

Olfactomedin 4 down-regulates innate immunity against *Helicobacter pylori* infection

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Olfactomedin 4 (OLFM4) is a glycoprotein that has been found to be up-regulated in inflammatory bowel diseases and *Helicobacter pylori* infected patients. However, its role in biological processes such as inflammation or other immune response is not known. In this study, we generated OLFM4 KO mice to investigate potential role(s) of OLFM4 in gastric mucosal responses to *H. pylori* infection. *H. pylori* colonization in the gastric mucosa of OLFM4 KO mice was significantly lower compared with WT littermates. The reduced bacterial load was associated with enhanced infiltration of inflammatory cells in gastric mucosa. Production and expression of proinflammatory cytokines/chemokines such as IL-1 β , IL-5, IL-12 p70, and MIP-1 α was increased in OLFM4 KO mice compared with infected controls. Furthermore, we found that OLFM4 is a target gene of NF- κ B pathway and has a negative feedback effect on NF- κ B activation induced by *H. pylori* infection through a direct association with nucleotide oligomerization domain-1 (NOD1) and -2 (NOD2). Together these observations indicate that OLFM4 exerts considerable influence on the host defense against *H. pylori* infection acting through NOD1 and NOD2 mediated NF- κ B activation and subsequent cytokines and chemokines production, which in turn inhibit host immune response and contribute to persistence of *H. pylori* colonization.

NF- κ B | nucleotide oligomerization domain-1 and -2

As a spiral, microaerophilic, Gram-negative bacteria that induces chronic gastritis, *Helicobacter pylori* (*H. pylori*) is a well-known risk factor for peptic ulcer and gastric cancer (1). Although *H. pylori* infection can persist for decades, only a fraction of colonized individuals ever develop clinical diseases (1). Clinical outcome is influenced by a balance between *H. pylori* virulence factors and the host immune response. However, the mechanisms by which bacterial and/or host factors cause disease remain unclear. Identification of immune response genes that regulate the *H. pylori*-host interactions will not only have diagnostic and therapeutic implications, but may also provide insights into other inflammation related cancer.

Olfactomedin 4 (OLFM4; also known as hGC-1 and GW112) is a member of olfactomedin domain-containing protein family that has a relatively diverse coil-coil domain in the amino terminus and a well-conserved olfactomedin domain in the carboxy-terminus (2). There are at least 13 members of olfactomedin domain-containing proteins in mammals (3). Recent studies revealed that olfactomedin-containing proteins play important roles in variety of aspects including neurogenesis, cell adhesion, cell cycle regulation, and tumorigenesis and may serve as modulators of critical signaling pathways such as Wnt and bone morphogenic proteins (BMP) (3). Mutation in two genes encoding myocilin (4) and olfactomedin 2 (5) has been implicated in pathogenesis of glaucoma and other genes belonging to this family may contribute to different human disorders including psychiatric disease (3). However, genetic knock-out mice for myocilin (6) and pancortin-2 (7) demonstrate no obvious phenotypes. OLFM4 is constitutively expressed in neutrophils and the gastrointestinal

tract epithelial cells (2, 8). A recent study showed that OLFM4 is a target gene of Notch pathway (9), suggesting a potential role for OLFM4 for Notch mediated cell differentiation, proliferation, and immune response to inflammation. As a target gene of important signal pathways implicated in cancer and inflammation, OLFM4 may emerge as a potential therapeutic target for these diseases. Recent publications have also reported increased expression of OLFM4 in gastrointestinal cancer (10–13) and inflammatory disease including inflammatory bowel disease (14) and *H. pylori* infection (15). However, the exact function of OLFM4 involvement in these diseases still remains elusive. The correlation of OLFM4 expression level with the severity of *H. pylori* infection has not been addressed yet. The association of OLFM4 with gastrointestinal inflammation and cancers suggest that it may play a role in inflammatory immune response and may be a potential target gene in inflammation-related cancer. The studies are done by generating OLFM4 KO mice and addressed this possibility in a *H. pylori* [Sydney strain 1 (SS1) strain]-infected mouse model. In this report, we provide evidence that OLFM4 is an important regulator of inflammatory and immune response in *H. pylori* induced gastritis.

Results

OLFM4 KO Mice Showed Normal Development and Hematopoiesis. To explore the in vivo function of OLFM4, we generated OLFM4 KO mice by genetic targeting (Fig. S1 A and B). The lack of OLFM4 transcripts and protein in the bone marrow of OLFM4 KO mice was confirmed by RT-PCR (Fig. S1C) and Western blotting (Fig. S1D). Mice heterozygous and homozygous for the null mutation in OLFM4 were observed for over a year and appeared to have normal development, fertility, and viability relative to WT mice. Histological examination of tissues including bone marrow, esophagus, small intestine, and colon did not reveal any discernable abnormalities in OLFM4 KO mice (Fig. S2). Therefore, OLFM4 does not appear to be essential for normal development and growth in mice.

OLFM4 Is Up-Regulated Following *H. pylori* Infection in Vivo and in Vitro. Up-regulation of OLFM4 mRNA in the gastric mucosa of patients with *H. pylori* infection (15) raises the possibility that OLFM4 may play a role in the gastric mucosa immune response. We investigated this potential function of OLFM4 in OLFM4

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KO mice following *H. pylori* infection. We observed an association of OLFM4 expression with *H. pylori* (strain SS1) infection of gastric mucosa. The up-regulation in the gastric mucosa (Fig. S3A) and neutrophils (Fig. S3B) of SS1-infected mice was confirmed by quantitative RT-PCR. We also investigated the OLFM4 mRNA expression in a mouse primary gastric epithelial cell line, GSM06, in response to *H. pylori*. Infection with either SS1 or US101 strains significantly increased OLFM4 transcripts (Fig. S3 C and D). We further examined if *H. pylori* could induce OLFM4 expression in macrophages, another important inflammatory cells. OLFM4 is not endogenously expressed in RAW264.7 cells, a mouse macrophage cell line, but SS1 and US101 infection induced significant OLFM4 expression (Fig. S3 E and F). Thus, the up-regulation of OLFM4 expression in gastric mucosa with *H. pylori* infection appears to be the direct action of *H. pylori* on epithelial cells and activated infiltrating inflammatory cells, such as neutrophils and macrophages.

Colonization of Gastric Mucosa by *H. pylori* Is Reduced in OLFM4 KO Mice. We next investigated whether endogenous OLFM4 is involved in host defense against *H. pylori* infection. OLFM4 KO mice and WT mice were orally challenged with SS1 organisms, killed 4, 8, and 20 wk postinfection, and the colonization level of SS1 organisms in the stomach of the mice determined by quantitative culture. A significantly reduced colonization in the gastric mucosa was observed in OLFM4 KO mice compared with WT mice after SS1 infection at all three time points, with the difference being the greatest at 20 wk (Fig. 1A). These data suggest an important function for OLFM4 in regulating host response to *H. pylori* infection.

Inflammatory Response to *H. pylori* Is Exacerbated in OLFM4 KO Mice. WT mice 4, 8, and 20 wk postinfection developed mild or moderate infiltration of inflammatory cells in the gastric mucosa, consisting of mononuclear cells and polymorphonuclear cells (Fig. 1B). The gastritis was more severe and extensive in OLFM4 KO mice than in WT mice (Fig. 1B). Semiquantitative analysis of the intensity of the gastric inflammation demonstrated a significant difference in the scores of mononuclear and polymorphonuclear cell infiltrates between OLFM4 KO and WT mice, with OLFM4 KO mice displaying more substantive infiltrates than WT mice at the two time points examined (Fig. 1C). Interestingly, larger numbers of eosinophils were observed in OLFM4 KO mice than in WT mice based on Luna staining (Fig. S4). These data demonstrated that the gastric damage associated with SS1 infection of OLFM4 KO mice was accompanied by enhanced infiltration of inflammatory cells.

Production and Expression of Proinflammatory Cytokines and Chemokines Is Enhanced in OLFM4 KO Mice Following *H. pylori* Infection. It is known that cytokines and chemokines play a critical role in the pathogenesis of mucosal inflammation. We investigated the level of 27 cytokines and chemokines in the serum of OLFM4 KO mice compared with WT mice in response to *H. pylori* infection using a high-throughput multiplex immunoassay. The levels of interleukin (IL)-1 β , IL-5, IL-12 (p70) and macrophage inflammatory protein (MIP)-1 α were significantly increased in OLFM4 KO mice compared with WT mice 2 wk after SS1 infection (Fig. 2A). Next, we examined local immune responses in stomach tissues to infection by monitoring mRNA expression of both these four differentially expressed cytokines and other selected cytokines associated with mucosa inflammation in gastric mucosa, using quantitative RT-PCR (Fig. 2B). The levels of IL-1 β , tumor necrosis factor (TNF)- α , IL-5, IL-12 α , MIP-1 α and monocyte chemoattractant protein (MCP)-1 mRNA were significantly higher in OLFM4 KO mice than in WT mice 2 wk postinfection (Fig. 2B). However, 8 wk after SS1 infection, only IL-5, IL-12 α , and MIP-1 α expression levels were higher in OLFM4 KO mice than in WT

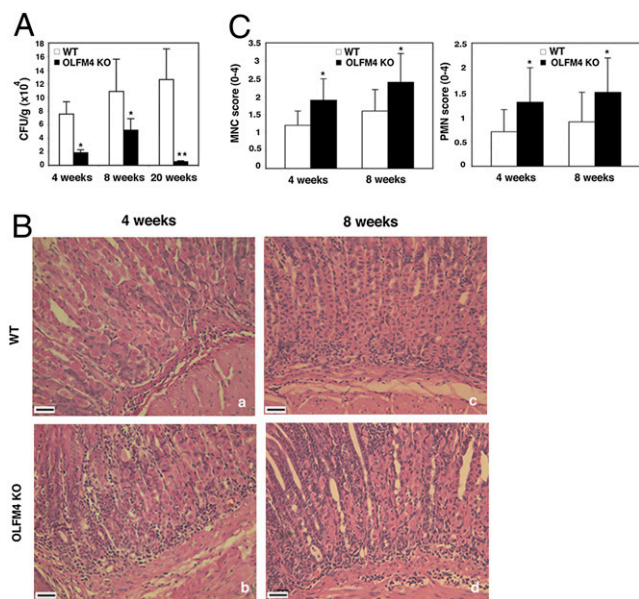


Fig. 1. *H. pylori* colonization in gastric mucosa is reduced and inflammation is exacerbated in *H. pylori*-infected OLFM4 KO mice. (A) Groups of OLFM4 KO and WT mice were orally challenged with 1×10^8 *H. pylori* SS1 organisms. Colony counts were determined for gastric tissue, including the entire length of the gastric mucosa, taken at 4, 8, and 20 wk after SS1 infection. At each time point, $n = 5$ for OLFM4 KO and WT mice. Data are expressed as mean \pm SD for each experimental group. * $P < 0.05$, ** $P < 0.01$ versus WT. (B) Representative histological findings in the gastric mucosa of SS1-infected WT and OLFM4 KO mice. (a) WT mice 4 wk postinfection with mild inflammation in the gastric antrum mucosa. (b) OLFM4 KO mice 4 wk postinfection with moderate inflammation in the gastric antrum mucosa. (c) WT mice 8 wk postinfection with moderate inflammatory infiltrates in the gastric antrum mucosa. (d) OLFM4 KO mice 8 wk postinfection with severe inflammation in the gastric antrum mucosa. Tissues sections were hematoxylin and eosin stained. Magnification 200 \times . (Scale bar, 50 μ m.) (C) Infiltration of polymorphonuclear (PMN) and mononuclear (MNC) cells was scored on a scale of 0–4 as described in *Materials and Methods*. $n = 5$ for each group of WT or OLFM4 KO mice were scored. Data are expressed as median \pm SD for each experimental group ($n = 5$). * $P < 0.05$ versus WT.

mice (Fig. 2B). At 20 wk postinfection, the expression level in gastric mucosa of all cytokines examined had decreased to the extent that differences were no longer observed between OLFM4 KO mice and WT mice (Fig. S5A). The mRNA expression of IL17, IL-23, IL-27, and MIP-2, IFN (IFN) γ , and granulocyte-macrophage colony-stimulating factor (GM-CSF) was not changed in OLFM4 KO mice compared with WT mice at all time points (Fig. 2B and Fig. S5 A and B). These results suggest that OLFM4 deficiency leads to an enhanced production and expression of some key inflammatory cytokines and chemokines both locally and systemically. We did not find differences in the levels of *H. pylori*-specific immunoglobins in OLFM4 KO mice and WT mice (Fig. S5C), suggesting that the enhanced immune response in OLFM4 KO mice was mainly mediated by cellular immunity rather than humoral immunity.

***H. pylori* Induces OLFM4 Expression Through the NF- κ B Pathway.** Our previous studies (16) showed that the OLFM4 proximal promoter, –101 from the transcription start site, is essential for maximal transcription and contains an NF- κ B responsive element (Fig. S6A). In this study, we investigated whether *H. pylori*-induced OLFM4 expression is mediated through the NF- κ B pathway or alternative pathways in the mouse gastric epithelial cell line, GSM06. First, we tested the possibility that SS1 infection stimulated NF- κ B activity in GSM06 cells. SS1 induced

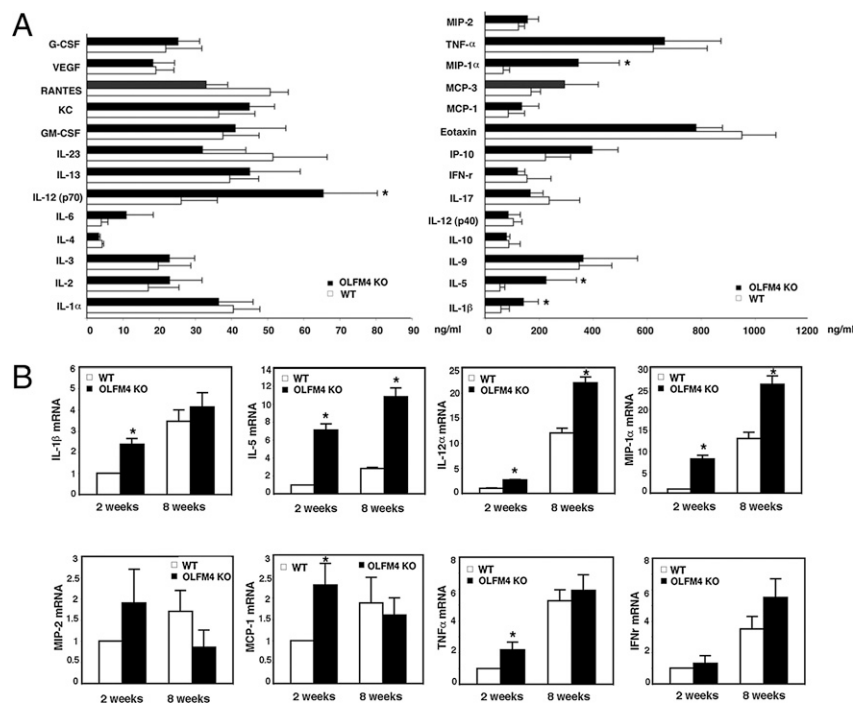


Fig. 2. Proinflammatory cytokine and chemokine levels in the serum and gastric mucosa are enhanced in OLFM4 KO mice. (A) Cytokine and chemokine levels in the serum of OLFM4 KO and WT mice 2 wk after SS1 infection were determined by high throughput immunoassay ($n = 10$ for OLFM4 KO and WT mice). Data represent mean \pm SD for each experimental group. * $P < 0.05$ versus WT. (B) Cytokine and chemokine mRNA levels in gastric mucosa of OLFM4 KO and WT mice 2 and 8 wk after SS1 infection were determined by quantitative RT-PCR ($n = 5$ for WT and OLFM4 KO mice). Data represent the mean \pm SD for cytokine or chemokine level relative to the mean level in WT mice 2 wk after infection, which was set to be 1. * $P < 0.05$ versus WT.

NF- κ B activation in a dose-dependent manner (Fig. S6B). Infection of GSM06 with SS1 induced an approximately 12-fold increase of OLFM4 promoter-driven luciferase transcription. Transcription was totally abolished when the NF- κ B binding site was mutated (Fig. S6C). Prior treatment of GSM06 cells with Bay11-7082 (an inhibitor of I κ B phosphorylation) and sulfasalazine (an inhibitor of I κ B kinase) resulted in a remarkable reduction of SS1-induced OLFM4 promoter-driven transcription (Fig. S6D). We also used dominant-negative mutants of I κ B α (S32A/S36A) and kinase-deficient mutants of IKK1 (K44M), IKK2 (K44A), and NIK (KK429/430AA) to examine whether these signaling intermediates participated in *H. pylori*-induced activation of NF- κ B. The expression of dominant-negative mutants of I κ B α and kinase-deficient mutants of IKK1, IKK2, and NIK effectively blocked SS1 induction of OLFM4 promoter-driven luciferase activity (Fig. S6E). These results confirmed that the NF- κ B pathway, via NIK-IKK signaling components, is involved in *H. pylori*-induced transcription of OLFM4 in normal gastric epithelial cells.

OLFM4 Down-Regulates *H. pylori*-Induced NF- κ B Activation. Recent studies showed that after NF- κ B activation, a complex network of negative feedback loops ensures the termination of the NF- κ B response (17). Therefore, we assessed whether OLFM4 might in turn affect NF- κ B activation. We tested this in GSM06 cells and found that SS1 stimulated NF- κ B activation, and OLFM4 overexpression was found to down-regulate NF- κ B activation in a concentration-dependent manner (Fig. 3A). To confirm a role for OLFM4 in the down-regulation of NF- κ B, a short hairpin RNA (shRNA) approach was used to selectively inhibit the expression of OLFM4. The effective knock-down (approximately 75%) of OLFM4 expression in GSM06 cells using lentiviral shRNA against OLFM4 in the presence or absence of SS1 infection was demonstrated by quantitative RT-PCR (Fig. 3B).

Use of OLFM4 shRNA significantly increased SS1-induced NF- κ B activation (Fig. 3C). The lack of an effect of a control shRNA on NF- κ B activity confirmed the specificity of this effect (Fig. 3C).

We examined NF- κ B activation in OLFM4 KO and WT mice. We assayed NF- κ B binding activities in nuclear extracts from mouse gastric mucosa. NF- κ B binding was significantly increased after SS1 infection in OLFM4 KO mice as compared with WT mice (Fig. 3D). To confirm the enhanced NF- κ B activity in OLFM4 KO mice, we performed immunohistochemistry on gastric mucosa tissues using NF- κ B/p65 antibodies that stain the activated NF- κ B p65 subunit in the nucleus. We observed more NF- κ B activation after SS1 infection in OLFM4 KO mice relative to WT mice (Fig. 3E and Fig. S7). Our results indicate that OLFM4 expression can inhibit NF- κ B activation via a negative feedback mechanism.

OLFM4 Binds to NOD1 and NOD2 and Inhibits NOD1/2-Mediated NF- κ B Activation and Cytokine Production. A previous study showed GRIM-19, a binding partner of OLFM4, is associated with nucleotide-binding oligomerization domain-2 (NOD2) and regulates NOD2-mediated NF- κ B activation (18). To investigate an effect of OLFM4 on NOD2 signaling, we first sought to find if a direct association between OLFM4 and NOD2 or NOD1, whose structure and function is similar to NOD2, could be demonstrated. A reciprocal coimmunoprecipitation assay in lysates of HEK 293T cells transfected with NOD1 or NOD2 and OLFM4 showed that OLFM4 associates with both NOD1 and NOD2 (Fig. 4A and B). We confirmed these interactions by examining this putative association of endogenous OLFM4 with NOD1 or NOD2 in GSM06 cells under physiological conditions. Endogenous OLFM4 weakly coprecipitated with NOD1 and NOD2 in GSM06 cells, but these associations were enhanced when cells were infected with SS1 for 16 h (Fig. 4C). Others have reported that the expression of NOD1 and NOD2 significantly

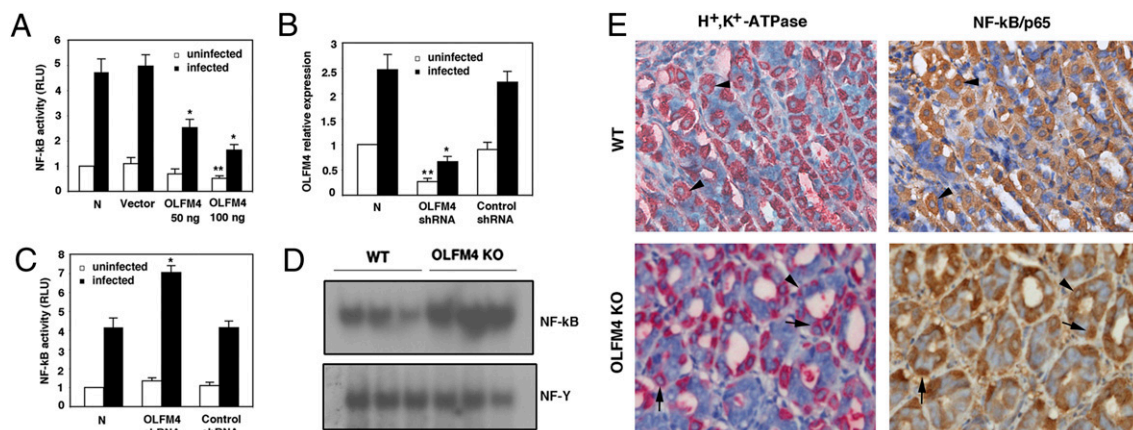


Fig. 3. OLFM4 negatively regulates NF- κ B activation. (A) GSM06 cells were transfected with OLFM4 plasmid and NF- κ B luciferase plasmids. The infection was performed as in Fig. S3C and luciferase activity was performed as in Fig. S6B. N, nontransfection with OLFM4 plasmid. Vector, transfection with empty vector. $n = 3$. * $P < 0.05$ versus infected N or Vector. ** $P < 0.05$ versus uninfected N or Vector. (B) GSM06 cells were infected with OLFM4 or control shRNA lentivirus particles and selected with puromycin. Quantitative RT-PCR was performed to detect OLFM4 expression. N, not treated with shRNA. $n = 3$. * $P < 0.05$ versus infected N. ** $P < 0.05$ versus uninfected N. (C) GSM06 cells were infected with OLFM4 or control shRNA lentivirus particles. Puromycin-resistant cells treated with control shRNA, or not infected with shRNA control cells (N) were transfected with NF- κ B plasmids 24 h before SS1 infection for 6 h. Luciferase activity assay was performed as in Fig. S6B. $n = 3$. * $P < 0.05$ versus infected N or control shRNA. (D) Nuclear extracts from gastric mucosa of WT and OLFM4 KO mice prepared 4 wk after SS1 infection were analyzed for NF- κ B DNA binding activity. Nuclear extract quality was monitored by measuring nuclear factor- γ (NF- γ) DNA binding. (E) Immunohistochemistry staining with NF- κ B/p65 antibody on gastric mucosa of WT and OLFM4 KO mice 4 wk after SS1 infection (Right, 400 \times). Arrowheads indicate cytoplasmic staining and arrows indicate nuclear staining. (Left) H⁺,K⁺-ATPase staining of corresponding sections to the right.

sensitizes HEK293 cells to *H. pylori*-induced NF- κ B activation (19). Therefore, we used this system to investigate whether OLFM4 affects NOD1 and NOD2-mediated NF- κ B activation induced by SS1 infection. Post-SS1 infection, NOD1 and NOD2-mediated NF- κ B activation in HEK 293T cells was significantly inhibited by OLFM4 overexpression in a dose-dependent manner (Fig. 5A). Similarly, NOD1 and NOD2-mediated NF- κ B activation induced by H-Ala-D-g-Glu-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) respectively, were also inhibited by OLFM4 expression in a dose-dependent manner (Fig. 5B). We next determined whether endogenous OLFM4 me-

diates negative feedback of NF- κ B activation after NOD1 and NOD2 stimulation. To test this, we used lentivirus OLFM4 shRNA, which specifically suppresses the expression of OLFM4 in GSM06 cells. NF- κ B activation induced by iE-DAP and MDP in GSM06 cells were enhanced by transfection of OLFM4 shRNA but not with control shRNA (Fig. 5C). To further confirm the inhibitory role of OLFM4 in NOD1 and NOD2 signaling in vivo, we injected WT and OLFM4 KO mice i.p. with NOD1 agonist (iE-DAP) or NOD2 agonist (MDP) and then determined the cytokine levels in the serum. The IL-1 β , IL-12(p70), MIP-1 α , and MCP-1 levels in iE-DAP treated OLFM4 KO mice were significantly higher than those in iE-DAP treated WT mice, and the IL-1 β , IL-12(p70), and MCP-1 levels in MDP treated OLFM4 KO mice were higher than those in MDP treated WT mice (Fig. 5D). In addition, we found that OLFM4 inhibition of NF- κ B reporter activity was specific to NOD 1 and NOD2, because overexpression of OLFM4 did not affect toll like receptor (TLR) 2 and TLR4-mediated NF- κ B activation in HEK 293T cells (Fig. S8). These results demonstrate that OLFM4 directly interacts with NOD1 and NOD2 and functions as a negative regulator of NOD1/2 signaling (Fig. S9).

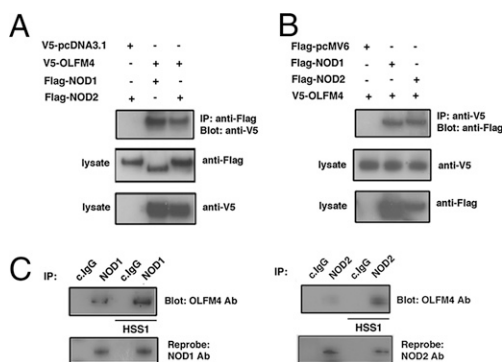


Fig. 4. OLFM4 associates with NOD1 and NOD2. (A) HEK 293T cells were transfected with Flag-NOD1 or Flag-NOD2 and V5-OLF4 or V5-pDNA3.1 plasmids. After 24 h, the total cell lysates were immunoprecipitated with anti-Flag antibody and probed with anti-V5 antibody (Top). Expression of Flag-NOD1 or Flag-NOD2 (Middle) and V5-OLF4 (Bottom) in the lysates was demonstrated by Western blot. (B) HEK 293T cells were transfected with V5-OLF4 and Flag-NOD1 or Flag-NOD2 or Flag-pCMV6 plasmids. After 24 h, the total lysates were immunoprecipitated with anti-Flag antibody and probed with anti-V5 antibody (Top). Expression of V5-OLF4 (Middle) and Flag-NOD1 or Flag-NOD2 (Bottom) in the lysates was demonstrated with Western blot. (C) Total cell lysates of GSM06 cells with or without SS1 infection for 16 h were immunoprecipitated with NOD1 antibody (Left) or NOD2 antibody (Right) or control antibody (c.IgG) and probed with OLFM4 antibody. The immunoprecipitates were reprobed with NOD1 antibody (Left) or NOD2 antibody (Right).

Discussion

In this report, we described the role of OLFM4 in mediating an immune response, inflammation and colonization following *H. pylori* infection. Our findings show: (i) OLFM4 deficiency leads to reduced *H. pylori* colonization; (ii) in contrast, such deficiency leads to enhanced immune response and inflammation; and (iii) OLFM4 inhibits NOD1 and NOD2-mediated NF- κ B activation. Persistent colonization of *H. pylori* in the gastric mucosa requires a balance between the virulence factor of the *H. pylori* and the immune response of the host. Several observations suggested the *H. pylori*-induced immune response is actively down-regulated (21). Our study provides evidence that the normal course of events during *H. pylori* infection involves suppression of the host anti-*H. pylori* response. We demonstrated that OLFM4 plays an important role in regulating innate (early) immune response against *H. pylori* infection. However, when *H. pylori* infection converts to a chronic state leading to persistence, we observe increased production of OLFM4. The ability of *H. pylori*

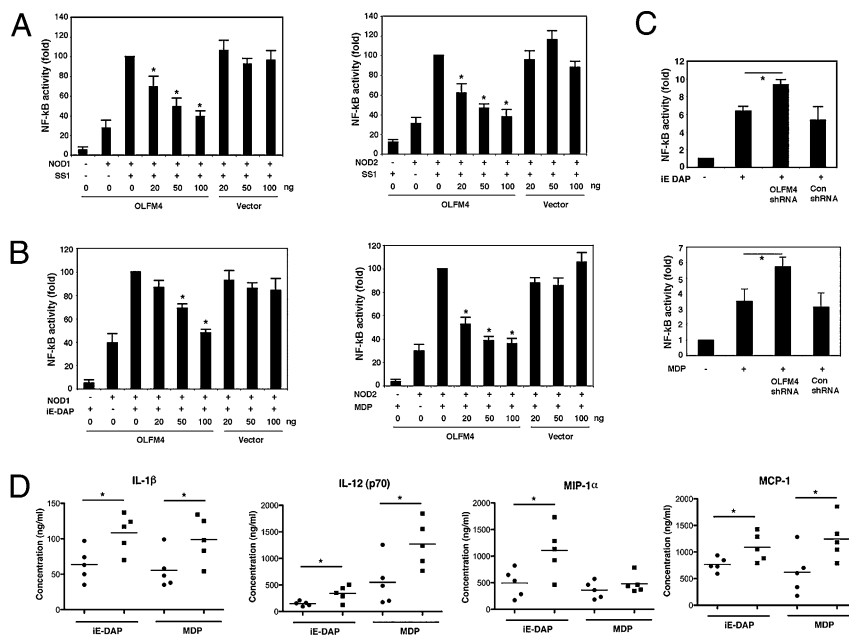


Fig. 5. OLFM4 inhibits NOD1 and NOD2-mediated NF- κ B activity and cytokine production. (A) HEK 293T cells were transfected with NF- κ B reporter with NOD1 (Left) or NOD2 (Right) vector and OLFM4 vector or empty vector were infected with or without SS1 (10^8 CFU) for 24 h. Luciferase assay was performed as in Fig. S6B. $n = 3$. * $P < 0.05$ versus cells transfected with NOD1 or NOD2 and treated with SS1. (B) Experiment was performed in the same fashion as in A except that iE-DAP (100 ng/mL) (Left) or MDP (1 μ g/mL) (Right) treatment for 6 h was added in lieu of SS1 infection. * $P < 0.05$ versus cells transfected with NOD1 treated with iE-DAP or NOD2 and treated with MDP. (C) GSM06 cells transfected with NF- κ B reporter were treated with or without lentiviral particles against OLFM4 shRNA or control shRNA and puromycin-resistant cells were then treated with iE-DAP (100 ng/mL) or MDP (1 μ g/mL) for 6 h. Luciferase assay was performed as in Fig. S6B. $n = 3$. * $P < 0.05$. (D) WT and OLFM4 KO mice ($n = 5$) were injected i.p. with DAP (2 mg/kg) and MDP (25 mg/kg), blood was collected from animals 2 h (DAP) or 4 h (MDP) after injection, and the cytokine levels in the serum of WT (circle) and OLFM4 KO (square) mice were measured by bead-based immunoassays. (Scale bars: mean.) * $P < 0.05$.

to promote the production of OLFM4 during chronic infection may contribute to the persistence of bacteria at the gastric mucosa. In this model, OLFM4 tips the balance toward *H. pylori* persistence from suppression of the host immune response.

One of the important functions of the NF- κ B pathway is the initiation and amplification of the innate immune system in mounting a powerful and fulminant reaction to combat infections (17). Activation of NF- κ B by *H. pylori* infection in gastric epithelial cells in vitro and in vivo has been well recognized (22, 23). Our study showed that OLFM4, as a target gene of NF- κ B, could have a negative feedback effect on NF- κ B activation. This was confirmed by increased NF- κ B binding activity and NF- κ B/p65 nuclear translocation in OLFM4 KO mice than in WT mice. Our results regarding NF- κ B-OLFM4 add to an already complex network of negative feedback loops to ensure the termination of the NF- κ B response. The loss of feedback inhibition of the NF- κ B pathway and the resulting enhanced NF- κ B activation in OLFM4 KO mice may explain the observed increase in inflammation and immune response and the subsequent reduction of bacteria. Our observations are consistent with recent reports suggesting that signals initiated during the induction phase also include a default termination procedure to temporally and spatially deactivate NF- κ B (17).

The findings that OLFM4 directly interacts with NOD1 and NOD2 and inhibits NOD1/2-mediated NF- κ B activation suggest a mechanism for OLFM4's inhibition of the NF- κ B pathway in the presence of *H. pylori* infection. Although it was generally hypothesized that Toll-like receptor (TLR) would be involved in the innate immune recognition of *H. pylori*, no conclusive evidence for a relevant TLR has been presented (19). Instead, it is proposed that NOD1 and NOD2 in vivo may play a critical role in the recognition of *H. pylori* (19). The intracellular NOD-like proteins or receptors are a family of sensors of intracellularly encountered microbial motifs that have emerged as critical com-

ponents of the innate immune response and of inflammation in mammals (24). The expression of NOD1 in gastric epithelial cells confers responsiveness to *H. pylori* and exerts a protective role against bacterial colonization in a mouse model of *H. pylori* infection (25). NOD2 also sensitizes epithelial cells to recognize *H. pylori* in vitro (19). NOD1 activity is regulated by its interacting proteins such as cIAP1/2 (26), SGT1 (27), CENTB (28), and Hsp90 (29). Multiple proteins, including TAK1 (30), GRIM-19 (18), Ipaf-1 (31), Erbin (32), and CENTB1 (28) interact with NOD2 and regulate NOD2-mediated NF- κ B activation. Both NOD1 and NOD2 subsequently activate NF- κ B through a common pathway involving RICK/RIP2 (33). These findings suggest that NOD1 and NOD2-mediated NF- κ B activation is highly controlled by several NOD protein interactors. We show that OLFM4 is a NOD1 and NOD2-associated protein. Unlike its known binding partner GRIM-19, whose interaction is necessary for the NOD2-mediated NF- κ B effect (18), OLFM4 downregulates NOD1 and NOD2-mediated NF- κ B activity stimulated by *H. pylori* infection. The loss of the inhibitory effect of OLFM4 on NOD1 and NOD2-mediated NF- κ B activation may lead to the observed increase in NF- κ B activity in OLFM4 KO mice. The involvement of OLFM4 with NOD1 and NOD2 mediated NF- κ B activation will provide a better understanding of the pathogenic mechanism in *H. pylori*-associated gastritis.

In conclusion, our results indicate that OLFM4 is an anti-inflammatory mediator in *H. pylori* infection. It plays an important role in the regulation of host resistance and gastric inflammatory response to *H. pylori* infection. We speculate that extrapolation of these results to human infection would suggest that the enhanced expression of OLFM4 seen in gastric mucosa of *H. pylori*-infected patients may actively confer a survival advantage to *H. pylori*, promoting gastric colonization.

Materials and Methods

Targeted disruption and generation of OLFM4 KO mice methods are provided in *SI Materials and Methods*.

Histopathology and Immunohistochemistry. To grade the severity of gastritis, we determined the presence of inflammatory cells in a semiquantitative fashion in reference to the updated Sydney system (0, none; 1, mild; 2, moderate; 3, marked; 4, marked and extensive) (34). Luna staining for specific eosinophils was performed as previously described (35). Immunohistochemistry with NF- κ B/p65 antibody (Thermo Scientific) and H⁺,K⁺-ATPase, α -subunit antibody (Calbiochem) on mouse gastric tissues was performed as previously described (13).

RT-PCR, Western Blot, and Immunoprecipitation. RT-PCR and quantitative RT-PCR to amplify OLFM4 were performed as previously described (8, 36). The primers and probes for cytokines and chemokines were all purchased from Applied Biosystems. Western blot and immunoprecipitation analysis were performed as previously described (36).

H. pylori Infection and Colonization. *H. pylori* growth, inoculations and colony recovery were performed as previously described (37). *H. pylori* SS1 was obtained from Drs. A. Lee and J. O'Rourke (University of South Wales, Sydney, Australia).

Cytokine Assay. High-throughput multiplex immunoassays were performed with the Procarta cytokine assay kit from Panomics according to the manufacturer's instructions. Samples were assayed and analyzed using Bio-plex (Bio-Rad). MIP-2 was measured using a mouse MIP-2 ELISA kit (Immuno-Biological Laboratories).

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