Interferon- γ -producing B cells induce the formation of gastric lymphoid follicles after *Helicobacter suis* infection

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Helicobacter (H.) suis is capable of infecting various animals including humans, and *H. suis* infections can lead to gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Recently, we reported that interferon- γ (IFN- γ) was highly expressed in the stomachs of *H. suis*-infected mice, but the direct relationship between the upregulation of IFN- γ expression and the formation of gastric lymphoid follicles after *H. suis* infection remains unclear. Here, we demonstrated that the IFN- γ produced by B cells plays an important role in the formation of gastric lymphoid follicles after *H. suis* infection. In addition, IFN- γ -producing B cells evoked gastric lymphoid follicle formation independent of T-cell help, suggesting that they are crucial for the development of gastric MALT induced by *Helicobacter* infection.

INTRODUCTION

Helicobacter (H.) suis is a Gram-negative bacterium that colonizes the stomachs of various animals such as pigs, dogs, cats, and monkeys.^{1–3} It has also been detected in the stomachs of humans with gastric disorders after endoscopic examination, indicating that the infection rate of *H. suis* in different countries is varying from 0.2 to 6%. In humans, *H. suis* infection may occur zoonotically through close contact with the above-mentioned animals^{4,5} and has been reported to associate with gastritis,^{6,7} peptic ulcers,⁸ and gastric cancer.^{9,10} Furthermore, gastric mucosa-associated lymphoid tissue (MALT) lymphoma occurs more frequently in *H. suis*-infected patients compared with *H. pylori*-infected patients and it can form in almost 100% of C57BL/6J mice at 6 months after infection, suggesting that *H. suis* has a greater tendency to induce gastric MALT lymphoma than other *Helicobacter* species.^{11–13}

Long-term antigenic stimulation by a *Helicobacter* infection results in the activation of innate and adaptive immune cells in the inflamed gastric mucosa, leading to the establishment of cytokine, chemokine, and adhesion molecule networks

that are critical for the recruitment of peripherally primed lymphocytes and ectopic lymphoid follicle organization.^{14,15} This tertiary lymphoid tissue has a similar structure to secondary lymphoid tissue and provides a histological background that can ultimately progress to gastric MALT lymphoma.^{12–15}

Helicobacter infection can induce the production of T helper type 1 (Th1) cytokines such as interferon (IFN)- γ , and T helper type 2 (Th2) cytokines such as interleukin (IL)-4 that are responsible for cellular immunity and humoral immunity, respectively.¹⁶ It is known that *H. pylori* infection predominately induces the Th1 response because gastric CD4⁺ T cells produce high levels of IFN- γ but not IL-4.^{17,18} Regarding the development of *H. pylori*-induced chronic gastritis, Peyer's patches, where coccoid *H. pylori* are captured by dendritic cells (DCs),¹⁹ are crucial induction sites, and the IFN- γ secreted by activated CD4⁺ T cells is indispensable.²⁰ Furthermore, multiple studies have revealed that *H. pylori*-specific CD4⁺ T cells are important for the activation and proliferation of mucosal B cells in gastric MALT lymphoma^{21,22} and that

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tumor-infiltrating CD4⁺ T cells predominantly produce IL-4.²³ In contrast, our previous studies reported that *H. suis* induced the development of gastric lymphoid follicles in mice independently of Peyer's patches.²⁴ In a previous study, severe gastritis involving neutrophil infiltration, mucosal atrophy, and intestinal metaplasia were absent in the stomachs of *H. suis*-infected mice although IFN- γ was highly expressed.²⁵ The upregulation of IFN- γ expression is necessary for the formation of gastric lymphoid follicles after *H. suis* infection, but IL-4 is dispensable.²⁵ These intriguing discrepancies might be because of the different pathogenic mechanisms of *H. suis* and *H. pylori*.

Generally, conventional and ectopic lymphoid tissues containing B cells and T cells are organized in a similar manner. CD4⁺ T cells provide specific help to antigen-activated B cells via costimulatory cytokines during lymphoid tissue development.^{26,27} However, isolated lymphoid follicles, which belong to gut-associated lymphoid tissue as tertiary lymphoid structures, also formed in T cell-deficient mice.²⁸ On the other hand, it has been widely reported that CD4⁺ T cells produce IFN- γ , but there is also evidence that other immune cells such as B cells, DCs, and natural killer cells have the ability to secrete IFN- γ .^{29–33} Therefore, it remains to be clarified whether T cells are required for the formation of gastric lymphoid follicles and which kinds of cells produce IFN- γ in the stomach after *H. suis* infection.

In this study, we found that *H. suis* infection induced the formation of gastric lymphoid follicles without help from T cells and, furthermore, the IFN- γ produced by B cells directly contributed to the development of gastric lymphoid follicles. Therefore, we suggest that B cell-produced IFN- γ is involved in the formation of gastric MALT after *Helicobacter* infection.

RESULTS

The *H. suis* infection-induced formation of gastric lymphoid follicles consisting of B cells, CD4⁺ T cells, DCs, and FDCs was dependent on IFN- γ

Previously, we reported that IFN- γ was highly expressed in the stomachs of *H. suis*-infected mice that might be associated with the formation of gastric lymphoid follicles.²⁵ In this study, to clarify whether IFN- γ is required for the formation of gastric lymphoid follicles, wild-type (WT) and IFN- γ knockout (KO) mice were infected with *H. suis* for 6 months. As a result, lymphoid follicles were detected in the fundic area and the cardiac region of all the *H. suis*-infected WT mice, but not the IFN- γ KO mice (**Figure 1a**). To elucidate which types of immunocompetent cells had infiltrated the follicles during the *H. suis* infection, immunofluorescent staining was performed with appropriate antibodies. Similar to gastric MALT lymphoma,^{23,26} B cells, CD4⁺ T cells, DCs, and follicular DC (FDCs) were present in the gastric lymphoid follicles of the *H. suis*-infected WT mice. However, the infiltration of these cells was completely suppressed in the *H. suis*-infected IFN- γ KO mice (**Figure 1b**). Germinal centers are unique microarchitectural units within B-cell

follicles and the majority of B-cell lymphomas originate from germinal center B cells³⁴ that express activation-induced cytidine deaminase (AID) contributing to antibody production and B-cell lymphomagenesis.³⁵ We found that AID was positive in the gastric lymphoid follicles of *H. suis*-infected WT mice (**Figure 1c**) that indicated the formation of germinal centers and might be involved in B-cell malignancy. In addition, natural killer cells are IFN- γ -producing cells,²⁹ but they were absent from the *H. suis*-infected mice at both 1.5 months (data not shown) and 6 months after infection (**Figure 1b**). These results suggest that B cells, CD4⁺ T cells, DCs, and FDCs might be important for the production of IFN- γ that is required for the formation of gastric lymphoid follicles.

IFN- γ was highly expressed in the stomachs of the WT mice after *H. suis* infection

The expression of IFN- γ mRNA in the gastric mucosa was examined by quantitative real-time PCR. Consistent with our previous report,²⁵ the upregulation of IFN- γ at 1.5 months after infection was not significant compared with that detected in the non-infected WT mice. At 6 months after *H. suis* infection, IFN- γ expression level was dramatically increased in the WT mice that was higher than 3 months after infection. Conversely, IFN- γ was undetectable in the IFN- γ KO mice (**Figure 2a** and **Supplementary Figure S1** online). Immunofluorescent staining revealed that IFN- γ expression was widely induced in the gastric lymphoid follicles and surrounding sites of the WT mice after *H. suis* infection (**Figure 2b**). These findings indicate that *H. suis* infection strongly induced the upregulation of IFN- γ expression in the stomachs of the WT mice.

H. suis was localized in the gastric mucosa, and the bacterial load was higher in the stomachs of the IFN- γ KO mice

At 6 months after infection, *H. suis* was detected in the stomachs of the mice by quantitative real-time PCR and immunofluorescent staining. As a result, *H. suis* was found in the gastric mucosa, and higher numbers of the bacteria were detected in the stomachs of the IFN- γ KO mice than in those of the WT mice (**Figure 3**), suggesting that IFN- γ is critical for the control of *Helicobacter* infections during the adaptive phase of the immune response.^{20,36}

The expression of IFN- γ activation-related genes was upregulated in the stomachs of the WT mice after *H. suis* infection

IL-12 is the major stimulator of IFN- γ production after bacterial infection. It is produced by monocytes, macrophages, DCs, neutrophils, and B cells.³⁷ T-bet is a transcription factor that directly activates IFN- γ gene expression.¹⁶ T cells, B cells and DCs stimulated by IL-12 can produce IFN- γ through an T-bet-IFN- γ receptor (IFNGR)-dependent pathway.^{16,31,37,38} Therefore, we examined the expression levels of these IFN- γ activation-related genes in the stomachs of the mice after *H. suis* infection. The mRNA level of IL-12 was significantly increased in the stomachs of both the WT and

Figure 1 Histological and immunohistological examinations of the gastric mucosae of mice at 6 months after *Helicobacter suis* infection. C57BL/6J wild-type (WT) mice ($n=5$) and interferon- γ knockout (IFN- γ KO) mice ($n=5$) were infected with *H. suis* for 6 months as described in the Methods. **(a)** Histological examinations of the gastric mucosae of WT and IFN- γ KO mice (each $n=5$) were performed at 6 months after *H. suis* infection using hematoxylin and eosin staining. (A) Uninfected WT mice; (B) *H. suis*-infected WT mice; (C) uninfected IFN- γ KO mice; (D) *H. suis*-infected IFN- γ KO mice. Original magnification: $\times 200$. The black bar indicates 100 μm . **(b)** Immunohistological examination of immunocompetent cells in the gastric mucosae of *H. suis*-infected WT and IFN- γ KO mice (each $n=5$) at 6 months after infection. Immunohistological examinations of B220-positive cells (B cells), CD4-positive cells (T helper cells), CD11c-positive cells (dendritic cells (DCs)), follicular DCs (FDCs), NK1.1-positive cells (natural killer (NK) cells), and phalloidin (F-actin) were performed using confocal laser scanning microscopy (red: B220 and CD4; green: CD11c, FDC-M₁, and NK1.1; blue: F-actin; original magnification: $\times 100$). (A–D) *H. suis*-infected WT mice; (E–H) *H. suis*-infected IFN- γ KO mice. The white bar indicates 100 μm . Isotype controls for positive staining are shown as the small insets. **(c)** Immunohistochemical staining of activation-induced cytidine deaminase (AID) in the stomach of WT mice at 6 months after *H. suis* infection. (A) Uninfected WT mice; (B) *H. suis*-infected WT mice. Original magnification: $\times 400$. The black bar indicates 100 μm . Isotype control for positive staining is shown as the small inset. Data are representative of at least three independent experiments and typical images are shown.

Figure 2 The expression of interferon- γ (IFN- γ) in the gastric mucosae of wild-type (WT) and IFN- γ knockout (KO) mice at 6 months after *Helicobacter suis* infection. **(a)** The IFN- γ mRNA expression levels in the gastric mucosae of WT and IFN- γ KO mice (each $n = 5$) at 6 months after *H. suis* infection were determined by quantitative real-time PCR and normalized to that of β -actin as an internal standard. Data are representative of at least three independent experiments and shown as scatter plots. The horizontal line represents the median value. *Significant difference ($P < 0.05$); ND, not detected. **(b)** The immunohistological examinations of IFN- γ expression in the gastric mucosae of WT mice ($n = 5$) and IFN- γ KO mice ($n = 5$) were performed using confocal laser scanning microscopy (green: IFN- γ ; blue: F-actin). Original magnification: $\times 100$. (A) Uninfected WT mice; (B) *H. suis*-infected WT mice; (C) uninfected IFN- γ KO mice; (D) *H. suis*-infected IFN- γ KO mice. The white bar indicates 100 μ m. Isotype control for positive staining is shown as the small inset. Data are representative of at least three independent experiments and typical images are shown. A full color version of this figure is available at the *Mucosal Immunology* journal online.

IFN- γ KO mice (Figure 4a). The mRNA expression levels of IFNGR and T-bet were upregulated in the *H. suis*-infected WT mice (as was IFN- γ), but the mRNA expression of these molecules was impaired in the IFN- γ KO mice (Figure 4b,c). These results suggest that the activation of these genes is involved in the production of IFN- γ in the stomach after *H. suis* infection.

***H. suis* induced the formation of gastric lymphoid follicles in TCR KO mice**

It has been reported that CD4⁺ T cells and the IFN- γ they produce play important roles in the pathogenesis of *H. pylori*-induced gastric diseases.^{17,20–22} On the other hand, the development of organized follicular structures, such as Peyer's patches, in the gut was impaired in T cell receptor β and δ

Figure 3 The colonization of *Helicobacter suis* in the gastric mucosae of wild-type (WT) and interferon- γ knockout (IFN- γ KO) mice at 6 months after infection. C57BL/6J WT mice ($n=5$) and IFN- γ KO mice ($n=5$) were infected with *H. suis* for 6 months as described in the Methods. (a) The expression level of *H. suis* 16S rRNA was estimated by real-time PCR using RNA samples extracted from the gastric mucosa homogenates of the WT and IFN- γ KO mice (each $n=5$) at 6 months after *H. suis* infection. The quantitative values were normalized to the mouse β -actin expression level in each sample. Data are representative of at least three independent experiments and shown as scatter plots. The horizontal line represents the median value. *Significant difference ($P<0.05$); ** Significant difference ($P<0.01$); ND, not detected. (b) The immunohistological examinations of the distribution of *H. suis* in the gastric mucosae of WT and IFN- γ KO mice (each $n=5$) were performed using confocal laser scanning microscopy (green: *H. suis*; blue: F-actin). Original magnification: $\times 100$. (A) Uninfected WT mice; (B) *H. suis*-infected WT mice; (C) uninfected IFN- γ KO mice; (D) *H. suis*-infected IFN- γ KO mice. The white bar indicates 100 μ m. Isotype controls for positive staining are shown as the small insets. Data are representative of at least three independent experiments and typical images are shown. A full color version of this figure is available at the *Mucosal Immunology* journal online.

Figure 4 The expression of interferon- γ (IFN- γ) activation-associated genes in the gastric mucosae of wild-type (WT) and IFN- γ knockout (KO) mice at 6 months after *Helicobacter suis* infection. The mRNA expression levels of (a) interleukin-12 (IL-12), (b) IFN- γ receptor (IFNGR), and (c) T-bet in the gastric mucosae of WT and IFN- γ KO mice (each $n = 5$) at 6 months after *H. suis* infection were determined by quantitative real-time PCR and normalized to that of β -actin as an internal standard. Data are representative of at least three independent experiments and shown as scatter plots. The horizontal line represents the median value. *Significant difference ($P < 0.05$); **significant difference ($P < 0.01$).

double knockout (TCR $\beta\delta$ DKO) mice that lacked peripheral T cells.³⁹ To clarify whether T cells are required for the formation of gastric lymphoid follicles, TCR $\beta\delta$ DKO mice were infected with *H. suis* for 6 months and then the number of follicles they possessed was determined by hematoxylin and eosin staining. Intriguingly, gastric lymphoid follicles were also observed in the stomachs of *H. suis*-infected TCR $\beta\delta$

DKO mice, and the number of follicles did not differ significantly from that seen in the *H. suis*-infected WT mice (Figure 5). These results suggest that T cells are not required for the formation of gastric lymphoid follicles after *H. suis* infection. Thus, the other cells that infiltrate into such follicles might secrete IFN- γ and be involved in gastric lymphoid follicle formation.

Figure 5 Histological examination of the gastric mucosae of *Helicobacter suis*-infected wild-type (WT) and T cell receptor β and δ double knockout (TCR $\beta\delta$ DKO) mice at 6 months after infection. C57BL/6J WT mice ($n=10$) and TCR $\beta\delta$ DKO mice ($n=10$) were infected with *H. suis* for 6 months as described in the Methods. (a) Histological examinations of the gastric mucosae of the WT and TCR $\beta\delta$ DKO mice (each $n=10$) were performed at 6 months after *H. suis* infection using hematoxylin and eosin staining. (A) *H. suis*-infected WT mice; (B) *H. suis*-infected TCR $\beta\delta$ DKO mice. Original magnification: $\times 40$. The black bar indicates 100 μ m. Data are representative of at least three independent experiments and typical images are shown. (b) The mean numbers of lymphoid follicles in the gastric mucosae of the *H. suis*-infected WT and TCR $\beta\delta$ DKO mice (each $n=10$) were determined by microscopically examining hematoxylin and eosin-stained specimens in a blinded manner. Data are representative of at least three independent experiments and shown as the mean $\pm$ s.d. NS, no significant difference ($P>0.05$). A full color version of this figure is available at the *Mucosal Immunology* journal online.

The formation of lymphoid follicles and the upregulation of IFN- γ had been restored at 3 months after infection in the stomachs of the IFN- γ KO mice that received WT B-cell transfers

Previous reports demonstrated that gastric MALT lymphomas are mainly composed of B cells, CD4 $^{+}$ T cells, DCs, and FDCs²³ that are also found in the gastric lymphoid follicles induced by *H. suis* infection (Figure 1b). To identify the IFN- γ -producing cells that trigger the formation of gastric lymphoid follicles, we purified B cells from spleen, DCs from cultured bone marrow cells (BMDC), and FDCs from spleen in noninfected WT mice, respectively, by fluorescence-activated cell sorting (FACS) (Figure 6a), and then these cells (viability $>90\%$) were transferred into each *H. suis*-infected IFN- γ KO mice equally. At 3 months after the *H. suis* infection, the formation of gastric lymphoid follicles and the induction of IFN- γ had been restored in all the IFN- γ KO mice that were administered WT B cells (Figures 6b and 7a). The lymphoid follicles in these mice contained B cells, CD4 $^{+}$ T cells, DCs, and FDCs, as was found in the lymphoid follicles of the infected WT mice (Figure 6d), although the number of follicles and the gastric IFN- γ expression level were lower than those encountered in the WT mice

(Figures 6c and 7a). In the DC- and FDC-administered IFN- γ KO mice, no gastric lymphoid follicle formation was observed, and the expression level of IFN- γ was low (Figures 6b,c and 7a). Moreover, IL-12 was significantly upregulated in the stomachs of the *H. suis*-infected mice, regardless of whether a cell transfer was performed (Figure 7b). The mRNA expression levels of IFNGR and T-bet mRNA were also increased in the *H. suis*-infected IFN- γ KO mice after B-cell transfer compared with the IFN- γ KO mice that were not given WT B cells (Figure 7c,d). These results indicate that the upregulation of IFN- γ expression in B cells is directly related to the formation of gastric lymphoid follicles after *H. suis* infection that might be induced by the stimulation of IL-12 and the IFNGR-T-bet pathway.

IFN- γ expression controlled the clearance of *H. suis* and was associated with the upregulation of CXCL13

The bacterial loads of *H. suis* in the stomachs of the mice that received cell transfers were evaluated at 3 months after infection by quantitative real-time PCR. In the stomachs of the B cell-administered IFN- γ KO mice, the bacterial load was similar to that seen in the WT mice, but lower than those detected in the IFN- γ KO mice and the DC- and FDC-administered IFN- γ KO mice (Figure 8a), indicating that there was an inverse relationship between the IFN- γ expression level and the bacterial load. A lack of IFN- γ might inhibit the immune response, decrease the clearance of *H. suis*, and result in the marked colonization of *H. suis* in the stomach.²⁵ On the other hand, we recently found that CXC chemokine ligand 13 (CXCL13), a B lymphocyte chemoattractant that is mainly produced by FDCs, is crucial for the development of gastric lymphoid follicles induced by *H. suis* infection because anti-CXCL13 treatment effectively inhibited gastric lymphoid follicle formation.⁴⁰ Interestingly, the CXCL13 expression level in the stomachs of *H. suis*-infected IFN- γ KO mice was significantly lower than the infected WT mice, and was restored by the transfer of WT B cells, but not BMDCs or FDCs (Figure 8b), suggesting that before cell transfer, B cells were not primed for migration in the stomachs of *H. suis*-infected IFN- γ KO mice because of the low expression level of CXCL13. After WT B-cell administration, the restored expression of IFN- γ might trigger the upregulation of CXCL13 and then evoke the formation of gastric lymphoid follicles.

The IFN- γ -producing cells that infiltrated the stomachs of the mice after *H. suis* infection were B cells

To further confirm the IFN- γ -producing cells in the stomach after *H. suis* infection, we performed intracellular staining for IFN- γ in the infiltrated cells from stomachs of *H. suis*-infected WT mice by flow cytometry. The results revealed that the IFN- γ -producing cells in the stomach after *H. suis* infection were B cells but not CD4 $^{+}$ T cells, DCs, and FDCs (Figure 9 and Supplementary Figure S2). Generally, B cells are divided into two major subsets: B1 cells (B220 $^{+}$ Mac-1 $^{+}$) that mainly reside in the body cavities, and B2 cells (B220 $^{+}$ Mac-1 $^{-}$) that are enriched in secondary lymphoid organs, both of which can produce cytokines and migrate to the

mucosal sites to involve the immune responses.^{32,41,42} To investigate which B-cell subset is involved in IFN- γ production after *H. suis* infection, Mac-1 expression was evaluated by flow cytometry, and the result indicated that the IFN- γ -producing B cells (B220⁺ CD19⁺ IFN- γ ⁺ cells) in the stomach were B2 cells (Mac-1⁻) (Figure 9). Furthermore, immunofluorescent staining showed that IFN- γ was positive in B cells that infiltrated in the gastric lymphoid follicles of *H. suis*-infected WT mice and B cell-administered IFN- γ KO mice, and this was also observed in the *H. suis*-infected TCR $\beta\delta$ DKO mice (Supplementary Figure S3). These data demonstrated that B cells had the ability to secrete IFN- γ after *H. suis* infection, even without the help of T cells. Finally, we isolated B cells from the stomachs of the noninfected WT mice and the *H. suis*-infected WT mice at 6 months after infection using FACS analysis. The infiltration of B cells into the stomach was markedly increased after *H. suis* infection (Figure 10a), and quantitative real-time PCR showed that the mean IFN- γ expression level of the B cells isolated from the *H. suis*-infected stomachs was significantly higher than that of the B cells collected from the noninfected

stomachs (Figure 10b), indicating that *H. suis* infection induced the aggregation of B cells, which produced IFN- γ and played an essential role in the formation of gastric lymphoid follicles, in the stomachs of the mice.

DISCUSSION

H. suis is the most common non-*Helicobacter pylori* *Helicobacter* species in humans and is associated with various gastric diseases, predominantly gastric MALT lymphoma.^{6–13} These bacteria obtained from pigs, monkeys, and humans can be maintained in the stomachs of C57BL/6J mice and universally induce the formation of gastric lymphoid follicles,^{24,25,43,44} indicating that this animal model is useful for investigating the pathogenesis of gastric MALT lymphoma.

Cytokines and chemokines control the development, differentiation, and migration of lymphoid cells and are involved in the shaping of lymphoid organs.⁴⁵ Their aberrant expression also plays a vital role in the etiology and progression of lymphoma, and the host response to tumors.^{46,47}

Figure 6 The formation of lymphoid follicles at 3 months after infection in the stomachs of mice that received cell transfers. (a) Purification of B cells, bone marrow-derived dendritic cells (BMDCs), and follicular dendritic cells (FDCs) from noninfected wild-type (WT) mice was performed using fluorescence-activated cell sorting (FACS). The appropriate cell populations were determined based on forward and side scatter. Doublets were excluded and propidium iodide (PI)-negative (live) cells were selected for further analysis with specific antibodies to purify CD19⁺ B220⁺ cells (A), CD11c⁺ MHCII⁺ cells (B), and FDC-M1⁺ cells (C), respectively. Isotype controls are shown (D–F). (b) Histological examinations of the gastric mucosae of the cell-administered mice (each $n=5$) were performed using hematoxylin and eosin staining. (A) *Helicobacter suis*-infected WT mice; (B) *H. suis*-infected interferon- γ knockout (IFN- γ KO) mice; (C) *H. suis*-infected IFN- γ KO mice administered WT B cells; (D) *H. suis*-infected IFN- γ KO mice administered WT DCs; (E) *H. suis*-infected IFN- γ KO mice administered WT FDCs. Original magnification: $\times 40$. The black bar indicates 100 μ m. Data are representative of at least three independent experiments and typical images are shown. (c) The mean numbers of lymphoid follicles in the gastric mucosae of the cell-administered mice (each $n=5$) were determined by microscopically examining hematoxylin and eosin-stained specimens in a blinded manner. Data are representative of at least three independent experiments and shown as mean $\pm$ s.d. *Significant difference ($P<0.05$); **significant difference ($P<0.01$); ND, not detected. (d) Immunohistological examination of immunocompetent cells in the gastric mucosae of the cell-administered mice (each $n=5$). The immunohistological examinations of B cells, CD4-positive T cells, DCs, FDCs, and F-actin were performed using confocal laser scanning microscopy (red: B220 and CD4; green: CD11c and FDC-M1; blue: F-actin; original magnification: $\times 100$). (A–C) *H. suis*-infected IFN- γ KO mice; (D–F) *H. suis*-infected IFN- γ KO mice administered WT B cells. The white bar indicates 100 μ m. Data are representative of at least three independent experiments and typical images are shown. FITC, fluorescein isothiocyanate; MHCII, major histocompatibility complex II; PE, phycoerythrin.

Figure 6 Continued.

The formation and maintenance of the functional architecture in different lymphoid tissues rely on distinct cytokines. For instance, IL-17 was expressed in the lungs of lipopolysaccharide-treated mice, and immunofluorescent staining revealed that the induction of IL-17 was detected in regions of inducible bronchus-associated lymphoid tissue that was mainly composed of B cells, T cells, and DCs. The development of inducible bronchus-associated lymphoid tissue is dependent on IL-17 produced by CD4⁺ T cells, and the administration of IL-17 antibody efficiently suppressed the formation of inducible bronchus-associated lymphoid tissue that is associated with pulmonary inflammation and infection.⁴⁸ Furthermore, the proliferation of malignant B cells in *H. pylori*-induced gastric MALT lymphoma is driven by Th2-polarized T cells that infiltrate into the tumor and mainly produce IL-4 that is critical

for efficient mucosal antibody responses.²³ In this study, the induction of IFN- γ was observed in the gastric lymphoid follicles and the surrounding sites (Figure 2b), suggesting that immunocompetent follicular cells are responsible for the production of IFN- γ after *H. suis* infection.

As a premalignant condition of gastric MALT lymphoma, local infection or chronic inflammation can result in the production of organized lymphoid follicles consisting of B-cell clusters composed of FDCs and surrounded by DCs and T cells.¹⁵ As a regular component of lymphoid tissue, T cells secrete cytokines and interact with B cells, leading to the progression of the germinal center B-cell response.²⁷ Interestingly, in our preliminary experiment using anti-CD4 antibody (GK1.5) we revealed that gastric lymphoid follicles can form in *H. suis*-infected mice in the absence of T helper

cells. In addition, the latter mice exhibited a comparable mean IFN- γ expression level to that seen in the mice that were not administered the antibody (data not shown). Consistent with these findings, in an experiment using TCR $\beta\delta$ DKO mice the present study showed that the formation of *H. suis* infection-induced gastric lymphoid follicles was T-cell independent (Figure 5). Thus, we considered that B cells, DCs, and/or FDCs, but not T cells, that infiltrate into the stomach after *H. suis* infection produce IFN- γ and, hence, initiate gastric lymphoid follicle formation.

The adoptive cell transfer experiment demonstrated that the formation of gastric lymphoid follicles and the induction of IFN- γ only occurred in the presence of naive WT B cells after *H. suis* infection (Figures 6b–d and 7a), indicating that the B cells from the WT mice secreted IFN- γ and induced the

formation of gastric lymphoid follicles. Moreover, the intracellular staining and cell isolation experiments further confirmed that B2 cells are able to produce IFN- γ in the stomach after *H. suis* infection, and this is consistent with the previous report that B2 cells (especially follicular B cells) but not B1 cells express high level of IFN- γ mRNA *in vitro*.³² Recently, IFN- γ -producing B cells were identified in mice that had been infected with pathogens such as *Toxoplasma gondii* and *Borrelia burgdorferi*, suggesting that B cells regulate the immune responses to infectious pathogens through their cytokine production.^{30,49} Moreover, naive B cells that are activated in the presence of IL-12 can produce IFN- γ via a T cell-independent pathway that initiates a positive feedback loop controlled by the IFN- γ receptor and T-bet that stimulates B cells to persistently secrete IFN- γ .³¹ In human *H. pylori*

Figure 7 The expression of interferon- γ (IFN- γ) and its activation-associated genes at 3 months after infection in the stomachs of mice that received cell transfers. (a–d) The mRNA expression levels of IFN- γ , interleukin-12 (IL-12), IFN- γ receptor (IFNGR) and T-bet in the gastric mucosae of the cell-administered mice (each $n=5$) were determined by quantitative real-time PCR and normalized to that of β -actin as an internal standard. Data are representative of at least three independent experiments and shown as scatter plots. The horizontal line represents the median value. *Significant difference ($P<0.05$); **significant difference ($P<0.01$); ND, not detected; NS, no significant difference ($P>0.05$). BMDC, bone marrow-derived dendritic cell; FDC, follicular dendritic cell.

Figure 7 Continued.

infected gastric mucosae, IL-12 expression was increased in order to promote the Th1 signaling pathway via the activation of T-bet and the subsequent induction of IFN- γ expression.⁵⁰ In

the present study, the upregulation of IL-12 and other IFN- γ activation-related genes was detected in the stomachs of *H. suis*-infected WT mice and IFN- γ KO mice administered WT B cells

Figure 8 The bacteria load and CXCL13 mRNA at 3 months after infection in the stomachs of mice that received cell transfers. **(a)** The expression level of *Helicobacter suis* 16S rRNA was estimated by real-time PCR using RNA samples extracted from the gastric mucosa homogenates of the cell-administered mice (each $n=5$). The quantitative values were normalized to the mouse β -actin expression level in each sample. Data are representative of at least three independent experiments and shown as scatter plots. The horizontal line represents the median value. *Significant difference ($P<0.05$); **significant difference ($P<0.01$); ND, not detected; NS, no significant difference ($P>0.05$). **(b)** The CXCL13 mRNA expression levels in the gastric mucosae of the cell-administered mice (each $n=5$) were determined by quantitative real-time PCR and normalized to that of β -actin as an internal standard. Data are representative of at least three independent experiments and shown as scatter plots. The horizontal line represents the median value. *Significant difference ($P<0.05$); **significant difference ($P<0.01$). BMDC, bone marrow-derived dendritic cell; FDC, follicular dendritic cell.

(Figures 4 and 7b–d) that demonstrated that follicular B cells have the potential to produce IFN- γ , even without T-cell help.

In addition, IFN- γ is able to upregulate the expression of T cell and monocyte chemoattractants such as CXCL9, CXCL10, CCL3, and CCL4 to induce the trafficking of these immune cells to sites of inflammation.⁵¹ Recently, it has been reported that recombinant IFN- γ significantly induced gene expression of CXCL13 in immune-related cells,⁵² and we found that activation of CXCL13 also plays an essential role in the formation of gastric lymphoid follicles after *H. suis* infection.⁴⁰ In addition, our present findings showed that the expression of CXCL13 in the murine stomach after *H. suis* infection was

closely related to the induction of IFN- γ (Figure 8b) that supports our speculation that IFN- γ triggers the expression of CXCL13 to recruit lymphocytes and contributes to the formation of gastric lymphoid follicles.

In conclusion, we found that follicular B cells produced IFN- γ and evoked lymphoid neogenesis in the stomachs of *H. suis*-infected mice that might have been related to the induction of CXCL13. Therefore, antibody blockade of IFN- γ is expected to effectively inhibit the development of ectopic lymphoid tissue and, hence, could be a feasible therapeutic strategy for *Helicobacter* infection-related human diseases, e.g., gastric MALT lymphoma.

Figure 9 Intracellular staining for interferon- γ (IFN- γ) in the gastric B cells of wild-type (WT) mice at 6 months after *Helicobacter suis* infection by flow cytometry. C57BL/6J WT mice were infected with *H. suis* for 6 months and the gastric cells collected after enzymatic dissociation were gated for lymphocyte population based on forward and side scatter. Doublets were excluded and then these cells were stained with B220 and CD19 antibodies (a), followed by intracellular staining for IFN- γ . The middle panels showed the IFN- γ staining result (b) and its isotype control (c) among B220⁺CD19⁺ cells. The right panel showed the Mac-1 staining result (d) and its isotype control (e) among B220⁺CD19⁺IFN- γ ⁺ cells. Isotype control for staining of B220⁺CD19⁺ cells is shown (f). Data are representative of three independent experiments and typical images are shown. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin. A full color version of this figure is available at the *Mucosal Immunology* journal online.

METHODS

Mice. All animal experiments were performed in accordance with Kobe University Animal Experimentation Guidelines (Permission No. P-120808 and P130106). C57BL/6J mice (WT mice) were purchased from CLEA Japan (Tokyo, Japan). IFN- γ KO mice that had been backcrossed with C57BL/6J mice at least 10 times were provided by Dr Iwakura (Tokyo University, Tokyo, Japan).⁵³ TCR $\beta^{-/-} \times \delta^{-/-}$ DKO mice on a C57BL/6J background were originally obtained from the Jackson Laboratory (Bar Harbor, ME)⁵⁴ and maintained at the National Center for Global Health and Medicine (Chiba, Japan). All mice were specific pathogen free (for Hemagglutinating virus of Japan, mouse hepatitis virus, *Mycoplasma pulmonis*, *Clostridium piliforme*, *Syphacia* spp) and bred under standard laboratory conditions.

***H. suis* infection.** *H. suis* was obtained from a cynomolgus monkey and maintained in the stomachs of WT mice as donor mice because we were unable to cultivate the bacterium at our laboratory. Six-week-old female C57BL/6J, IFN- γ KO, and TCR $\beta\delta$ DKO mice were infected with *H. suis* after orally administering the same amount of gastric mucosal homogenate from donor mice that has been confirmed

to contain only *H. suis* but not other *Helicobacter* species in the stomach.⁴⁴ The control mice were administered an equal volume of phosphate-buffered saline (PBS).

Histological examination. The histological examination was performed according to our previous reports.^{24,25,40,44} Briefly, C57BL/6J, IFN- γ KO, and TCR $\beta\delta$ DKO mice were killed by cervical dislocation under anesthesia at the desired time point after *H. suis* infection. Then, their stomachs were resected, opened at the outer curvature, and sliced longitudinally from the esophagus to the duodenum. Half of the stomach was embedded in paraffin wax, a quarter of the stomach was used to extract RNA as described below, and the remaining part of the stomach was frozen in OCT compound (Sakura Finetek, Tokyo, Japan). The paraffin-embedded tissues were sliced into three specimens longitudinally and stained with hematoxylin and eosin. Each section included both the corpus and antrum. The number of gastric lymphoid follicles identified in three specimens from each mouse was determined in a blind manner.

Antibodies. The following antibodies were used in this study: purified polyclonal rabbit anti-*H. pylori* antibody (DAKO, Tokyo, Japan),

purified monoclonal rat anti-mouse CD45R/B220 antibody (BD Bioscience (San Jose, CA) and Abcam (Cambridge, UK)), CD4 antibody (BD Bioscience), purified monoclonal rat anti-mouse FDC-M₁ antibody (BD Bioscience), Alexa488-conjugated anti-mouse NK1.1 antibody (BioLegend, San Diego, CA), purified polyclonal rabbit anti-mouse AID antibody (Santa Cruz Biotechnology, Dallas, TX), fluorescein isothiocyanate (FITC)-conjugated monoclonal rat anti-mouse CD19 antibody (BD Bioscience), phycoerythrin-conjugated monoclonal rat anti-mouse CD45R/B220 antibody (BD

Bioscience), phycoerythrin-conjugated monoclonal rat anti-mouse CD4 antibody (BD Bioscience), FITC-conjugated monoclonal hamster anti-mouse TCR β chain antibody (BD Bioscience), FITC-conjugated monoclonal rat anti-mouse major histocompatibility complex II antibody (BD Bioscience), phycoerythrin-conjugated monoclonal hamster anti-mouse CD11c antibody (BD Bioscience), FITC-conjugated monoclonal hamster anti-mouse CD11c antibody (BD Bioscience), FITC-conjugated polyclonal goat anti-rat IgG (BD Bioscience), allophycocyanin-conjugated monoclonal rat anti-mouse

Figure 10 The mRNA expression level of interferon- γ (IFN- γ) in B cells isolated from the stomachs of wild-type (WT) mice at 6 months after *Helicobacter suis* infection. (a) Sorting of B cells from the stomachs of C57BL/6J WT mice was performed using fluorescence-activated cell sorting (FACS). Gastric cells were gated for lymphocyte population based on forward and side scatter. Doublets were excluded and then these cells were used to purify CD19⁺ B220⁺ cells from the stomachs of uninfected mice (A) and *H. suis*-infected mice (B). Isotype controls are shown (C, D). (b) The IFN- γ mRNA expression levels of B cells isolated from the stomachs of uninfected WT mice and *H. suis*-infected WT mice at 6 months after infection were determined by quantitative real-time PCR and normalized to that of β -actin as an internal standard. Data are representative of at least three independent experiments and shown as scatter plots. The horizontal line represents the median value. **Significant difference ($P < 0.01$). FITC, fluorescein isothiocyanate; PE, phycoerythrin. A full color version of this figure is available at the *Mucosal Immunology* journal online.

IFN- γ antibody (BD Bioscience), brilliant violet (BV) 421-conjugated monoclonal rat anti-mouse Mac-1 antibody (BD Bioscience), purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block), purified monoclonal rat anti-mouse IFN- γ antibody (Abcam), polyclonal rabbit anti-mouse IFN- γ antibody (Life Technologies, Grand Island, NY), Alexa488-conjugated polyclonal goat anti-rabbit IgG antibody (Invitrogen, Eugene, OR), Alexa488-conjugated polyclonal goat anti-rat IgG antibody (Invitrogen), Alexa546-conjugated polyclonal goat anti-rat IgG antibody (Invitrogen), horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Abcam). The nuclei and F-actin in the sections were stained with Alexa642-conjugated TO-PRO (Invitrogen) and Alexa647-conjugated phalloidin (Invitrogen). Corresponding isotype control antibodies were also used.

Immunofluorescent staining. The paraffin-embedded sections were deparaffinized in xylene and ethanol. Antigen retrieval was performed with Tris-HCl/EDTA buffer at 105 °C for 5 min. The frozen sections were dried for 2 h using a slide glass dryer, fixed in acetone for 5 min (IFN- γ staining was performed without acetone fixation). Then, all of the sections were blocked in 10% goat serum for 30 min. After being washed with PBS, the sections were incubated with appropriate antibodies overnight (the IFN- γ staining involved 3 days of incubation) at 4 °C and then reacted with the corresponding secondary antibodies for 1 h at room temperature.^{24,25,40} Then, the sections were observed using a confocal laser-scanning microscope (Zeiss LSM 5PASCAL, Carl Zeiss, Jena, Germany).

Immunohistochemistry staining. At 6 months after *H. suis* infection, the gastric sections from WT mice were incubated with AID antibody at 4 °C for 3 days after blocking as described above. Then, the sections were immersed in 0.3% H₂O₂ in distilled water for 15 min to block the endogenous peroxidase activity before reacting with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The chromogenic reaction was developed by using 3,3'-diaminobenzidine Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

Quantitative real-time PCR. The mucosal and submucosal layers of the stomach were homogenized with 1 ml of TRIZOL reagent (Invitrogen). RNA was extracted from the homogenates and isolated stomach cells according to the manufacturer's instructions, before being subjected to a reverse transcription reaction using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. 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.^{24,25,40} The following specific primer pairs (Hokkaido System Science, Sapporo, Japan) were used for the real-time PCR: *H. suis*-specific 16S rRNA gene: 5'-AGACAAAGCCTCCCAACAAC-3' and 5'-ATCACTGACGCTGATTGCAC-3'; IFN- γ : 5'-GCGTATTGAATCACACCTG-3' and 5'-TGAGCTCATTGAATGCTTGG-3'; IL-12: 5'-AGCA GTAGCAGTTCCCTGA-3' and 5'-AGTCCCTTTGGTCCAGTGTG-3'; IFNGR-1: 5'-GTATTGTCGCTTCTGGCTCCTT-3' and 5'-AAGGCTAGCCGAGGCAAAAC-3'; IFNGR-2: 5'-GTTGGGCATCTTCGCATTG-3' and 5'-TATTTGAGGAACAGGGTGAAGCA-3'; T-bet: 5'-ACCTGGACCCAACCTGTCAAC-3' and 5'-AACTGTGTTCCCGAGGTGTC-3'; CXCL13: 5'-CATAGATCGGATTCAAGTTACGCC-3' and 5'-TCTTGGTCCAGATCACAACCTTCA-3'; β -actin: 5'-AAGGCCAACCCTGAAAAGAT-3' and 5'-GTGGTACGACCAGAGGCATAC-3'. To allow comparisons of relative gene expression levels, the comparative C_T ($\Delta\Delta C_T$) method was used and measurements were normalized using β -actin complementary DNA as an endogenous control.

Splenocyte preparation. Spleens were collected from noninfected WT mice, rinsed in RPMI-1640 medium, and disrupted mechanically. After the tissue had been filtered and centrifuged at 420 $\times$ g for 5 min,

5 ml ACK buffer⁵⁵ was added to lyse the red blood cells for 3 min under slight agitation, and then 10 ml PBS was added. After being centrifuged, the splenocytes were resuspended in cold stain buffer (BD Bioscience) at a concentration of 2×10^7 per ml and used in the staining experiment described below.

Culturing of bone marrow cells. The culturing of BMDCs was performed according to the methods used in previous reports.^{55,56} Briefly, femurs, tibias, and humeri were collected from noninfected WT mice and placed in ice-cold RPMI-1640 medium. Bone marrow cells were then obtained by flushing the bones with RPMI-1640 medium. After being filtered, the obtained bone marrow cells were cultured in 30 ml RPMI-1640 medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 10 ng ml⁻¹ each of IL-4 and granulocyte-macrophage colony-stimulating factor (PEPROTECH, Rocky Hill, NJ), before being incubated in 5% CO₂ at 37 °C. Additional IL-4 and granulocyte-macrophage colony-stimulating factor (final concentrations of both: 10 ng ml⁻¹) were added on day 4 after the culture medium had been changed. On day 9, loosely adherent and nonadherent cells were collected and resuspended in cold stain buffer at a concentration of 2×10^7 cells per ml and used in the staining experiment described below.

Enzymatic dissociation of gastric cells. The infiltrating cells in the noninfected and *H. suis*-infected stomachs of the WT mice were isolated via a modified version of a previously described technique.⁵⁷ Briefly, the stomachs were resected, washed in cold PBS, stored in collection medium (calcium- and magnesium-free Hank's Balanced Salt Solution containing 5% fetal bovine serum and 1% penicillin plus streptomycin), cut into small pieces, rinsed in collection medium containing 1 mM dithiothreitol and 1 mM EDTA, and agitated for 1 h at 37 °C, before epithelial cells were removed from the suspension. The specimens were washed with complete RPMI-1640 medium and then treated with collagenase at 1 mg ml⁻¹ (SIGMA ALDRICH, Steinheim, Germany) in complete RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C for 3 h under agitation. Undigested tissue was removed by filtration, and the collected cells were resuspended in cold stain buffer at a concentration of 2×10^7 cells per ml and used in the staining experiment described below.

Antibody staining and cell isolation by FACS. The cells collected using the above method were suspended in cold stain buffer at a final concentration of 2×10^7 cells per ml. To purify the cells from the spleen and bone marrow, propidium iodide staining solution (BD Bioscience) was used to identify viable cells. Appropriate antibodies or their isotype control antibodies were added at the optimal concentration and then incubated for 30 min on ice protected from light. B cells were stained with B220 antibody and CD19 antibody. DCs were stained with CD11c antibody and major histocompatibility complex II antibody. For FDC labeling, the cells were first stained with FDC-M₁ antibody on ice for 1 h, followed by FITC-conjugated goat anti-rat IgG. The cells were then washed with stain buffer to remove any unbound antibodies, resuspended at a final concentration of 2×10^7 cells per ml, and analyzed with a BD FACS Aria II cell sorter and BECKMAN COULTER cell sorter (MoFlo XDP, Fullerton, CA) to collect purified cells. The viability of purified cells was assessed by Trypan blue exclusion after sorting.

Cell transfer. Noninfected WT mice were killed as donor mice to purify splenic B cells (4×10^7 cells were harvested from 10 mice), BMDCs (5×10^7 cells were harvested from 20 mice), and splenic FDCs (5×10^7 cells were harvested from 20 mice) using FACS. Then, each kind of purified cells were resuspended in sterile PBS and equally transferred into the five *H. suis*-infected IFN- γ KO mice at 2 weeks after infection via intraperitoneal injection. Some infected and uninfected WT and IFN- γ KO mice were intraperitoneally injected with an equal volume of PBS instead of the above-mentioned cells. After 3 months, all the mice were killed, and gastric tissue samples were collected.

Intracellular staining for IFN- γ in the gastric cells. The gastric cells collected after enzymatic dissociation were first stained by appropriate antibodies for cell surface antigens after blocking Fc receptors. Then, the cells were incubated with Fixation/Permeabilization solution at 4 °C for 20 min. After being washed two times in 1 $\times$ BD Perm/Wash buffer, allophycocyanin-conjugated IFN- γ antibody or its isotype control antibody was added and incubated at 4 °C for 30 min in the dark. Finally, the cells were resuspended in stain buffer before flow cytometric analysis.

Statistical analysis. All results are shown as mean $\pm$ s.d. Student's *t*-test was used for comparisons between two groups, and nonrepeated measures analysis of variance followed by the Bonferroni test was used for comparisons among three or more groups to analyze statistical significance.⁴⁰ A level of probability of 0.05 or 0.01 was regarded as the significance criterion.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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