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Research Article

RESEARCH ARTICLE

High-fat-diet-induced obesity upregulates the expression of lymphoid chemokines and promotes the formation of gastric lymphoid follicles after *Helicobacter suis* infection

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One sentence summary: Obesity promotes the formation of gastric MALT induced by *Helicobacter suis*.
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ABSTRACT

Helicobacter suis colonizes the stomachs of a variety of animals, including humans, and is more likely than other *Helicobacter* species to induce gastric mucosa-associated lymphoid tissue lymphoma. Obesity is a low-grade chronic inflammatory state in which the induction of a chemokine network contributes to a variety of diseases. However, the effect of obesity on the development of gastric MALT in the presence of *H. suis* infection remains unclear. Here, we reveal that high-fat-diet-induced obesity upregulates the expression of lymphoid chemokines in the stomach and accelerates the *H. suis* infection-induced formation of gastric lymphoid follicles, potentially via a mechanism that involves the activation of the nuclear factor kappa B (NF-κB) signaling pathway. These findings provide novel insight into the pathogenesis of obesity-related diseases, especially those induced by *Helicobacter* infection.

Keywords: obesity; *Helicobacter suis*; gastric lymphoid follicles; chemokine

INTRODUCTION

Helicobacter suis belongs to the non-*H. pylori* Helicobacters and was originally named '*H. heilmannii*' type 1 (O'Rourke *et al.* 2004b). *Helicobacter suis* colonizes the stomach of more than 60% of slaughter pigs and causes chronic gastritis and ulcers of the pars esophagea (Queiroz *et al.* 1996; Roosendaal *et al.* 2000; Hellemans *et al.* 2007; Haesebrouck *et al.* 2009). It has also been associated with human gastric diseases, including gastritis, peptic ulcer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Morgner *et al.* 2000; Okiyama *et al.* 2005; Matsumoto *et al.* 2014). In humans, the rate of infection of *H. suis* ranges

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between 0.2% and 6% (Okiyama *et al.* 2005; Haesebrouck *et al.* 2009). However, in experimental murine models, gastric MALT lymphoma-like lesions were induced by long-term infection with *H. suis* in nearly 100% of C57BL/6J mice (Nakamura *et al.* 2007), suggesting that this bacterium is more closely related than other *Helicobacter* species to gastric lymphomagenesis.

Unlike the gut, the gastric mucosa does not normally contain MALT, and the increased frequency of lymphoid neogenesis at this site represents a condition with a high-risk of generating MALT-type gastric lymphoma (Mazzucchelli *et al.* 1999). Previous reports showed that *H. suis* derived from pig stomachs colonized the stomachs of C57BL/6J mice and subsequently induced the formation of gastric lymphoid follicles consisting of B cells, $CD4^+$ T cells, dendritic cells, and follicular dendritic cells (FDCs) (Yamamoto *et al.* 2011; Yang *et al.* 2015). These cells are regarded as ectopic or tertiary lymphoid tissues that have the potential to progress to gastric MALT lymphoma when stimulated by the persistent presence of bacterial antigens (Carragher, Rangel-Moreno and Randall 2008; Wharry, Haines and Carroll 2009).

In recent years, some of the key molecular determinants that contribute to the generation of tertiary lymphoid tissues have been identified. These include tumor necrosis factor (TNF) family members and lymphotoxin (LT)-$\alpha 1\beta 2$ ($LT\alpha_1\beta_2$). Chronic antigen stimulation leads to the persistent activation of innate and adaptive immune cells in inflamed tissues and to the increased expression of $LT\alpha_1\beta_2$ by activated B and T cells and lymphoid chemokines by resident stromal cells. When $LT\alpha_1\beta_2$ and TNF-α bind to their respective receptors, lymphotoxin-beta receptor ($LT\beta R$) and TNFR1, respectively, the nuclear factor kappa B (NF-κB) signaling pathway is activated and subsequently induces a set of chemokines known as lymphoid or homeostatic chemokines (i.e. CC-chemokine ligand 19 (CCL19), CCL21, CXC-chemokine ligand 12 (CXCL12) and CXCL13) that regulate lymphocyte homing and compartmentalization in ectopic lymphoid tissues (Aloisi and Pujol-Borrell 2006; Wharry, Haines and Carroll 2009). Furthermore, we found that the B lymphocyte chemoattractant CXCL13 was crucial for the development of *H. suis* infection-induced gastric lymphoid follicles because treatment with an anti-CXCL13 factor effectively inhibited the formation of gastric lymphoid follicles (Yamamoto *et al.* 2014).

Obesity is an important public health problem around the world. It increases the risk of many chronic diseases, such as cardiovascular diseases and diabetes. Moreover, obesity is correlated with an increased risk of certain tumors, including gastric cancer and malignant lymphoma (Kanda *et al.* 2010; Lichtman 2010; Song *et al.* 2015). For instance, high-fat-diet-induced obesity increased proinflammatory immune responses and accelerated gastric carcinogenesis in *H. felis*-infected mice (Ericksen *et al.* 2014), and a gastric MALT lymphoma in an obese patient entered complete remission after the patient lost 20 kg of weight (Helman *et al.* 2011). Additionally, obesity is recognized as a low-grade chronic inflammatory state that involves a chemokine network that contributes to a variety of diseases. High-fat-diet-induced obesity increased both CCL19 and CCL21 levels in the lymph nodes (LN) of mice (Jung *et al.* 2015), and diet-induced obese mice exhibited a robust increase in CXCL12 expression in white adipose tissue (Kim *et al.* 2014). Based on these findings, we hypothesized that obesity might increase the expression of lymphoid chemokines in the stomach and thereby promote *H. suis* infection-induced gastric MALT formation. In this study, we investigated the effect of high-fat-diet-induced obesity on the formation of gastric lymphoid follicles in the stomachs of mice infected with *H. suis*.

MATERIALS AND METHODS

Mice

All procedures and animal experiments were approved by the Animal Care and Use Committee of Qingdao University based on current ethical standards. C57BL/6 wild-type (WT) mice (18–20 g) were purchased from Peng Yue Experimental Animal Breeding Ltd. (Jinan, China). All mice were specific pathogen-free and bred under standard laboratory conditions.

Helicobacter suis infection and mouse diet

Helicobacter suis from pig stomachs with natural infections was maintained in the stomachs of C57BL/6 WT mice according to the methods used in previous reports (Baele *et al.* 2008; Yamamoto *et al.* 2011) because this bacterium cannot be reliably cultivated *in vitro* in our laboratory. Briefly, six pig stomachs were obtained from a slaughterhouse and incubated with 1% HCl for 1 h. Then the gastric mucosal homogenates from red lesions in pig stomachs were collected by scraping with a glass slide. Six-week-old female C57BL/6J mice were infected with *H. suis* by oral gavage with this homogenates. The resulting *H. suis*-infected donor mice were confirmed to carry only *H. suis* and no other *Helicobacter* species (i.e. *H. pylori*, *H. rodentium*, *H. typhlonium*, *H. hepaticus* and *H. muridarum*) in their stomachs and the infection condition was checked every 6 months by PCR according to the methods described in a previous report (Yamamoto *et al.* 2011). Forty C57BL/6 female (8 weeks old) WT mice were randomly divided into the four following groups containing 10 mice each: normal control (NC) group, *H. suis*-infected (HS) group, high-fat diet (HFD) group and *H. suis*-infected plus high-fat diet (HS + HFD) group. The same amount of gastric mucosal homogenate was obtained from *H. suis*-infected donor mice and orally administered to the mice in the HS and HS + HFD groups to initiate infection. The mice in the NC and HFD groups were given an equal volume of gastric mucosal homogenate obtained from non-infected WT mice. The mice in the HFD and HS+HFD groups were fed a high-fat diet (45% of calories from fat, Research Diets 12451, New Brunswick, NJ), and the mice in the NC and HS groups were fed a standard chow diet (13% of calories from fat) beginning 1 week post-infection. The body weights of the mice in each group were measured every week.

Histological examination

At 24 weeks after each distinct diet was begun, all of the mice were sacrificed using cervical dislocation while under anesthesia. The stomachs were then resected, opened at the outer curvature, and sliced longitudinally from the esophagus to the duodenum. Half of each stomach was embedded in paraffin wax, one-quarter of each stomach was used for RNA extraction, and the remaining quarter of each specimen was used to extract protein, as described below. The number and major axis length of clearly identifiable gastric lymphoid follicles were determined in three specimens from each mouse in a blinded manner using a microscope. The major axis of the lymphoid follicles was measured using the scale bar of a microscope. A fraction that was $<10\ \mu$m was rounded down (Nobutani *et al.* 2010).

Urease Activity Assay

The stomachs in HS group and HS+HFD group were collected and sliced longitudinally from the esophagus to the duodenum. Half of each stomach was weighed and then homogenized in

phosphate buffered saline (PBS) with protease inhibitors. After centrifugation at 10 000× *g* for 10 min at 4°C, the supernatant fluid in each group was collected and adjusted to the same volume. The urease activity was detected according to the protocol of Urease Activity Assay Kit (BioVision, Milpitas, CA).

Quantitative real-time PCR

The mucosal and submucosal layers of the mouse stomachs in each group and the human B cells collected as described above were homogenized using TRIZOL reagent (Invitrogen, Carlsbad, CA). RNA was extracted from these homogenates and subjected to reverse transcription using a cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and an ABI Prism 7500 Real Time PCR system (Applied Biosystems) according to the manufacturer's instructions. The following specific primer pairs were used for real-time PCR.

Helicobacter suis-specific 16S rRNA gene: 5′-AGACAAAGCC TCCCAACAAC-3′ and 5′-ATC ACTGACGCTGATTGCAC-3′; CXC L13: 5′-CATAGATCGGATTCAAGTTACGC C-3′ and 5′-TCTTGG TCCAGATCACAACTTCA-3′; CXCR5: 5′-AACATCCTGG TGCTG GTAATCC-3′ and 5′-GGCTACTGCGAGGTGGAACA-3′; CCL19: 5′-C CTGGGAACATCGTGAAAG-3′ and 5′-AGCCCCTTAGTGTGGT GAACAC-3′; CCL21: 5′-AGGAAGAACCGGGAACCTC-3′ and 5 ′-AGGGCTGTGTCTGTTCA GTTCTC-3′; CCR7: 5′-CAAGAACCAAA AGCACAGCC-3′ and 5′-GACAAGGA GAGCCACCACC-3′; CXCL12: 5′-TGACGGTAAACCAGTCAGCC-3′ and 5′-C TTGCATCT CCCACGGATGT-3′; CXCR4: 5′-ATCTGTGACCGCCTTTACCC-3′ and 5′-AACAGGAGAGGATGACGATGC-3′; CXCR7: 5′-AACCTCTTTGG GAG CATCTTCTT-3′ and 5′-GGTGCCGGTGAAGTAGGTGAT-3′; TNF-α: 5′-CATC TTCTCAAAATTCGAGTGACAA-3′ and 5′-TGGGAGTAGACAAGGTACAACC C-3′; TNFR1:5′-TCCGCTTG CAAATGTCACA-3′ and 5′-GGCAACAGCACCGC AGTAC-3′; LTα: 5′-GCTTGGCACCCCTCCTGTC-3′ and 5′-GATGCCATGGGT CAAGT GCT-3′;LTβ: 5′-CCAGCTGCGGATTCTACACCA-3′ and 5′-AGCC CTT GCCCACTCATCC-3′; LTβR: 5′-CCAGATGTGAGATCCAG GGC-3′ and 5′-GAC CAGCGACAGCAGGATG-3′; NF-kB$_1$ gene: 5′-CGGGATAGTGACAGCGTCT GT-3′ and 5′-CAGTAAGAGACTCT GTAAAGCTGAGTTTG-3′; NF-kB$_2$ gene: 5′-GCCCGAGCGTTGCT GGACTA-3′ and 5′-CGCCGAGGTACTGAGCGTGA T-3′; and β-actin: 5′-AAGGCCAACCGTGAAAAGAT-3′ and 5′-GTGGTACGACCAG AGGCATAC-3′. To compare relative gene expression levels, the comparative CT ($\Delta\Delta$CT) method was used, and all measurements were normalized using β-actin cDNA as an endogenous control.

Western Blot analysis

Cytoplasmic and nuclear proteins were extracted from the stomachs of mice in each group using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific Inc., Bremen, Germany) in the presence of Protease and Phosphatase Inhibitors (Abcam, Cambridge, UK), and the lysates were then boiled with the appropriate amount of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Equal amounts of lysates were separated using SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were soaked in a blocking solution (1% skim milk and 0.05% Tween 20-PBS) for 1 h and then incubated with primary antibodies (purchased from Santa Cruz Biotechnology (Dallas, Texas) and Abcam) for p65, phospho-p65, IκBα, phospho-IκBα, p100, p52 and β-actin for 2 h. After the membranes were washed with Tween 20-PBS, they were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Specific bands were visualized using ECL Western Blot Detection Reagents (GE Healthcare, Little Chalfont, UK).

Statistical analysis

All results are shown as the means ± SD. Student's *t*-test was used for comparisons between two groups, and non-repeated measures analysis of variance was used for comparisons between three or more groups to analyze statistical significance. A level of probability of 0.05 or 0.01 was regarded as the significance criterion.

RESULTS

Body weights were significantly higher in the mice in the high-fat-diet groups than in those fed a standard chow diet

The body weights of the mice in each group were measured every week and found to have gradually increased in parallel with the length of infection (Fig. 1A). At 24 weeks after each distinct diet was begun, the body weights of the mice in the high-fat-diet groups were significantly higher than the body weights of the mice fed a standard chow diet. There were no significant differences between the infected and uninfected groups fed the same diet (Fig. 1B), suggesting that infection with *Helicobacter suis* did not itself significantly affect body weight.

Diet-induced obesity promotes the formation of gastric lymphoid follicles in *Helicobacter suis*-infected mice by upregulating lymphoid chemokines but does not affect the bacterial load in the stomach

At 24 weeks after each distinct diet was begun, gastric lymphoid follicles were observed in the *H. suis*-infected mice but not the non-infected mice. The mean number of follicles and mean major axis size of the follicles were higher in the stomachs of *H. suis*-infected mice fed a high-fat diet than in those fed a standard chow diet (Fig. 2A–C). The relative number of *H. suis* and urease activity in the stomachs of the mice was evaluated using real-time PCR with a specific 16s rRNA primer and Urease Activity Assay Kit, respectively. The results showed that there was no significant difference in bacterial load and urease activity between the HS and HS + HFD groups (Fig. 3A and B). The mRNA expression levels of the lymphoid tissue formation-related chemokines CXCL13, CCL19, CCL21 and CXCL12 and their receptors, CXCR5 (for CXCL13), CCR7 (for CCL19 and CCL21), CXCR4 and CXCR7 (for CXCL12), in the stomachs of mice in the HS + HFD group were higher than the levels observed in the HS group (Fig. 3C–E). These data indicate that high-fat-diet-induced obesity upregulated lymphoid chemokines in the stomach and promoted the formation of gastric lymphoid follicles in mice infected with *H. suis* but did not affect the bacterial load in the stomach.

The expression levels of TNF-α, LT and their receptors and the activation of the NF-κB signaling pathway in the stomach were enhanced by high-fat-diet-induced obesity in mice infected with *Helicobacter suis*

Previous studies have demonstrated that the activation of NF-κB is mediated by LTs and that TNF-α is essential for the

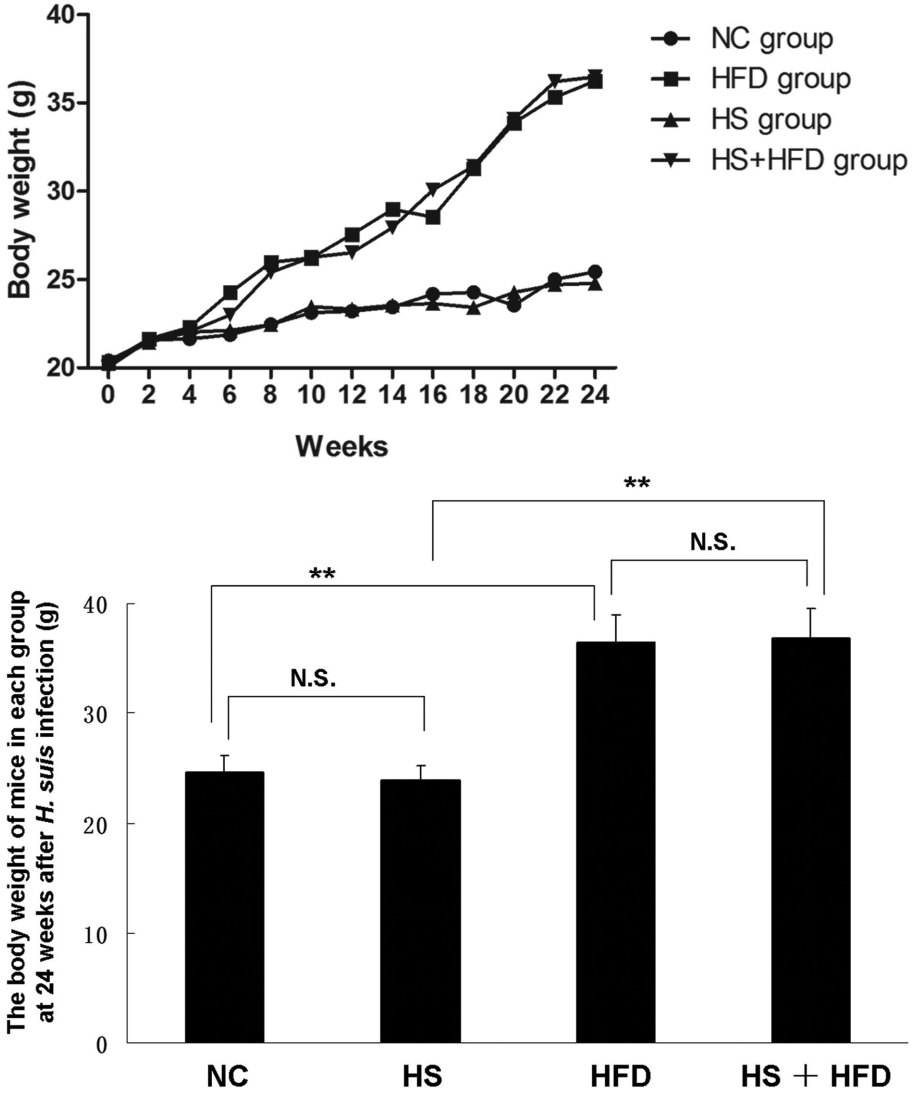

Figure 1. The relationship between changes in body weight in each group and infection time and body weights at 24 weeks after *H. suis* infection. Forty C57BL/6 female (8 weeks old) wild-type mice were randomly divided into the following 4 groups of 10 mice each: normal control (NC), *H. suis*-infected (HS), high-fat diet (HFD) and *H. suis*-infected plus high-fat diet (HS+HFD). The mice in the NC and HS groups were fed a standard chow diet, and the mice in the HFD and HS+HFD groups were fed a high-fat diet beginning 1 week post-infection. The body weights of the mice in each group were measured every week. Changes of the body weights of the mice in each group are shown according to the length of infection (**A**) and were compared at 24 weeks post-infection (**B**). The data are shown as the mean or mean ± SD (n = 10). **Significant difference ($P < 0.01$); N.S., no significant difference ($P > 0.05$).

organogenesis and construction of secondary lymphoid tissues (Mebius 2003; Weih and Caamaño 2003; Hoffmann and Baltimore 2006). The NF-κB family consists of five members, including RelA, RelB, cRel, NF-κB_1 (p50) and NF-κB_2 (p52). In the canonical pathway, the TNF receptor (TNFR) transmits a signal that induces the phosphorylation and degradation of inhibitor of NF-κB (IκB), triggers the translocation of the RelA–p50 complex from the cytoplasm to the nucleus (canonical pathway) and initiates the expression of a broad array of inflammatory genes (Hoffmann, Leung and Baltimore 2003; Hoffmann and Baltimore 2006). Via an alternative non-canonical pathway, the LTβR triggers the phosphorylation and processing of p100 (a NF-κB_2 gene product), and this generates p52, which activates the RelB–p52 complex (non-canonical pathway), which is known to regulate a restricted set of genes that includes CXCL13, CCL19, CCL21 and CXCL12 (Dejardin *et al.* 2002; Hoffmann and Baltimore 2006; Wharry, Haines and Carroll 2009). Importantly, the activation of both the canonical and non-canonical NF-κB pathways is essential to induce CXCL13 (Suto *et al.* 2009). Therefore, we analyzed the expression of factors upstream of the NF-κB signaling pathway, including TNF-α, LTα, and LTβ, their receptors, TNFR1 and LTβR, and the NF-κB_1 and NF-κB_2 genes, using real-time PCR. The results showed that the expression levels of these markers were higher in the stomachs of mice in the HS + HFD group than in the stomachs of mice in the HS group (Fig. 4A–C). Next, we performed western blot analysis using antibodies for NF-κB signaling pathway-related proteins, and the results showed that the expressions of p65 in nuclear and phospho-p65, phospho-IκBα, p100 and p52 in cytoplasm were upregulated in the stomachs of *H. suis*-infected mice and enhanced by high-fat-diet-induced obesity (Fig. 5A and B). These findings indicated that diet-induced obesity enhanced the activation of TNFR1/LTβR

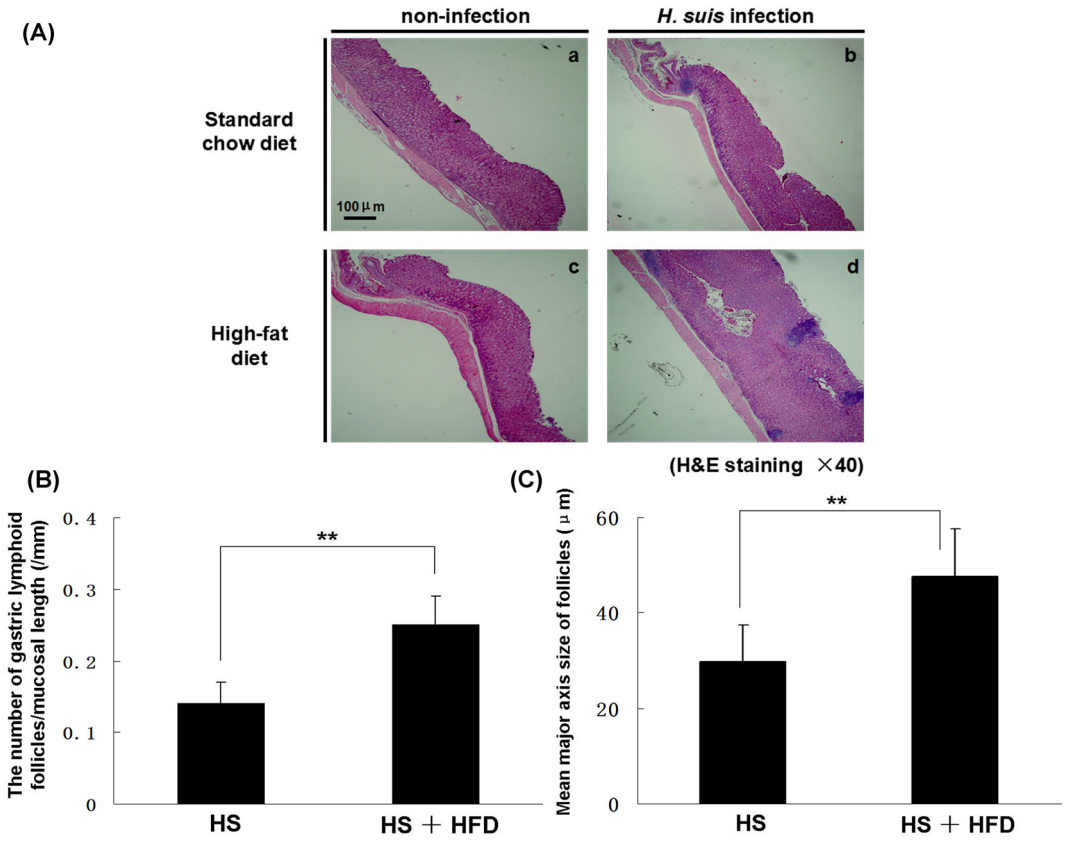

Figure 2. The formation of lymphoid follicles in the gastric mucosa and the bacterial load in the stomachs of mice in each group at 24 weeks post-*H. suis* infection. (A) Histological examinations of the gastric mucosa in each group (n = 10) were performed using hematoxylin and eosin staining at 24 weeks post-*H. suis* infection. (a) NC group; (b) HS group; (c) HFD group; (d) HS+HFD group. Original magnification: ×40. The black bar indicates 100 μm. The mean number of lymphoid follicles (**B**) and the mean major axis size of the lymphoid follicles in the gastric mucosae of mice in the HS and HS+HFD groups (**C**) were determined by examining hematoxylin and eosin-stained specimens under a microscope in a blinded manner. The data shown are representative of at least three independent experiments and are shown as the mean ± SD (n = 10). **Significant difference ($P < 0.01$).

and the downstream NF-κB signaling pathway in mice infected with *H. suis*, and these effects contributed to the upregulation of lymphoid chemokines and the formation of gastric lymphoid follicles.

DISCUSSION

Helicobacter suis is the most prevalent gastric NHPH species in humans and is transmitted to humans most likely via direct or indirect contact with pigs, which are their main reservoir (Haesebrouck *et al.* 2009). Compared to *H. pylori*, which is known to cause predominantly metaplastic/dysplastic changes, *H. suis* rarely induces gastric adenocarcinoma, and *H. suis*-associated gastritis appears to be less active and less severe. However, the risk of developing MALT lymphoma is higher in *H. suis* infections (Morgner *et al.* 2000; O'Rourke *et al.* 2004a; Nakamura *et al.* 2007). The formation of *Helicobacter*-induced MALT lymphoma is a less common outcome in *H. pylori* infection because it requires a prolonged and complex interaction among the bacteria, the host epithelium and the host immune system. Therefore, *H. suis*-infected animals are ideal models for investigating the mechanism underlying gastric MALT formation and the pathogenesis of gastric MALT lymphoma.

There is an increasing recognition that being overweight and obese impacts the development of a variety of cancers that can occur at a diversity of sites. Obesity is associated with an increased incidence of colorectal cancer, esophageal adenocarcinoma, and cancers of the gastric cardia, gallbladder, pancreas, liver, kidney, and thyroid as well as non-Hodgkin lymphoma (Larsson and Wolk 2011; Wolin, Carson and Colditz 2013). Furthermore, obesity is a condition involving chronic low-grade inflammation that is characterized by abnormal cytokine production, immune activation, and increased inflammatory signaling (Alemán *et al.* 2014). Numerous chemokines, including CCL19 and CXCL12, are expressed by adipocytes under obesity-associated chronic inflammation conditions, and their expression is predominantly regulated by NF-κB, which is the main mediator of TNF-α-induced chemokine expression (Tourniaire *et al.* 2013; Kim *et al.* 2014). However, obesity also affects the responsiveness of immune cells in some organs to chemokines. For instance, obesity increased lymphocyte hyper-responsiveness to chemokines in the liver and is directly responsible for a higher level of CXCL12- and CXCL13-induced chemotaxis in $CD4^+$ T and B cells (Bigorgne *et al.* 2008). Many studies have focused primarily on the direct effect of adipose-derived factors. However, little is known regarding the effect of obesity on the expression of chemokines in gastric tissues or the formation of chronic inflammation-induced acquired MALT. In this study, we provide the first evidence showing that high-fat-diet-induced obesity enhances the expression of lymphoid chemokines, including CXCL13, CCL19, CCL21 and CXCL12, and their receptors in the stomachs of animals infected with *H. suis*, which also have

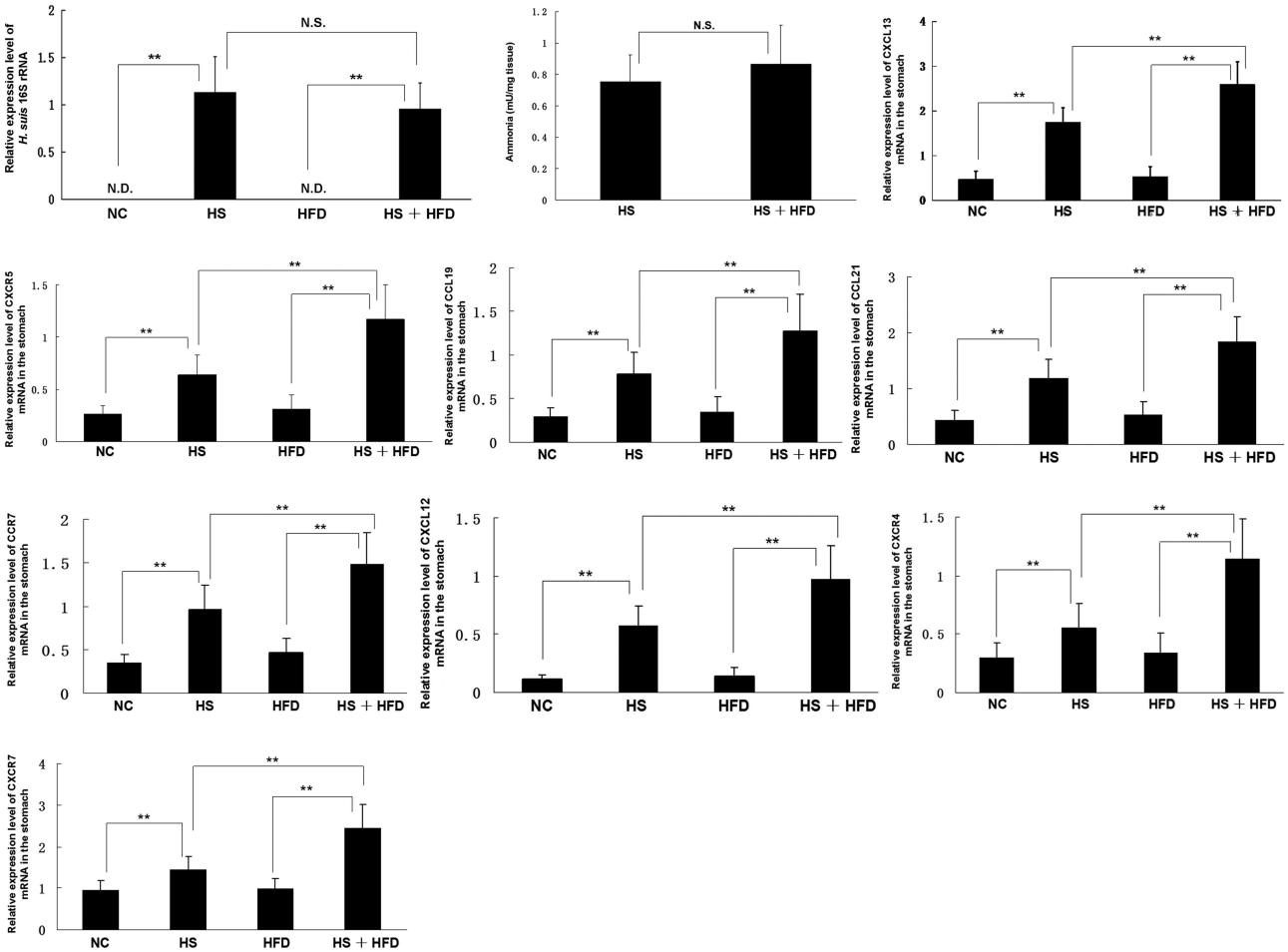

Figure 3. The relative bacterial load, urease activity and the expression of lymphoid chemokines and their receptors in the stomachs of mice in each group at 24 weeks post-*H. suis* infection. The relative expression level of the *H. suis* 16S rRNA (**A**), urease activity (**B**) and the mRNA expression levels of lymphoid chemokines and their receptors (**C**–**E**) in the stomachs of mice in each group at 24 weeks post-*H. suis* infection were estimated using real-time PCR and Urease Activity Assay Kit, respectively. The quantitative values were normalized to the level of mouse β-actin expression in each sample. The data shown are representative of at least three independent experiments and are shown as the mean ± SD (n = 10). **Significant difference ($P < 0.01$); N.D., not detected; N.S., no significant difference ($P > 0.05$).

more and larger gastric lymphoid follicles, but did not affect the bacterial load in the stomach. These data demonstrate that obesity promotes the upregulation of lymphogenesis-related factors and contributes to the formation of gastric MALT.

It has been reported that the NF-κB signal transduction pathway plays an essential role in the development of secondary lymphoid organs and the recruitment of cells to these tissues (Weih and Caamaño 2003). The activation of NF-κB by the LT$\alpha_1\beta_2$-LTβR-NIK-IKKα pathway is crucial to the development and maintenance of a normal spleen architecture, FDC maturation and the organogenesis of the LN, Peyer's patches and nasopharyngeal-associated lymphoid tissue. However, TNF-TNFR1-IKKγ/β signaling is required for the proper cellular and structural organization of B-cell follicles and the development of FDC networks (Weih and Caamaño 2003). Furthermore, the engagement of LTβR by LT$\alpha_1\beta_2$ results in the transmission of a signal that is required to induce CXCL13 by promoting cooperation among multiple signaling pathways, including the canonical and non-canonical NF-κB pathways, and the latter is also responsible for the production of CCL19, CCL21 and CXCL12 (Dejardin *et al.* 2002; Hoffmann and Baltimore 2006; Suto *et al.* 2009; Wharry, Haines and Carroll 2009). Interestingly, we found that both the canonical and non-canonical NF-κB signaling pathways are activated in the stomach in animals in which obesity was induced by a high-fat diet. This activation induced the upregulation of lymphoid chemokines and accelerated the development of gastric MALT in animals infected with *H. suis*.

In summary, in this study, we reveal that the *H. suis*-induced formation of gastric lymphoid follicles is accelerated by high-fat-diet-induced obesity. The mechanism underlying this relationship might involve the activation of the NF-κB signaling pathway and the induction of lymphoid chemokines in the stomach. Because multiple mechanisms are implicated in the relationship between obesity and neoplasia, including inflammatory changes, altered adipokine profiles, insulin resistance and adipose tissue hypoxia, we expect that future investigations will identify the specific pathogenesis involved in obesity-related gastric diseases, especially those induced by *Helicobacter* infection.

FUNDING

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Conflict of interest. None declared.

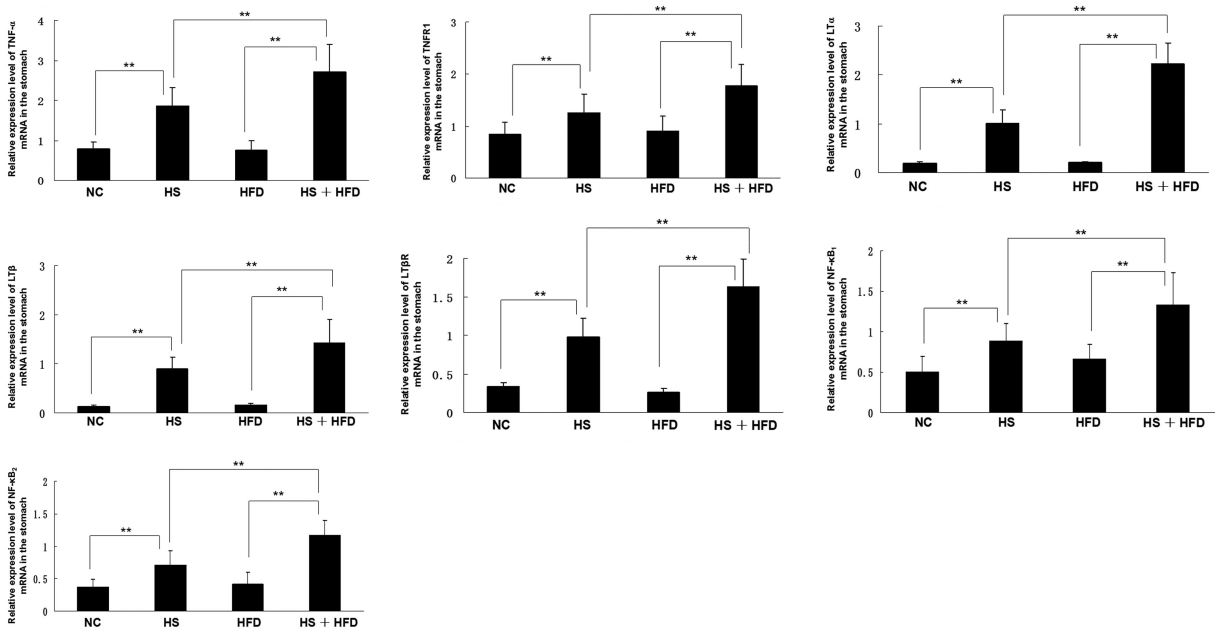

Figure 4. The expression levels of TNF-α and lymphotoxin and their receptors, NF-κB$_1$ and NF-κB$_2$, in the stomachs of mice in each group at 24 weeks post-*H. suis* infection. The mRNA expression levels of TNF-α and its receptor, TNFR1 (**A**), and lymphotoxin and its receptor, LTβR (**B**), in addition to the expression levels of NF-κB$_1$ and NF-κB$_2$ (**C**) in the stomachs of mice in each group were estimated using real-time PCR at 24 weeks post-*H. suis* infection. The quantitative values were normalized to the level of mouse β-actin expression in each sample. The data shown are representative of at least three independent experiments and are shown as the mean ± SD (n = 10). **Significant difference ($P < 0.01$).

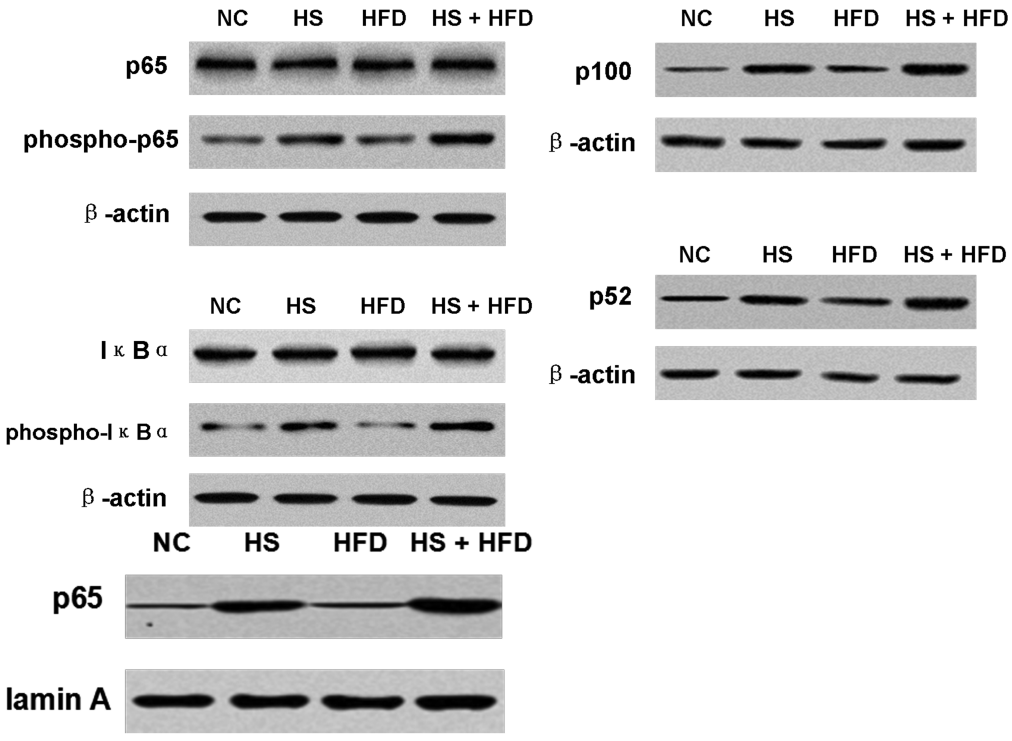

Figure 5. The activation of the NF-κB signaling pathway in the stomachs of mice in each group at 24 weeks post-*H. suis* infection. The expressions of p65, phospho-p65, IκBα, phospho-IκBα in cytoplasm and p65 in nuclear (**A**) and the expressions of p100 and p52 in cytoplasm (**B**) in the stomachs of mice in each group at 24 weeks post-*H. suis* infection was detected using western blot analysis with corresponding antibodies. The data shown are representative of at least three independent experiments and typical images are shown.

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