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Anti-CXCL13 antibody can inhibit the formation of gastric lymphoid follicles induced by *Helicobacter* infection

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Helicobacter suis **infects the stomachs of both animals and humans, and can induce gastric mucosa-associated lymphoid tissue (MALT) lymphomas. It is known that CXC chemokine ligand 13 (CXCL13) is highly expressed in the *Helicobacter*-infected mice and gastric MALT lymphoma patients, but the pathway that links the activation of CXCL13 and the formation of gastric MALT lymphomas remains unclear. In this study, we examined whether CXCL13 neutralization would interfere with the formation of gastric lymphoid follicles including B cells, CD4 + T cells, dendritic cells (DCs), and follicular DCs (FDCs) in germinal centers to determine the role of CXCL13 in the formation of B-cell aggregates after *H. suis* infection. Moreover, the expression of genes associated with the lymphoid follicle formation was also effectively suppressed by anti-CXCL13 antibody treatment. These results suggest that the upregulation of CXCL13 has an important role in the development of gastric MALT lymphomas and highlight the potential of anti-CXCL13 antibody for protection against *Helicobacter*-induced gastric diseases.**

INTRODUCTION

Helicobacter suis is a Gram-negative, spiral-shaped bacterium belonging to the *Helicobacter* family that also includes *H. pylori*. It is found in the stomachs of various animals including cats, dogs, and pigs, and has also been observed in humans.[1–3] Therefore, it is suspected that humans may be infected with *H. suis* through contact with these animals via zoonotic infection.[4,5] Recently, it has been reported that *H. suis* was implicated in inducing gastric mucosa-associated lymphoid tissue (MALT) lymphoma.[6] Multiple studies have revealed that the prevalence of gastric MALT lymphoma in *H. suis*-infected patients is higher than in *H. pylori*-infected patients.[7] Therefore, *H. suis* is considered to be a potent inducer of gastric MALT lymphoma.

To date, gastric MALT lymphoma is thought to develop as a result of a long-term infection with the Gram-negative gastric bacterium *Helicobacter*,[8–10] which is detectable in a large majority of MALT patients.[8,10,11] Persistent infection with *Helicobacter* causes chronic gastritis that, in some cases, can develop into gastric MALT lymphoma. Gastritis begins as an antigen-dependent disease in its initial stage and develops histological similarity to Peyer's patches in the small intestine.[12] Direct antigenic stimulation by *Helicobacter* infection results in the proliferation of lymphocytes and the formation of lymphoid follicles in the gastric mucosa that ultimately leads to gastric MALT lymphoma.[5,13,14]

Lymphoid follicles are B-cell-rich compartments of lymphoid organs that function as the sites of B-cell exposure to antigens and their subsequent differentiation. It has also been demonstrated that B lymphocyte chemoattractant CXCL13 (CXC chemokine ligand 13; i.e., BLC in mouse and[15] BCA1 in human[16]) and its receptor CXCR5 are needed for B-cell homing to follicles in lymph nodes and spleen.[17,18] CXCL13 was produced in a lymphotoxin (LT)-dependent manner by follicular stromal cells such as follicular dendritic cells (FDCs),[15,19] and the mice lacking CXCL13 or its receptor,

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CXCR5, failed to form lymphoid follicles.[17,18] CXCL13 is a pivotal chemokine responsible for the formation and maintenance of B lymphocyte follicles and germinal centers (GCs) in spleen and lymph nodes,[17] and has been identified in inflammation and autoimmune disease-associated tertiary lymphoid organs.[20–22] Transgenic CXCL13 expression in normal mouse islets is sufficient to induce formation of ectopic lymphoid aggregates, which is a LT-dependent process.[23] Previous studies have shown that the activation of CXCL13 is closely associated with various diseases, such as rheumatoid arthritis, Sjogren's syndrome, and gastritis.[24–29] Moreover, CXCL13 and CXCR5 are highly expressed in *Helicobacter*-induced MALT lymphoma patients,[30] and we previously reported that the formation of gastric lymphoid follicles at 1 month after *H. suis* infection, which are identified as clusters of mononuclear cells, were observed at the lamina propria of the gastric mucosa. At 3 months after infection, the follicles were >1 month, although their number was almost similar.[31] The amount of *H. suis* and the expression level of CXCL13 mRNA were increased at 3 months after infection compared with 1 month.[31] However, the detailed mechanism that links activation of CXCL13 and the formation of gastric lymphoid follicles after *Helicobacter* infection remains to be fully revealed.

In this study, we considered that it is suitable and easy to evaluate inhibitory effects of anti-CXCL13 antibody treatment on the follicles formation and *H. suis* clearance at the late stage (3 month infection) compared with at the earlier stage (1 month infection), because the amount of *H. suis* and the expression level of CXCL13 mRNA were increased at 3 months after infection compared with 1 month. Therefore, we examined the relationship between the activation of CXCL13 and the formation of gastric lymphoid follicles in C57BL/6J mice at 3 months after *H. suis* infection treated with a neutralizing anti-CXCL13 antibody. We show that the formation of gastric lymphoid follicles after *H. suis* infection is inhibited by anti-CXCL13 antibody treatment, suggesting a novel therapeutic approach for *Helicobacter* infection-related diseases.

RESULTS

Formation of gastric lymphoid follicles is inhibited by anti-CXCL13 antibody treatment

In previous studies, it has been reported that *H. suis* infection induced the formation of lymphoid follicles and MALT lymphoma in the stomachs of mice accompanied by the upregulation of CXCL13.[6,31,32] To investigate the role of CXCL13 in the induction of lymphoid follicles, *H. suis*-infected C57BL/6J mice were administered a neutralizing anti-CXCL13 antibody. As a result, the gastric lymphoid follicles were observed in the fundic area and the cardiac region of all the mice stomach ($n=10$) after isotype control antibody treatment (**Figure 1a**). However, the number of gastric lymphoid follicles after *H. suis* infection was significantly reduced by anti-CXCL13 antibody treatment (**Figure 1a** and **b**). In addition, to examine whether CXCL13 in the stomachs of *H. suis*-infected mice could be neutralized by anti-CXCL13 antibody treatment, the expression levels of CXCL13 mRNA in the stomachs were assayed by real-time PCR and the gastric tissues were immunostained with anti-CXCL13 and anti-B220 antibody. At 3 months after *H. suis* infection and weekly anti-CXCL13 antibody treatment starting on day 7, the expression levels of CXCL13 mRNA (**Figure 2a**) and CXCL13 protein levels were suppressed along with the number of gastric lymphoid follicles compared with the isotype control antibody-treated mice (**Figure 2b** and **c**). These results indicate that anti-CXCL13 antibody has a suppressive effect on the formation of gastric lymphoid follicles after *H. suis* infection.

Development of GCs, including B cells, CD4 + T cells, dendritic cells, and FDCs, after *H. suis* infection is inhibited by anti-CXCL13 antibody treatment

Next, we stained the cells in gastric lymphoid follicles induced by *H. suis* to characterize cell infiltrates. It has been reported that FDCs, B cells, CD4 + T cells, and dendritic cells (DCs) constitute the lymphoid follicles of the spleen and Peyer's patches.[33] A large number of each of these immunocompetent cells was observed in the gastric lymphoid follicles after *H. suis* infection and their infiltration was markedly reduced by anti-CXCL13 antibody treatment (**Figure 3a** and **c**).

Activation of NF-κB2 and IκBα after *H. suis* infection is suppressed by anti-CXCL13 antibody treatment

In a previous study, it has been reported that NF-κB2 regulates the expression of a restricted set of genes, including the gene encoding CXCL13, a crucial chemokine for the formation of the lymph nodes.[34] We examined the relationship between NF-κB2 activation and the formation of gastric lymphoid follicles after *H. suis* infection using immunostaining technique. NF-κB2 was strongly induced in the gastric lymphoid follicles where NF-κB2 staining coincides with the staining for FDC (**Figure 4a**). Moreover, NF-κB2 activation was also detected by Western blot analysis in the lymphoid follicles formed in the stomach along with the activation of inhibitor κBα (IκBα) (**Figure 4b**). These observations suggest that NF-κB2 is activated in the gastric lymphoid follicles formed in the stomach after *H. suis* infection through IκB activation, and this results in strongly inducing CXCL13.

Expression of lymphoid follicle-related genes is suppressed by anti-CXCL13 antibody treatment

It has been previously reported that the formation of lymphoid follicles is associated with the expression of LTA, LTB, tumor necrosis factor receptor 1 (TNFR1), LT B receptor (LTBR), CXCR5, and interferon-γ (IFNγ).[7,35–38] Therefore, we examined whether the expression of these lymphoid follicle-related genes is affected by anti-CXCL13 antibody treatment using real-time PCR. We observed that the mRNA expression levels of the LTA, LTB, CXCR5, TNFR1, LTBR, and IFNγ genes were significantly suppressed in anti-CXCL13 antibody-treated mice (**Figure 5a–f**). These results suggest that the formation of gastric lymphoid follicles after *H. suis* infection is closely associated with the activation of the lymphoid follicle-related genes, and that inhibition of such gene expression by anti-CXCL13 antibody treatment leads to the suppression of gastric lymphoid follicle formation.

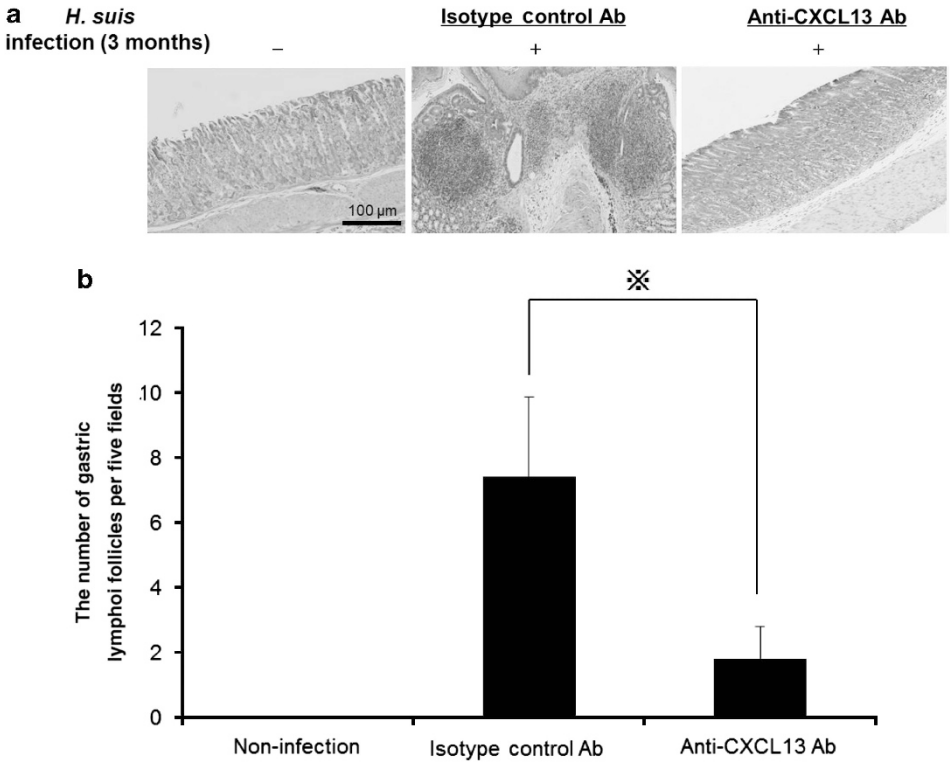

Figure 1 Suppression of the formation of gastric lymphoid follicles after anti-CXCL13 antibody treatment. The C57BL/6J mice (each $n=10$) were treated with anti-CXCL13 antibody or the isotype control antibody weekly for 3 months starting 1 week after *H. suis* infection as described in Methods. (**a**) Histological examination of the gastric mucosa in anti-CXCL13 antibody or the isotype control antibody-treated mice 3 months after *H. suis* infection was performed by hematoxylin and eosin staining. Original magnification $\times 200$. Data are representative of at least three independent experiments for each mouse ($n=10$) and typical images are shown. (**b**) The total number of lymphoid follicles per stomach per mouse was determined macroscopically and shown as the mean ± s.d. ($n=10$). $^{※}P<0.05$ according to the Student's *t*-test.

Deep invasion of *H. suis* into the gastric mucosa is limited by anti-CXCL13 antibody treatment

To detect the presence of *Helicobacter spp.* in *H. suis*-infected gastric homogenate, we performed PCR analysis for *H. pylori*, *H. felis*, *H. bizzozeronii*, *H. salomonis* (multiplex PCR analysis), and *H. suis* 16S rRNA. In gastric homogenates, only *H. suis* rRNA was detected (**Figure 6a**). Expression levels of *H. suis*-specific 16S rRNA gene were significantly reduced in the anti-CXCL13 antibody-treated group compared with the isotype control antibody-treated group (**Figure 6b**). The confocal microscopic analysis of *H. suis* in the gastric sections was performed using polyclonal rabbit anti-*H. pylori* antibody that has been reported to have the cross-reactivity with *H. suis*.[39,40] *H. suis* was located in the gastric mucosa of *H. suis*-infected mice that was inhibited by the treatment of anti-CXCL13 antibody (**Figure 6c**). These results suggest that anti-CXCL13 antibody might protect against the deep invasion of *H. suis* into the gastric mucosa.

Anti-*H. suis* IgA is significantly increased in the gastric juice of *H. suis*-infected mice after anti-CXCL13 antibody treatment

We have confirmed our previous observations[41] that anti-*H. suis*-specific immunoglobulin-G (IgG) is strongly induced in gastric juice of *H. suis*-infected mice (data not shown). In contrast, the level of anti-*H. suis* IgA in the gastric juice of the anti-CXCL13 antibody-treated mice is significantly higher than in the isotype control antibody-treated mice (**Figure 7a**). We confirmed by immunohistochemistry the presence of IgA in the stomachs of anti-CXCL13 antibody as well as isotype control antibody-treated mice, and also that anti-CXCL13 antibody treatment results in an increase in the IgA level in the stomach of both *H. suis*-infected and non-infected mice (**Figure 7b**).

Expression of TGF-β and interleukin-6 is induced by anti-CXCL13 antibody treatment

Various studies have demonstrated that transforming growth factor-β (TGF-β) is the major cytokine involved in promoting IgA class switching, and that IgA synthesis is enhanced by interleukin-6 (IL-6).[42,43] Real-time PCR results showed that expression of TGF-β and IL-6 was induced in the stomachs of anti-CXCL13 antibody-treated mice (**Figure 7c** and **d**), and there were significantly increased levels of TGF-β and IL-6 in anti-CXCL13 antibody-treated mice as compared with isotype control antibody-treated *H. suis*-infected mice. In addition, anti-CXCL13 antibody treatment significantly increased gastric TGF-β and IL-6 expression in the absence of *H. suis*

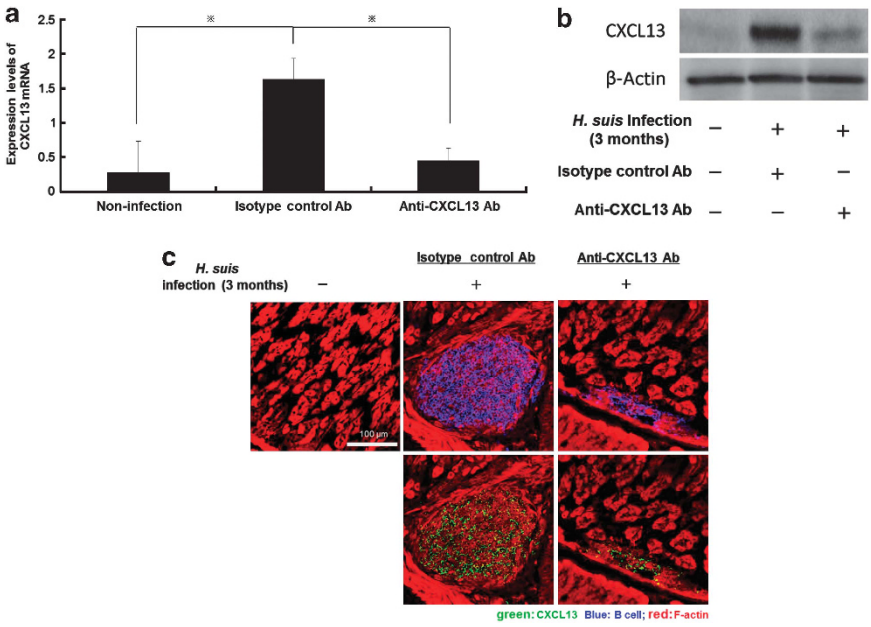

Figure 2 Suppression of expression of CXCL13 in the stomachs of *H. suis*-infected mice after anti-CXCL13 antibody treatment. The C57BL/6J mice (each $n = 10$) were treated with anti-CXCL13 antibody or the isotype control antibody weekly for 3 months starting 1 week after *H. suis* infection as described in Methods. (**a**) The mRNA expression levels of CXCL13 in the gastric mucosa were determined by real-time quantitative PCR. The mRNA expression levels were normalized to those of β-actin as an internal standard. Data are representative of at least three independent experiments for each mouse and shown as the mean ± s.d. ($n = 10$). $^{※}P < 0.05$ according to the Student's *t*-test. (**b**) The proteins were obtained from the stomach of the anti-CXCL13 antibody or isotype control antibody-treated mice with *H. suis* infection, and were subjected to immunoblotting using anti-CXCL13 antibody or anti-β-actin antibody. Data are representative of at least three independent experiments for each mouse ($n = 10$) and typical images are shown. (**c**) Immunohistological examination of the gastric mucosa was performed by confocal laser scanning microscopy, and the selected serial sections were stained with anti-CXCL13 antibody in a blind manner. Green: CXCL13; blue: B220; and red: F-actin. Original magnification × 200. Bar = 100 μm. Data are representative of at least three independent experiments for each mouse ($n = 10$) and typical images are shown.

infection, although not to the same high levels as in the presence of *H. suis* (**Figure 7d** and **e**).

DISCUSSION

Compared with *H. pylori, H. suis* is known to infect both animals and humans, and causes zoonotic disease.[1–4,11,44] In addition, we have determined that *H. suis* infection can induce the formation of gastric lymphoid follicles that[45] are believed to develop into gastric MALT lymphomas.[11] Interestingly, the expression of CXCL13 is significantly upregulated in the gastric MALT lymphomas in both humans and mice.[30,31] Therefore, we hypothesized that chemokine CXCL13 is critically involved in the formation of B-cell aggregates.

In this study, we have demonstrated that, in the stomachs of *H. suis*-infected mice, the formation of gastric lymphoid follicles consisting of B cells, CD4 + T cells, DCs, and FDCs is associated with the upregulation of CXCL13 and was inhibited by anti-CXCL13 antibody treatment. The present and previous studies have demonstrated the increased expression of CXCL13 in the FDC within the gastric mucosae of humans and mice with *Helicobacter*-induced MALT lymphoma, and found that these increases were induced via activation of the NF-κB2 pathway.[30,31,46] In this study, we have shown that in the gastric lymphoid follicles of *H. suis*-infected mice the upregulation of CXCL13 and its modulator NF-κB2 was significantly inhibited by anti-CXCL13 antibody treatment (**Figures 2a** and **b**, and **4a** and **b**). Thus, it seems that *H. suis* infection induces local CXCL13 biosynthesis and/or secretion in the gastric lymphoid follicles, and that CXCL13 may be a key regulator of tertiary lymphoid tissue formation during the course of *Helicobacter*-induced gastric B-cell aggregates.

In addition, the anti-CXCL13 antibody-treated mice displayed a markedly lower number of *H. suis* in their stomachs compared with the isotype control antibody-treated mice (**Figure 6b** and **c**), suggesting that anti-CXCL13 antibody induces a robust immunological response to protect against *H. suis* infection in addition to inhibit the formation of gastric lymphoid follicles. Secretory immunoglobulins such as IgA and IgG that are present in mucosal surfaces have an important role as the first line of defense against microorganisms in mucosal tissues. They help clear the invading pathogens and reduce subsequent colonization of respiratory epithelium.[47,48] IgA is the most abundant immunoglobulin isotype in mammals that can be produced in the absence of any organized follicular structures.[49–51] TGF-β is the major cytokine promoting IgA class switching, and IgA synthesis is enhanced by IL-6.[42,43] In this study, we found that IgA was observed in the gastric mucosa and submucosa but not in the gastric lymphoid follicles after CXCL13 antibody treatment (**Figure 7b**), which was consistent with a previous report that IgA^+ cells were mainly scattered in the lamina propria and corpus submucosa of *H. suis*-infected C57BL/6 mice.[52] No immunoglobulin-secreting

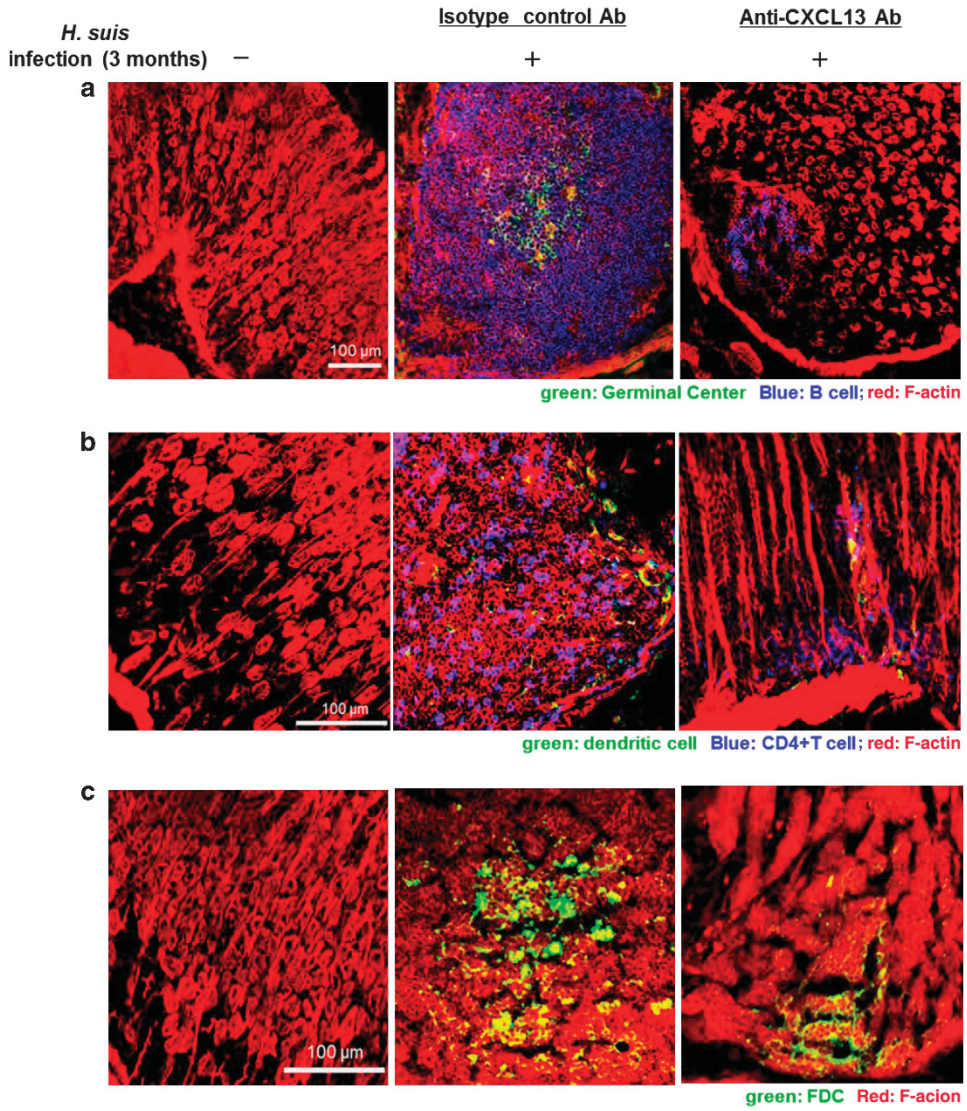

Figure 3 Immunohistological observation of B cells, CD4 + T cells, dendritic cells (DCs) and follicular DCs (FDCs) including germinal center in the stomachs of *H. suis*-infected mice after anti-CXCL13 antibody treatment. (**a**) The immunohistological examination of germinal center, B cells and F-actin was performed using confocal laser scanning microscopy (green: germinal center; red: B220; and blue: F-actin. Original magnification × 100). (**b**) The immunohistological examination of CD4-positive cells, DCs, and F-actin was performed by confocal laser scanning microscopy (blue: CD4; green: DC; and red: F-actin. Original magnification × 200). (**c**) The immunohistological examination of FDCs and F-actin was performed using confocal laser scanning microscopy (green: FDC and red: F-actin. Original magnification × 200). Bar = 100 μm. Data are representative of at least three independent experiments for each mouse ($n = 10$) and typical images are shown.

cells were found in gastric lymphoid follicles.[52] Furthermore, it has also been reported that in *H. pylori*-infected mice, most of the IgA in gastric secretion, either antigen-specific or non-specific, was derived from swallowed saliva, suggesting that salivary glands had an important role in the induction and maintenance of gastric immunity to *H. pylori* in mice.[53] Therefore, although fewer B cells were infiltrated in the stomach after CXCL13 antibody treatment, B cells consisting of gastric lymphoid follicles might not contribute to antibody production after *Helicobacter* infection. Taken together, we consider that most of the IgA might be secreted from the IgA^+ B cells in salivary glands, which can be promoted in the present of the TGF-β, IL-6, and interleukin-10 (IL-10) activation after anti-CXCL13 antibody treatment. Moreover, we demonstrated that anti-*H. suis*-specific IgG was strongly induced in gastric juice by *H. suis* infection (data not shown), and that there were no differences in the levels of anti-*H. suis* IgG in gastric juice of anti-CXCL13 antibody treated as compared with isotype control antibody-treated mice. However, the level of anti-*H. suis*-specific IgA was significantly increased in the gastric juice of anti-CXCL13 antibody-treated mice (**Figure 7a**). Recently, Cerutti *et al.* and He *et al.* reported that the presence of IL-10, TGF-β, and IL-6 can further emphasize to accumulate the IgA production.[54,55] On the other hand, Zheng *et al.*[25] reported that anti-CXCL13 antibody treatment can induce the upregulation of IL-10 production and contributes to the suppression of

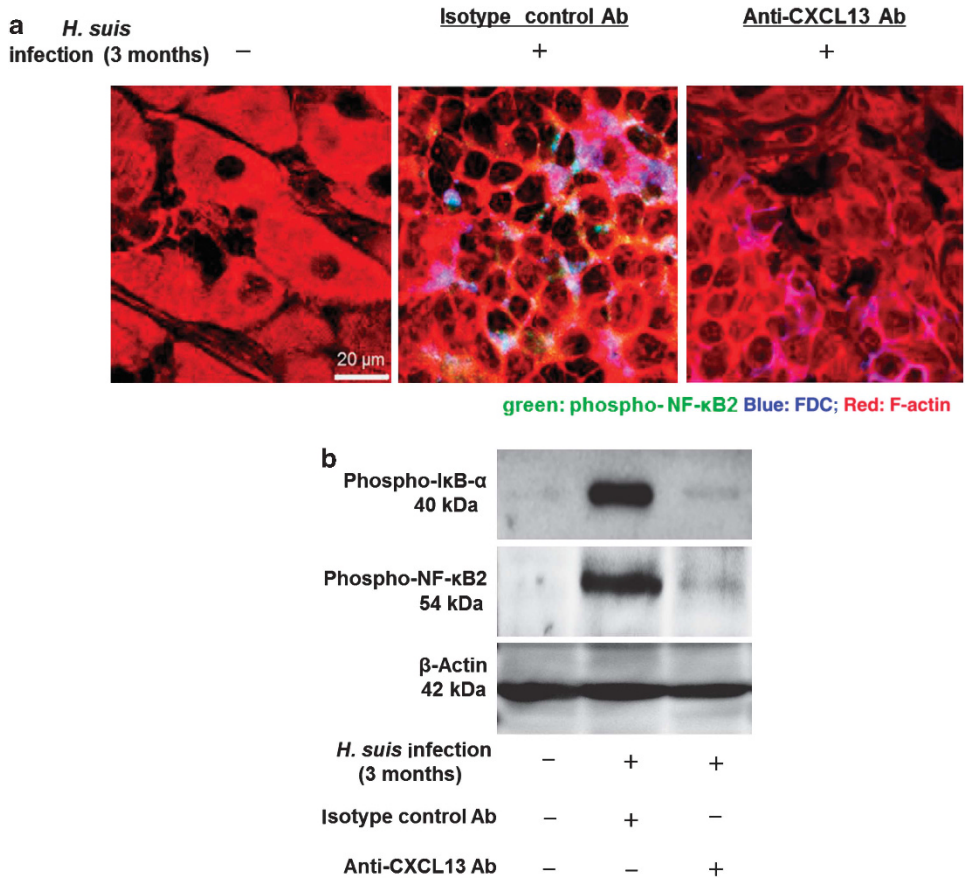

Figure 4 Suppression of NF-κB2 activation in the stomachs of *H. suis*-infected mice after anti-CXCL13 antibody treatment. (**a**) The immunohistological examination of NF-κB2, FDCs, and F-actin was performed using confocal laser scanning microscopy (green: NF-κB2; blue: FDC and red: F-actin. Original magnification × 1,000). Bar = 20 μm. The purified monoclonal rat anti-mouse FDC M1 antibody (BD Biosciences) was used to detect the FDCs in the gastric lymphoid follicles. (**b**) The proteins were obtained from the stomach of the anti-CXCL13 antibody or isotype control antibody-treated mice with *H. suis* infection, and were subjected to immunoblotting using anti-phosphorylated I-κBα antibody, anti-phosphorylated anti-NF-κB2 antibody, or anti-β-actin antibody. Data are representative of at least three independent experiments for each mouse ($n = 10$) and typical images are shown.

collagen-induced arthritis in anti-CXCL13 antibody-treated mice.[25] In our study, we also found that the anti-CXCL13 antibody treatment on the *H. suis*-infected mice leads to the activation of TGF-β and IL-6, which can promote the production of anti-*H. suis*-specific IgA in the stomach (**Figure 7a**, **c** and **d**). Thus, the administration of anti-CXCL13 antibody to the *H. suis*-infected mice may inhibit *H. suis* colonization by increasing the *H. suis*-specific IgA level via the induction of TGF-β, IL-6, and IL-10 expression, and thereby reducing formation of inflammatory follicles that can lead to suppressing gastric B-cell aggregates. In addition, the levels of IgA in the stomachs of anti-CXCL13 antibody-treated mice were increased regardless of *H. suis* infection (**Figure 7b**), indicating that regulation of CXCL13 might be a valuable therapeutic tool in the treatment of bacterial infection-related diseases including *Helicobacter*-related diseases.

In conclusion, our findings show that the formation of gastric lymphoid follicles after *H. suis* infection is efficiently suppressed by the administration of anti-CXCL13 antibody. The neutralization of CXCL13 might provide a new therapeutic approach for bacterial infection-related diseases including *Helicobacter* infection.

METHODS

Ethics statement and mice. All animal studies were performed in accordance with the guideline and law by the Ministry of Education, Culture, Sports, and Science and Technology, and the Ministry of Health, Labour and Welfare in Japan. This study was approved by the Institutional Animal Care and Use Committee (permission number: P110609) and carried out according to the Kobe University Animal Experimentation Regulations. C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and bred under standard laboratory conditions.

***H. suis* infection and confirmation**. C57BL/6J mice are orally infected with *H. suis*, which was originally obtained from a cynomolgus monkey. One week after the *H. suis* infection, weekly administration of 0.6 mg IP of anti-CXCL13 antibody (Vaccinex, Rochester, NY) or the isotype control antibody (Vaccinex) was started. Twelve weeks after *H. suis* infection, the mice were killed, and then *H. suis* infection was confirmed by PCR of DNA samples extracted from gastric mucosal homogenates using the primers for *H. suis* 16S rRNA sense 5′-TTGGGAGGCTTTGTCTTTCCA-3′ and antisense

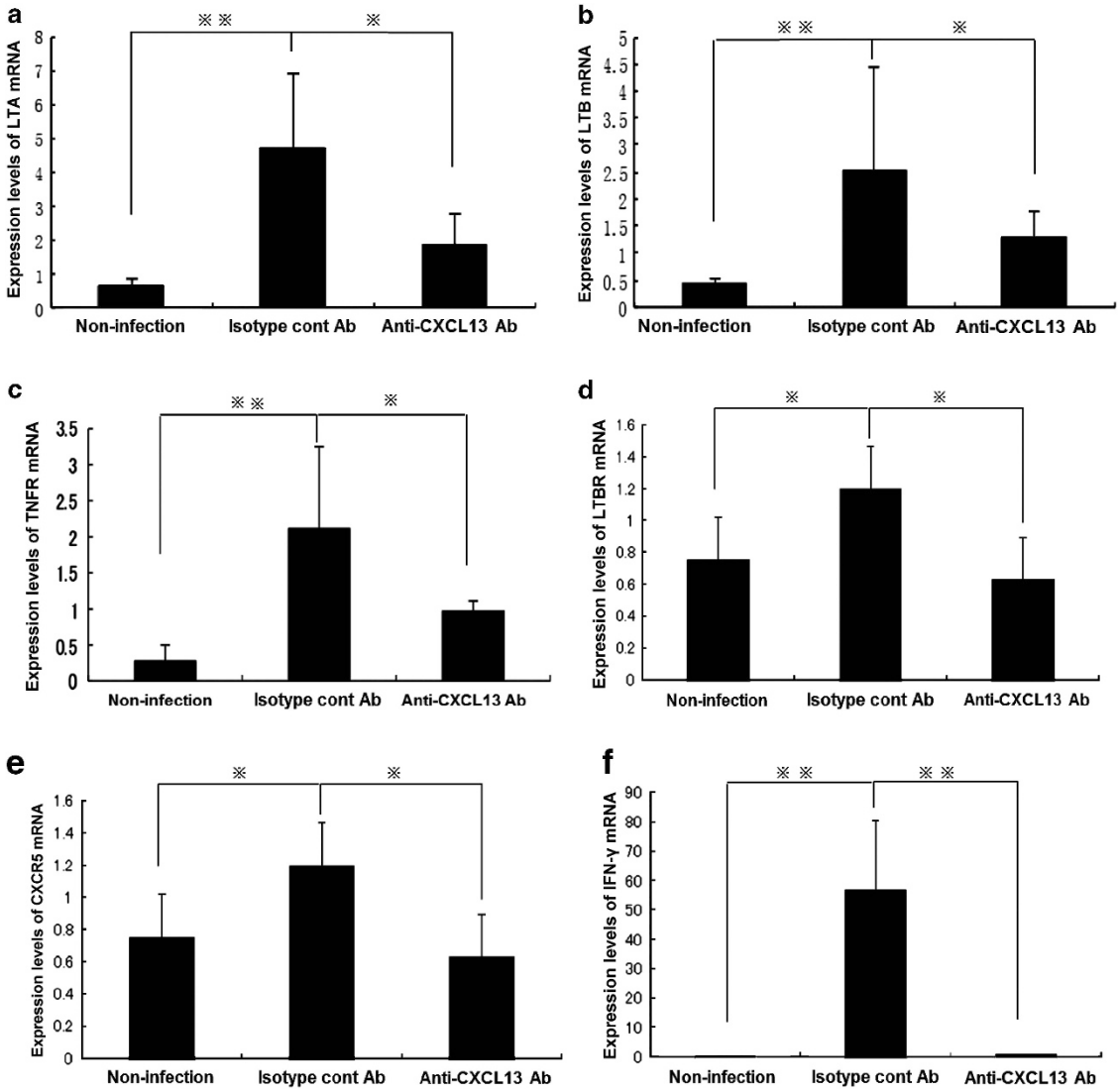

Figure 5 The expression level of lymphoid follicles associated genes in the stomachs of *H. suis*-infected mice after anti-CXCL13 antibody treatment. The C57BL/6J mice (each $n = 10$) were treated with anti-CXCL13 antibody or the isotype control antibody weekly for 3 months starting 1 week after *H. suis* infection as described in Methods. The mRNA expression levels of lymphotoxin A (**a**), lymphotoxin B (**b**), CXCR5 (**c**), tumor necrosis factor receptor 1 (TNFR1) (**d**), lymphotoxin B receptor (LTBR) (**e**), and interferon-γ (IFNγ) (**f**) in the gastric mucosa were determined by real-time quantitative PCR. The mRNA expression levels were normalized to those of β-actin as an internal standard. Data are representative of at least three independent experiments for each mouse and shown as the mean ± s.d. ($n = 10$). $^{※}P < 0.05$; $^{※※}P < 0.01$ according to the Student's *t*-test.

5′-GATTAGCTCTGCCTCGCGGCT-3′. The control experiment was performed using primers for *H. pylori* 16S rRNA sense 5′-TGCGAA GTGGAGCCAATCTT-3′ and antisense 5′-GGAACGTATTCACC GCAACA-3′; the multiplex PCR primers for *H. felis*, *H. bizzozeronii* and *H. salomonis*-specific 16S rRNA sense 5′-TGCGTAGGCGGGGT TGTAAG-3′ and antisense 5′-CAGAGTTGTAGTTTCAAATGC-3′, (see refs. 41, 56). To confirm the *H. suis* localization, immunostaining experiments were also carried out using a cross-reacting anti-*H. pylori* antibody as reported previously.[41]

PCR amplification. The PCR amplification reactions involved 1 × reaction buffer (20 mM Tris/HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween-20, 0.5% Nonidet P40, and 50% glycerol) containing 1 unit of Taq DNA polymerase (Toyobo, Osaka, Japan); 10 nmols of each deoxynucleotide triphosphate; 10 pmols of each oligonucleotide primer; and 1 μl of the diluted DNA, which was usually prepared by 1:10 dilution of the original samples with a DNA concentration of ∼20–100 ng μl^{-1} in a final volume of 50 μl. The cycling conditions for the *H. suis*, *H. pylori*-specific 16S rRNA reactions, and the multiplex PCR analysis for *H. felis*-, *H. bizzozeronii*-, and *H. salomonis*-specific 16S rRNA reactions involved 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The PCR products were separated on agarose mini-gels in TAE buffer (40 mM Tris/acetate and 1 mM EDTA) and then were photographed under UV transillumination after being stained with ethidium bromide.

Histological examination. Twelve weeks after *H. suis* inoculation and antibody treatment (i.e., anti-CXCL13 antibody or isotype control antibody), the infected mice were killed by cervical dislocation under anesthesia. The stomachs were resected and opened at the outer curvature. The stomachs were then sliced longitudinally from the esophagus to the duodenum. Half of the stomach was embedded in paraffin wax; one quarter of the stomach was used for DNA and RNA extraction, as described below; and the remaining specimen was frozen

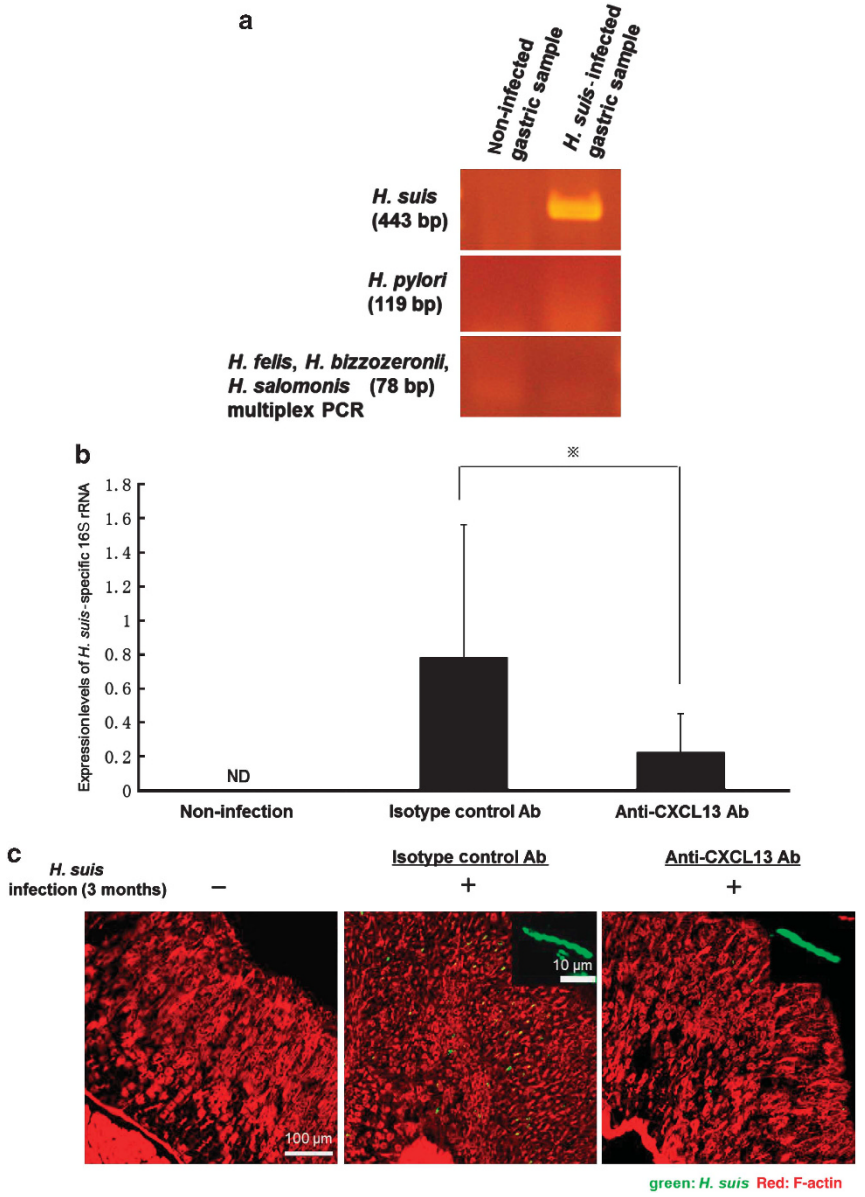

Figure 6 The colonization of *H. suis* in the stomach of mice. (**a**) The PCR analysis of DNA samples extracted from homogenates of gastric mucosa for the *H. suis* 16S rRNA gene, the *H. pylori* 16S rRNA gene, and multiplex 16S rRNA genes from *H. felis*, *H. bizzozeronii*, and *H. salomonis*. Data are representative of at least three independent experiments for each mouse ($n = 10$) and typical images are shown. (**b**) The level of *H. suis* 16S rRNA was evaluated by real-time PCR using RNA samples extracted from homogenates of gastric mucosa of the *H. suis*-infected mice with and without anti-CXCL13 antibody treatment. The quantitative values were normalized with mouse β-actin expression levels in each sample. 'NS' indicates no significant difference. Ten of *H. suis*-infected mice were used to detect the colonization of *H. suis* in the stomach of mice. Data are representative of at least three independent experiments for each mouse and shown as the mean ± s.d.. ($n = 10$). $^{※}P < 0.05$ according to the Student's *t*-test. (**c**) Immunohistochemical staining of *H. suis* in the stomach of *H. suis*-infected mice with and without the treatment of anti-CXCL13 antibody or the isotype control antibody. The selected serial sections were stained with polyclonal rabbit anti-*H. pylori* antibody in a blind manner. Green: *H. suis* and red: F-actin. Original magnification: × 100. Bar = 100 μm. Enlarged view; green: *H. suis*. Original magnification: × 1,000. Bar = 10 μm. Data are representative of at least three independent experiments for each mouse ($n = 10$) and typical images are shown.

in OCT compound (Sakura Finetek, Tokyo, Japan). The paraffin-embedded tissues were longitudinally sliced into three specimens and then stained with hematoxylin and eosin. All section samples include both the corpus and the antrum, and the total number of the gastric lymphoid follicles identified in three specimens from each mouse was counted in a blinded manner. In graphs of the experimental results, the *y* axis shows the average number of follicles identified in each group ($n = 10$).

Antibodies. The following antibodies were used: purified monoclonal rat anti-mouse CD45R antibody (BD Biosciences, Franklin Lakes, NJ), purified monoclonal rat anti-mouse FDC M1 antibody (BD Biosciences), purified monoclonal rat anti-mouse CD4 antibody (BD Biosciences), FITC-conjugated monoclonal hamster anti-mouse CD11c antibody (BD Biosciences), goat anti-CXCL13 (R&D Systems, Minneapolis, MN), goat anti-mouse IgA antibodies (Southern Biotech, Birmingham, AL), rabbit anti-mouse NF-κB phospho ser865 (Abcam,

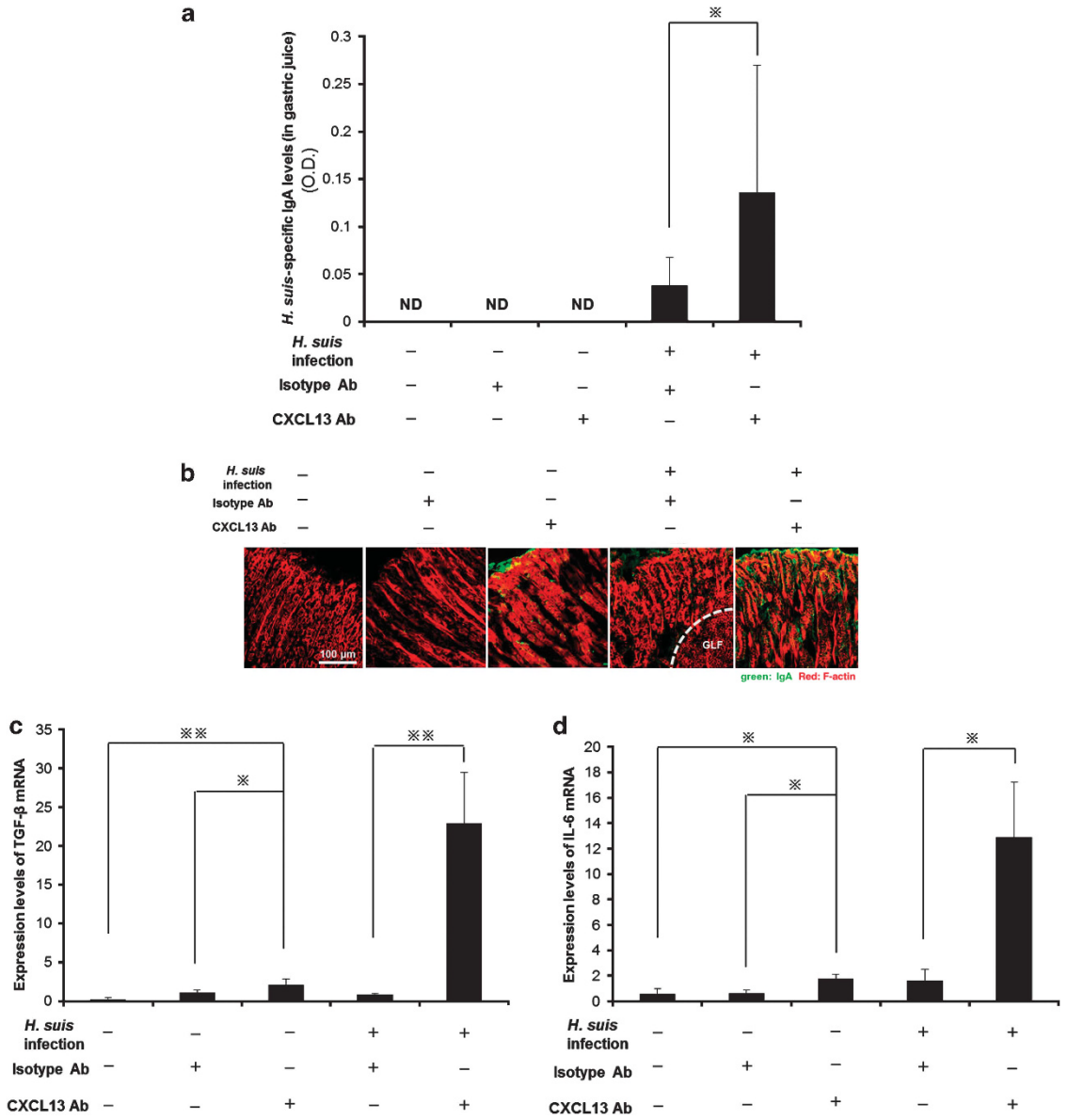

Figure 7 The *H. suis*-specific IgG and IgA levels in gastric juice via the induction of IgA class switch-related genes of *H. suis*-infected mice after anti-CXCL13 antibody treatment. The C57BL/6J mice (each $n=10$) were treated with anti-CXCL13 antibody or the isotype control antibody weekly for 3 months starting 1 week after *H. suis* infection as described in Methods. The levels of the anti-*H. suis* IgA in gastric juice of the mice (**a**) were measured by enzyme-linked immunosorbent assay. Data are representative of at least three independent experiments for each mouse and shown as the mean ± s.d. ($n=10$). $^{※}P<0.05$ according to the Student's *t*-test. (**b**) Immunohistological examination of the gastric mucosa was performed by confocal laser scanning microscopy, and the selected serial sections were stained with anti-IgA antibody in a blind manner. Green: IgA and red: F-actin. Original magnification: × 100. Bar = 100 μm. Data are representative of at least three independent experiments for each mouse ($n=10$) and typical images are shown. The mRNA expression levels of TGF-β (**c**) and interleukin (IL)-6 (**d**) in the gastric mucosa were determined by real-time quantitative PCR. The mRNA expression levels were normalized to those of β-actin as an internal standard. Data are representative of at least three independent experiments for each mouse and shown as the mean ± s.d. ($n=10$). $^{※}P<0.05$ and; $^{※※}P<0.01$ according to the Student' s *t*-test.

Cambridge, UK), biotinylated peanut agglutinin (Vector Laboratories, Burlingame, CA), Alexa488-conjugated polyclonal goat anti-rat IgG antibody (Invitrogen, Eugene, OR), Alexa642-conjugated polyclonal goat anti-rat IgG antibody (Invitrogen), Alexa642-conjugated polyclonal chicken anti-rat IgG antibody (Invitrogen), Alexa488-conjugated polyclonal goat anti- rabbit IgG antibody (Invitrogen), Alexa488-conjugated polyclonal rabbit anti-goat IgG antibody (Invitrogen), and DyLIGHT 488 Streptavidin (Jackson ImmunoResearch Labs, West Grove, PA). The F-actin in the sections was stained with Alexa546-conjugated phalloidin (Invitrogen).

Immunofluorescence staining. A fluorescence immunohistological examination was carried out using frozen sections obtained from the stomachs of mice at 12 weeks after *H. suis* infection and the antibody treatment. The sections were air-dried, fixed in acetone for 5 min, and then blocked with 10% goat serum or 1% horse serum for 30 min. After

being washed with PBS, the sections were incubated with appropriate antibodies for overnight at 4 °C before being treated with the corresponding secondary antibodies for 60 min at room temperature. These sections were then observed using a confocal laser scanning microscope (LSM 5 PASCAL, Carl Zeiss, Jena, Germany).

Immunoblotting. Cytoplasmic proteins from the stomachs of mice were extracted using NE-PER cytoplasmic extraction reagents (Thermo Scientific, Bremen, Germany), and the lysates were subjected to SDS-PAGE and subsequent electrotransfer onto nitrocellulose membranes. The membranes were blocked with Blocking One-P (Nacalai Tesque, Kyoto, Japan), and were subsequently treated with the primary antibodies for phospho-NF-κB2, phospho-IκBα, and β-actin.

Quantitative real-time PCR. The mucosal and submucosal layers of the stomach were homogenized with 1 ml of TRIZOL reagent (Invitrogen), and RNA was extracted from the homogenates according to the manufacturer's instructions. RNA was subjected to the reverse transcription reaction using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols, and quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7500 Real Time PCR system (Applied Biosystems) according to the manufacturer's instructions. Specific primer pairs (Hokkaido System Science, Sapporo, Japan) used for real-time PCR were as follows: LTA sense 5′-GCTTGGCACCCC TCCTGTC-3′ and antisense 5′-GATGCCATGGGTCAAGTGCT-3′; LTB sense 5′-CCAGCTGCGGATTCTACACCA-3′ and antisense 5′-AGCCCTTGCCCACTCATCC-3′; LTBR sense 5′-CCAGATGTG AGATCCAGGGC-3′ and antisense 5′-GACCAGCGACAGCAGG ATG-3′; TNFR1 sense 5′-TCCGCTTGCAAATGTCACA-3′ and antisense 5′-GGCAACAGCACCGCAGTAC-3′; CXCR5 sense 5′-AACATCCTGGTGCTGGTAATCC-3′ and antisense 5′-GGCTA CTGCGAGGTGGAACA-3′; IFNγ sense 5′-GCGTCATTGAAT CACACCTG-3′ and antisense 5′-TGAGCTCATTGAATGCTTGG-3′; CXCL13 sense 5′-CATAGATCGGATTCAAGTTACGCC-3′ and antisense 5′-TCTTGGTCCAGATCACAACTTCA-3′; TGF-β sense 5′-CACTGATACGCCTGAGTGR-3′ and antisense 5′-GTGAGCG CTGAATCGAAA-3′; IL-6 sense 5′-GAGGATACCACTCCCAAC AGACC-3′ and antisense 5′-AAGTGCATCATCGTTGTTCATA CA-3′; *H. suis*-specific 16S rRNA gene sense 5′-AGACAAAGCCTCC CAACAAC-3′ and antisense 5′-ATCACTGACGCTGATTGCAC-3′; and β-actin sense 5′-AAGGCCAACCGTGAAAAGAT-3′ and antisense 5′-GTGGTACGACCAGAGGCATAC-3′. To allow a relative comparison of RNA expression levels, the data from real-time PCR were normalized to the amount of β-actin cDNA as an endogenous control.

Enzyme-linked immunosorbent assay. To detect *H. suis*-specific IgG and IgA in the serum and gastric juice, the gastric juice was centrifuged at 16,000 *g* for 5 min at 4 °C, and the resultant supernatant was collected. The serum was separated from the blood by centrifugation at 15,000 *g* for 10 min at 4 °C. Ninety six-well plates were coated overnight at 4 °C with 100 µl of a bicarbonate solution (pH 9.6) containing 100 µg ml^{-1} *H. pylori* lysate, and blocked by the addition of 1.5% (wt/vol) BSA in PBS for 1 h at 37 °C. The serum and gastric juice, which were diluted at 1:200 and 1:15, respectively, were added to the plates, followed by addition of 100 µl of HRP-conjugated goat anti-mouse IgG antibody (Bio-Rad Laboratories, Hercules, CA) or HRP-conjugated goat anti-mouse IgA antibody (Southern Biotech) diluted at 1:5,000 in PBST with 0.05% (wt/vol) Tween-20 containing 0.2% (wt/vol) BSA. The bound antibody was detected by addition of *o*-phenylenediamine substrate, and measurement of absorbance at 490 nm was carried out.

Statistical analysis. All results are expressed as mean ± s.d. Statistical significance was analyzed using the Student's *t*-test for comparisons between two groups, and non-repeated measures analysis of variance followed by the Bonferroni test for comparisons among three or more groups. A level of $P = 0.05$ or $P = 0.01$ was used as the criterion of significance.

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DISCLOSURE

Ekaterina Klimatcheva, Tracy Pandina, and Maurice Zauderer are employees of Vaccinex that is developing an antibody to CXCL13. This does not alter our adherence to all Mucosal Immunology policies on sharing data and materials. The remaining authors declare no conflict of interest.

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