of trans-membrane proteins (5). Previous studies have demonstrated that Tim-3 serves a role in the differentiation of T helper (Th) 1 cells from Th2 cells; upon activation, Tim-3...
downregulates Th1 cell function (5-8). Several studies have also demonstrated that TLR4 and Tim-3 are expressed on macrophages and that they participate in the regulation of cytokine secretion from macrophages (9-11), suggesting that Tim-3 impacts macrophage function by interacting with the TLR4 signaling pathway. However, it is unclear to date how H. pylori infection impacts the interaction of Tim-3 and TLR4 in macrophages.

Based on these previous studies, an investigation of the interaction between Tim-3 and TLR4 signaling pathways in H. pylori-associated inflammation was undertaken. The present study aimed to provide theoretical and experimental evidence to support Tim-3 as a potential therapeutic target for the control and prevention of H. pylori infection-related diseases.

Materials and methods

Cell lines and bacteria

The murine macrophage RAW264.7 cell line was obtained from the Gastroenterology Institute of Jiangxi Province (Nanchang, China) and was cultured in DMEM with 10% FBS, 100 U/ml penicillin (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 µg/ml streptomycin (Gibco, Thermo Fisher Scientific, Inc.) under 5% CO₂ at 37°C in a humidified atmosphere. The H. pylori standard strain SS1 (CagA+), containing 10% sheep blood, under infection was grown in serum-free medium alone.

RAW264.7 cells were plated at a density of 2x10⁵ cells/ml in a 6-well plate, grown overnight, and transferred to serum-free medium. The pLVX-IRES-ZsGreen1-Tim-3 plasmid was constructed as follows: Tim-3, forward 5’-ACT CTA CCT ACA TCT GGG TGG CTG CTG GCT GT-3’. The PCR products were purified and cloned into pLVX-IRES-ZsGreen1 (Clontech Laboratories, Inc., Mountainview, CA, USA) according to the manufacturer’s protocols. Cells were transfected with 2.5 µg pLVX-IRES-ZsGreen1-Tim-3 plasmid or empty vector using Lipofectamine LTX and Plus Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocols. Cells were observed for green fluorescent protein using fluorescence microscopy after 42 h transfection. The control cells were grown in serum-free medium alone. Tim-3 overexpression was confirmed by dual-endonuclease digestion and sequencing.

Reverse transcription (RT)-PCR

Total RNA was extracted from RAW264.7 cells in 6-well plate using an E.Z.N.A. Total RNA kit II (Omega BioTek, Inc., Norcross, GA, USA) according to the manufacturer's protocols. RNA quality was evaluated by 1% agarose gel electrophoresis. Using a Quantscript RT Kit (Tiangen Biotech Co., Beijing, China), single-strand cDNA was synthesized and then used as a template for PCR amplification of Tim-3, TLR4, myeloid differentiation factor 88 (MyD88) and β-actin (used for normalization). The 2−ΔΔCq method (12) was used to determine the relative quantities of products. Primer sequences were as follows: Tim-3, forward 5’-ACTCTACCTCATCTGGG ACACT-3’ and reverse 5’-TAGTTCATGGATCCTCAG-3’; TLR4, forward 5’-AGAGACGGCAACCTGGACCT-3’ and reverse 5’-GGCCCTAGCTTCTTCTC-3’; MyD88, forward 5’-CTGGCCTTGTAGACCGTGTA-3’ and reverse 5’-TCC AAAAGTTCCGGCTTTG-3’; and β-actin, forward 5’-GAG ACCTTCAACCCCCAGC-3’ and reverse 5’-ATGTCACCG ACGATTCCC-3’. Each 25 µl PCR consisted of 10 pmol of each primer, 10 µl 2xTaq Master Mix, 3 µl template, and 10 µl ddH₂O. β-actin and MyD88 were amplified at 94°C for 90 sec (1 cycle); 94°C for 30 sec, 61°C for 30 sec and 72°C for 1 min (30 cycles); and 72°C for 5 min (1 cycle). Tim3 and TLR4 were amplified at 94°F for 90 sec (1 cycle); 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min (30 cycles); 72°C for 5 min (1 cycle). The PCR products were visualized following electrophoresis on 2% agarose gels. Densitometric analysis of the bands was carried out using a ChemiDoc MP System with Image Lab™ software version 5.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

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Results are expressed as the ratio of the intensity of the band for Tim-3, TLR4, and MyD88 to the intensity of the band for β-actin.

Western blot analysis. Total protein was extracted from cells using cell lysis buffer (cat no. P0013; Beyotime Biotechnology, Shanghai, China). Protein concentrations were measured using a bicinchoninic acid (BCA) protein assay (Generay Biotech Co., Ltd., Shanghai, China), according to the manufacturer's protocols. Equal amounts of proteins (10 µg) of cell lysates were separated on a 10% SDS-PAGE and electro-transferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked in Tris buffered saline Tween containing 5% fat-free dry milk and incubated overnight at 4°C with primary antibodies, including rabbit anti-Tim-3 antibody (M-171; sc-292390; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse anti-TLR4 antibody (ab22048; 1:1,000; Abcam, Cambridge, UK), rabbit anti-MyD88 antibody (ab2068; 1:1,000; Abcam), rabbit anti-phosphorylated (p-) nuclear factor κB (NF-κB) p65 antibody (3033; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse anti-β-actin antibody (ab1801; 1:1,000; Abcam). Goat anti-rabbit horseradish peroxidase (HRP)–conjugated immunoglobulin G (IgG; ZDR-5306; 1:2,000; ZSGB-BIO, Beijing, China) or rabbit anti-mouse HRP-IgG (ZDR-5109; 1:2,000; ZSJB-BIO) were used as secondary antibodies. Proteins were detected using a ChemiDoc MP System with Image Lab™ Software version 5.1 (Bio-Rad Laboratories, Inc.). Data were normalized to β-actin levels.

ELISA. The concentrations of interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin
10 (IL-10) in the cell culture supernatants of RAW264.7 cells were determined by ELISA, using a commercial human multiplex kit (IL-6, TNF-α, IFN-γ and IL-10; 88-5083; Aushon Biosystems, Inc., Wuxi, China) according to the manufacturer's protocol.

Statistical analysis. Data are expressed as the means ± standard deviation of three independent experiments. Data were analyzed by paired or unpaired t-tests when comparing differences between paired samples or two independent groups respectively. One-way analysis of variance with the LSD post hoc test was used for multiple comparisons. Data analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of H. pylori infection on Tim-3, TLR4 and MyD88 mRNA expression in macrophages. H. pylori infection of macrophages was performed by co-culturing H. pylori with RAW264.7 cells for 12 h at various bacteria/cell ratios, i.e. multiplicities of infection (MOI). H. pylori infection significantly elevated Tim-3, TLR4 and MyD88 mRNA expression levels in RAW264.7 macrophages compared with control uninfected macrophages (P<0.01 compared with control; Fig. 1). Tim-3, TLR4 and MyD88 mRNA levels increased in a MOI-dependent manner (MOI range tested 25-200; Fig. 1). Tim-3 mRNA expression was significantly higher in the infected cells compared with the control cells in all MOI tested (P<0.01; Fig. 1), and no significant difference was observed between different MOI effects (P>0.05; Fig. 1). TLR4 mRNA expression was significantly higher at MOI ratios 50-200 compared with control uninfected cells (P<0.01; Fig. 1). Lastly, MyD88 mRNA expression was significantly higher at MOI ratios 100 and 200 compared with control uninfected cells (P<0.01; Fig. 1).

Construction of Tim-3-overexpressing macrophages. RAW264.7 cells were either left untransfected (control) or were transfected with the pLVX-IRES-ZsGreen1-Tim-3 expression plasmid, in order to establish Tim-3 overexpression in macrophages. Tim-3 mRNA (Fig. 2A) and protein (Fig. 2B) expression levels were significantly higher in transfected RAW264.7 cells than in the control cells (P<0.05; Fig. 2), suggesting that RAW264.7 cells were successfully transfected.

Effect of H. pylori infection on Tim-3 expression and TLR4 pathway proteins in Tim-3-overexpressing macrophages. RAW264.7 cells were either left untransfected (control), or were transfected with empty plasmid (mock) or Tim-3 overexpression plasmid (Tim-3), then cultured for an additional 12 h either alone or with H. pylori at an MOI of 100. In the control macrophages, Tim-3, TLR4, MyD88 mRNA and protein levels, and p-NF-κBp65 protein levels, were significantly higher in the H. pylori-infected cells compared with the H. pylori-negative cells (P<0.05; Fig. 3). In the Tim-3 overexpressing macrophages, TLR4 and MyD88 mRNA expression was increased following infection (P<0.01; Fig. 3A), but no significant change was observed in TLR4, MyD88 and p-NF-κBp65 protein levels between the H. pylori-infected and uninfected cells (P>0.05; Fig. 3B). H. pylori infection increased Tim-3 mRNA and protein expression in the Tim-3-overexpressing cells compared with the untransfected cells (P<0.05, Fig. 3). In addition, infected by H. pylori, TLR4 and MyD88 mRNA expression levels were significantly lower in the Tim-3-overexpressing cells compared with the untransfected cells (P<0.01; Fig. 3A), but no significant change was observed in TLR4, MyD88 and p-NF-κB p65 protein levels (P>0.05; Fig. 3B).

Effect of H. pylori infection on inflammatory cytokine secretion in Tim-3-overexpressing macrophages. RAW264.7 cells were either left untransfected (control), or were transfected with empty plasmid (mock) or Tim-3 overexpression plasmid (Tim-3), then either cultured alone or infected with H. pylori at MOI 100 for 12 h. The culture supernatants were subsequently assayed by ELISA for levels of the inflammatory cytokines TNF-α, IL-6, IFN-γ and IL-10. H. pylori infection increased TNF-α, IL-6 and IFN-γ secretion in the control and mock-transfected cells compared with the H. pylori-negative
It has been reported that *H. pylori* lipopolysaccharide (LPS) activates NF-κB through the TLR4 signaling pathway, which leads to the release of pro-inflammatory cytokines, including IL-6, interleukin 1β (IL-1β) and TNF-α, and to the promotion of inflammatory reactions (19). However, its regulation and the exact mechanism by which it acts remain unclear. To determine the effect of *H. pylori* infection on the TLR4 signaling pathway, the expression of various proteins downstream of TLR4 was examined in macrophages that were infected with different *H. pylori* concentrations. In addition, the release of inflammatory cytokines from the macrophages was also evaluated. The results revealed that *H. pylori* infection at MOIs 25-100 significantly increased TLR4 and MyD88 mRNA expression levels. When cells were infected with *H. pylori* at MOI 100 for 12 h, the protein expression levels of TLR4, MyD88 and p-NF-κBp65 were significantly upregulated. *H. pylori* infection also significantly increased the secretion of pro-inflammatory cytokines, including IL-6, IFN-γ and TNF-α, but reduced the secretion of the anti-inflammatory cytokine IL-10. The present results suggested that *H. pylori* infection activated the TLR4-mediated and MyD88-dependent signaling pathway, activated NF-κB, and promoted inflammatory reactions. Pathak *et al.* (20) reported that *H. pylori* 0175 proteins come directly in contact with the extracellular domain of TLR4 and that they activate the MAPK and NF-κB signaling pathways, thus leading to IL-6 secretion from macrophages. Maeda *et al.* (21) reported that *H. pylori* activated the NF-κB signaling pathway and increased TNF-α release from human monocytes THP-1 cells but failed to activate the NF-κB signaling pathway in macrophages derived from TLR4 mutant mice. Mandell *et al.* (22) reported that *H. pylori* infection increases the secretion of TNF-α and IL-6 from THP-1 cells in a concentration-dependent manner. However, macrophages lacking TLR4 expression do not respond to *H. pylori* LPS stimulation. In summary, TLR4 signaling pathway activation in macrophages is important in the abnormal inflammatory reaction that is caused by *H. pylori* infection, but the exact regulatory mechanism remains unclear.

Tim-3 was the first, and is presently the only, surface molecule shown to specifically identify Th1 cells in mice and humans (23). Numerous studies have demonstrated that Tim-3 is also expressed in innate immune cells, including dendritic cells, macrophages and natural killer cells, but the effect of Tim-3 on immune cells is different than on other cells (5,23). Tim-3 has been demonstrated to be important in innate immune cells, including macrophages/monocytes (24). The present study revealed that *H. pylori* infection upregulated the mRNA and protein expression of Tim-3 in macrophages in a concentration-dependent manner. In a previous study by this group, it was demonstrated that following 12 h of *H. pylori* stimulation, there was a marked increase in Tim-3 production by mouse spleen lymphocytes (25). Tim-3 expression was also confirmed *in vivo* to be higher in the gastric mucosa of *H. pylori*-infected mice than of uninfected mice (25). However, the function of Tim-3 in *H. pylori* infection remains unclear.

TLR4 and Tim-3 are commonly expressed in various immune cells, and their interaction is closely related to the development of many diseases (10,11). However, no report to date has examined the effect of Tim-3 overexpression on the activation of the TLR4 signaling pathway and the
Figure 3. Effect of *Helicobacter pylori* infection on expression of Tim-3 and TLR4 signaling pathway proteins in macrophages. RAW264.7 cells were either left untransfected (control), or were transfected with empty plasmid (Mock) or Tim-3 overexpression plasmid (Tim-3), and then they were cultured for 12 h either alone (*H. pylori*- ) or with *H. pylori* at MOI 100 (*H. pylori*+). (A) mRNA expression was measured for Tim-3, TLR4 and MyD88 by reverse transcription-quantitative polymerase chain reaction and gel electrophoresis. (B) Protein expression was measured for Tim-3, TLR4, MyD88 and p-NFκBp65 mRNA by western blot analysis. Densitometric quantification and representative images of the blots are shown. Quantification for mRNA and protein was relative to β-actin. n=3 independent experiments performed in duplicate. *P<0.05 and **P<0.01, with comparisons indicated by lines. Tim-3, T-cell immunoglobulin and mucin-domain-containing molecule-3; TLR4, Toll-like receptor 4; MOI, multiplicity of infection; MyD88, myeloid differentiation factor 88; p-NFκBp65, phosphorylated nuclear factor κB p65 subunit.
**H. pylori**-induced inflammatory reactions in macrophages. The present study revealed that mRNA expression of TLR4 and MyD88, and secretion of IL-6, IFN-γ and TNF-α, in Tim-3-overexpressing macrophages infected with **H. pylori** were significantly lower than control macrophages. These results suggest that Tim-3 inhibited the activation of macrophages by negatively regulating the TLR4 signaling pathway. A previous study from this group (26) demonstrated that blocking Tim-3 with an inhibitory antibody upregulated TLR4 and MyD88 expression, promoted NF-κB activation, decreased the number of CD4+CD25+Foxp3+Treg cells, and upregulated Th1 immune responses, resulting in intensified inflammation of the gastric mucosa of **H. pylori**-infected mice. Frisancho et al (11) reported that, in a mouse model of inflammatory heart disease, blocking Tim-3 significantly increased TLR4 expression and cardiac inflammation, indicating that Tim-3 is a negative regulator of the TLR4 signaling pathway. The present study also demonstrated that following **H. pylori** infection, Tim-3 overexpression in macrophages not only inhibited the release of IL-6, IFN-γ and TNF-α, but also suppressed the release of IL-10.

**Figure 4.** Effect of *Helicobacter pylori* infection on cytokine secretion in Tim-3-overexpressing macrophages. RAW264.7 cells were either left untransfected (control), or were transfected with empty plasmid (Mock) or Tim-3 overexpression plasmid (Tim-3), then cultured for 12 h either alone (**H. pylori**-) or with **H. pylori** at MOI 100 (**H. pylori**+). Levels of the inflammatory cytokines (A) TNF-α, (B) IFN-γ, (C) IL-6 and (D) IL-10 were detected in the culture supernatants by ELISA. n=3 independent experiments performed in duplicate. *P<0.05 and **P<0.01, with comparisons indicated by lines. Tim-3, T-cell immunoglobulin and mucin-domain-containing molecule-3; MOI, multiplicity of infection; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-6, interleukin 6; IL-10, interleukin 10.

In conclusion, **H. pylori** infection in RAW264.7 macrophages activated the TLR4 signaling pathway, upregulated Tim-3 expression and increased secretion of pro-inflammatory cytokines (TNF-α, IL-6 and IFN-γ), while decreasing secretion of an anti-inflammatory cytokine (IL-10). Under
conditions of *H. pylori* infection, however, Tim-3 overexpression inhibited the TLR4 pathway activation and the secretion of pro-inflammatory cytokines. Taken together, the present study indicated that Tim-3 may represent a novel therapeutic target for treating *H. pylori* infection-related diseases.

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