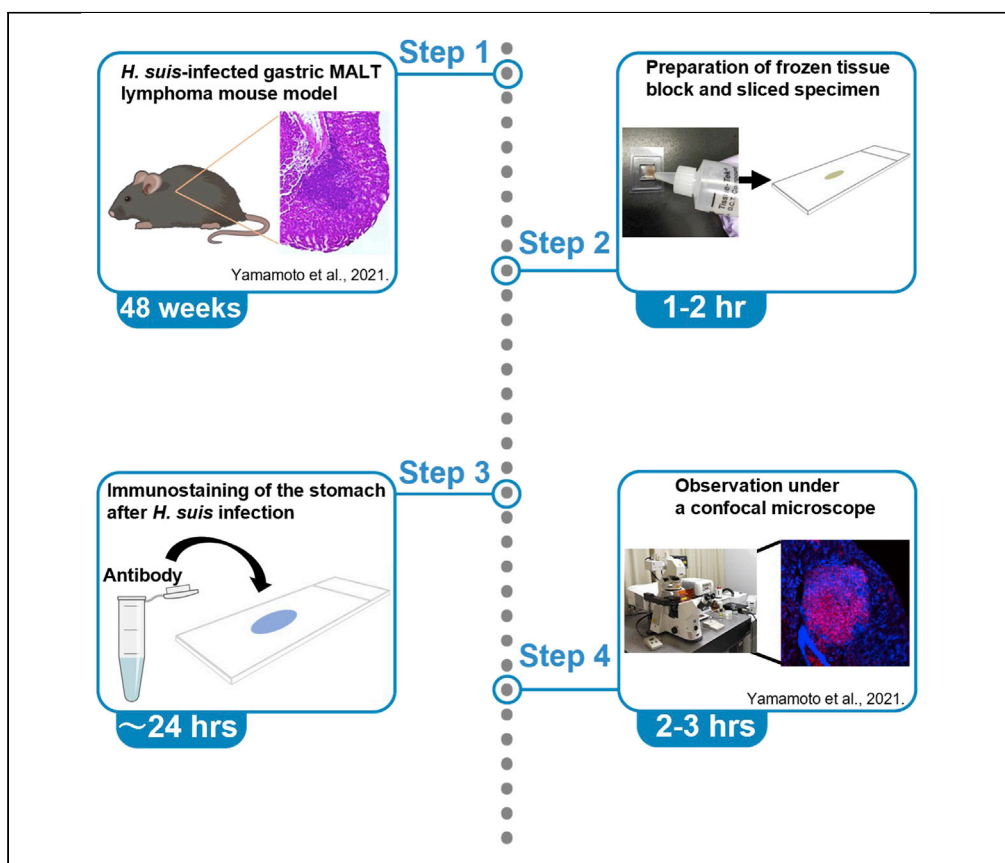


Protocol

Protocol for generating a mouse model of gastric MALT lymphoma and the identification of MALT lymphoma cell populations by immunostaining



Koji Yamamoto,
Yasuyuki Kondo,
Toshiro Sugiyama,
Naoya Sakamoto

kyama@pop.med.
hokudai.ac.jp

Highlights

Protocol uses
Helicobacter suis to
induce gastric MALT
lymphoma

Protocol details the
generation of a
gastric MALT
lymphoma mouse
model

Protocol describes an
immunostaining
approach to identify
MALT lymphoma cell
populations

Protocol is applicable
in mice with genetic
alterations

Helicobacter suis, a zoonotic infection-related bacterium, induces gastric mucosa-associated lymphoid tissue (MALT) lymphoma in humans and animals. However, a lack of suitable animal models complicates the detailed analysis of this disease. Here, we describe the generation of a gastric MALT lymphoma mouse model. We then detail the use of this model combined with an immunostaining protocol to identify the cell populations that constitute gastric MALT lymphoma. This protocol can be used to identify the constituent cells of human MALT lymphoma.

Yamamoto et al., STAR
Protocols 3, 101155
March 18, 2022 © 2022 The
Author(s).
[https://doi.org/10.1016/
j.xpro.2022.101155](https://doi.org/10.1016/j.xpro.2022.101155)

Protocol

Protocol for generating a mouse model of gastric MALT lymphoma and the identification of MALT lymphoma cell populations by immunostaining

Koji Yamamoto,^{1,2,4,5,*} Yasuyuki Kondo,³ Toshiro Sugiyama,² and Naoya Sakamoto¹¹Department of Gastroenterology and Hepatology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido 060-8638, Japan²Research Division of Molecular Targeting Therapy and Prevention of GI Cancer, Hokkaido University Hospital, Sapporo, Hokkaido 060-8638, Japan³Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0017, Japan⁴Technical contact⁵Lead contact*Correspondence: kyama@pop.med.hokudai.ac.jp
<https://doi.org/10.1016/j.xpro.2022.101155>

SUMMARY

Helicobacter suis, a zoonotic infection-related bacterium, induces gastric mucosa-associated lymphoid tissue (MALT) lymphoma in humans and animals. However, a lack of suitable animal models complicates the detailed analysis of this disease. Here, we describe the generation of a gastric MALT lymphoma mouse model. We then detail the use of this model combined with an immunostaining protocol to identify the cell populations that constitute gastric MALT lymphoma. This protocol can be used to identify the constituent cells of human MALT lymphoma.

For complete details on the use and execution of this profile, please refer to Yamamoto et al. (2021).

BEFORE YOU BEGIN

All animal experiments were performed in accordance with the Guidelines for Animal Experimentation at Kobe University (Permission No. P130105 and P130106), Hokkaido University (Permission No. 17-0094), and the ARRIVE guidelines. Six-week-old female C57BL/6J mice were purchased from CLEA Japan (C57BL/6JJcl; Tokyo, Japan) and Japan SLC (C57BL/6JJmsSlc; Shizuoka, Japan).

Animals

1. All mice should be maintained under barrier conditions, in microisolator cages, containing either compressed paper or aspen chip bedding placed on free-standing shelves or individually ventilated cage racks.
2. All KO mice should be co-housed to homogenize the gut microbiota.
3. The animals should be maintained under specific pathogen-free conditions in a clean room under a controlled environment at the Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine, or at Kobe University Graduate School of Medicine.
4. The temperature should be maintained at $22 \pm 3^\circ\text{C}$, humidity at 40–60%, with a 12 h light/dark cycle.
5. All mice should have free access to food (standard chow) and water (Yamamoto et al., 2021). Preparation 1 should be conducted in the safety cabinet of a BSL2-compatible infectious animal laboratory. The Preparation 2 should be conducted in the safety cabinet of a laboratory facility,



which had permission for BSL2-compatible pathogen handling. The Preparations 3 and 4 should be conducted in the laboratory safety cabinet.

Preparation 1. Procedure for establishing an *H. suis*-infected gastric MALT lymphoma mouse model

⌚ Timing: 24 weeks

The *H. suis* was originally obtained from pig stomach (Yamamoto et al., 2011) and maintained in C57BL/6J mice stomachs for 3–6 months following the infection (as donors). These methods are established and have been used in several studies (Mimura et al., 2011; Ben Suleiman et al., 2012; Yamamoto et al., 2014; Yang et al., 2015). Perform the following experiments using these mice.

6. Resect the stomach of an *H. suis*-infected or an uninfected mouse, opening at the outer curvature; wash it with 1× PBS (Figures 1A and 1B).
7. Transfer the opened stomach to a Petri dish.
8. Prepare a gastric homogenate from the stomach using 1 mL of 1× PBS on a glass slide.
9. Use this homogenate as the bacterial inoculum to propagate *H. suis* infection.
10. Infect wild-type mice orally with the gastric homogenate.

⚠ **CRITICAL:** Use a glass slide to exert a sufficient pressure for preparing the gastric homogenate (Figure 1C).

11. Administer 0.2 mL of gastric homogenate using a 20G oral sonde to each uninfected mouse.

⚠ **CRITICAL:** Suspend one stomach in 1 mL of 1× PBS; administer 0.2 mL of homogenate per mouse. The homogenate from one stomach could be administered to up to 5 animals.

12. Use an aliquot of the gastric homogenate to confirm *H. suis* infection using PCR analysis. (Figure 1C, Preparation 2, Preparation 3, and key resources table).
13. After confirming *H. suis* infection, continue maintaining the infected mice for 6 months to develop a gastric MALT lymphoma model. In this study, the end-point of the experiment was at 6 months.

Preparation 2. Extraction of DNA from the stomach of *H. suis*-infected mice

⌚ Timing: 2 days

14. Add the specified amounts of Tris-HCl, SDS, and Proteinase K (Table 1) to the gastric homogenate to enable cell lysis.
15. Incubate the mixture at 55°C for 12–24 h in a heat block.
16. Add 400 µL of phenol/chloroform/isoamyl alcohol (PCI) (25:24:1) to the lysate and centrifuge at 13200×g at 4°C for 5 min.

⚠ **CRITICAL:** The PCI mixture is a non-medicinal toxic substance and is always stored in the dark at 4°C in locked shelves.

17. After centrifugation, collect 300 µL of supernatant and add 30 µL of 3 M sodium acetate (pH 5.2) and 825 µL of 99% ethanol.
18. Centrifuge the mixture at 15500×g at 4°C for 10 min.
19. After centrifugation, discard the supernatant and add 1 mL of 75% ethanol, taking care to not break the pellet.
20. Centrifuge the mixture at 15500×g at 4°C for 5 min.

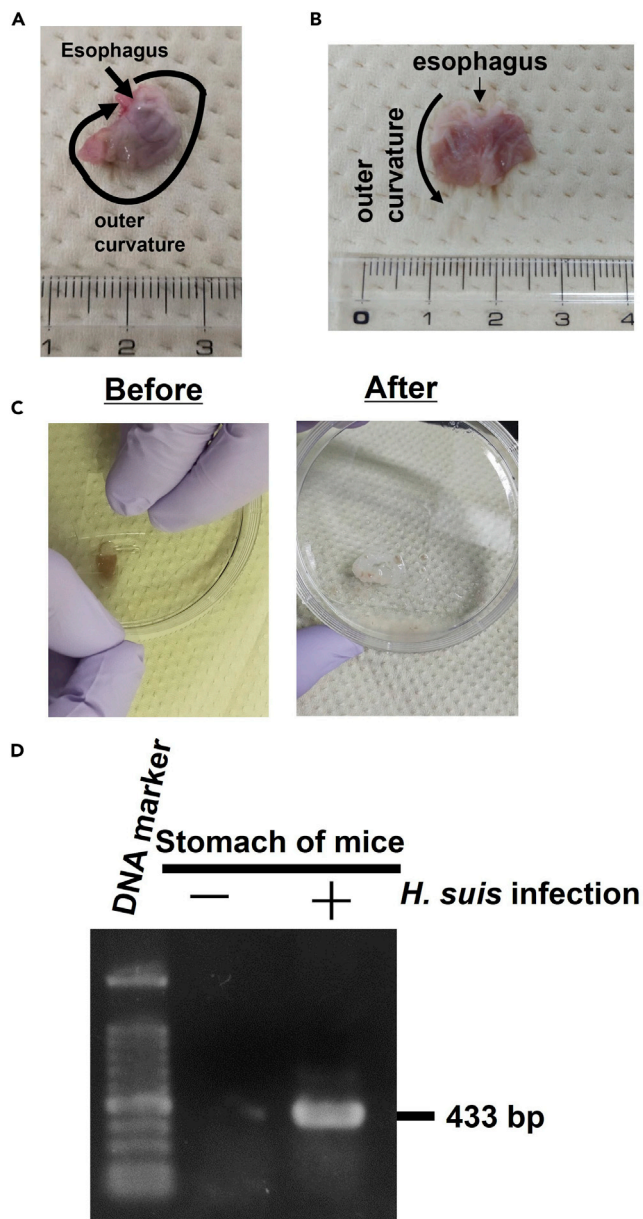


Figure 1. Confirmation of *H. suis* infection using PCR analysis of the stomach homogenates of *H. suis*-infected mice

(A) Macroscopic observation of mouse stomach before being opened at the outer curvature from the esophagus. 1 scale; 0.1 cm.

(B) Macroscopic observation of mouse stomach after being opened at the outer curvature from the esophagus. 1 scale; 0.1 cm.

(C) Preparation of mouse gastric homogenate. Before: Photograph before preparing the gastric homogenate on cover glass. After: Photograph after preparing the stomach homogenate with cover glass. In the figure on the right, the gastric epithelial tissue is peeled off by the cover glass.

(D) PCR analysis with *H. suis* 16S rRNA-specific primers.

21. After centrifugation, discard the supernatant, and place the tubes on a bench with the lid open and air dry the pellet at room temperature for 10 min.
22. Add Tris-EDTA (TE) buffer (200–500 μ L; pH 8.0) to the pellet and incubate at 37°C, in a heat block for 5–10 min.

Table 1. Lysis of mouse-derived stomach

Reagent	Final concentration	Amount
1 M Tris-HCl (pH 7.5)	10 mM	4 μ L
10% SDS	1%	40 μ L
Proteinase K (20 mg/mL)	200 μ g/mL	4 μ L
Gastric homogenate	–	352 μ L
Total	–	400 μ L

23. Use the extracted DNA for PCR analysis.

Δ **CRITICAL:** DNA concentration and quality is evaluated using a spectrophotometer (Nanodrop). If the A260/230 ratio is lower than 1, it indicates the presence of contaminants, such as phenol. If the A260/230 ratio is less than 1, purify the DNA again. Ensure that the A260/A280 ratio is between 1.5 and 2.0; ideally, it should be 1.8. The above evaluation is highly recommended because the quality of DNA affects the PCR. After measuring the concentration of the extracted DNA, the DNA is diluted to 1000 ng/ μ L, and 1000 ng of DNA is used for the PCR analysis.

Preparation 3. PCR analysis conditions for confirming *H. suis* infection

\odot Timing: 3–4 h

24. Preparation of PCR amplification mix

The details of the reagents used and the PCR cycling conditions are shown in [Tables 2](#) and [3](#).

Preparation 4. Gel electrophoresis

\odot Timing: 1 h

25. Prepare agarose mini-gel

26. Separate the PCR products on 1.5% agarose mini-gels in TAE buffer (40 mM Tris-acetate and 1 mM EDTA).

27. Conduct electrophoresis at 100 V, using a 100 bp DNA ladder as the standard.

28. Following electrophoresis, stain the gels with a DNA binding dye, such as ethidium bromide.

29. Place the gel in ethidium bromide buffer and stain for 15 min.

Δ **CRITICAL:** Ethidium bromide is a powerful mutagen; therefore, be careful when storing and handling it. Wear disposable gloves when handling. Ethidium bromide solution should be stored at room temperature (20°C–25°C) used for staining agarose gels, and disposed immediately after inactivation. For inactivating the liquid waste after ethidium bromide

Table 2. Preparation of PCR reaction mix

Reagent	Final concentration	Amount
PCR-Grade Water	–	40 μ L
10 \times Advantage 2 PCR Buffer	10 \times	5 μ L
50 \times dNTP Mix (10 mM)	50 \times	1 μ L
5' primer (10 μ M)	10 μ M	1 μ L
3' primer (10 μ M)	10 μ M	1 μ L
50 \times Advantage 2 Polymerase Mix	50 \times	1 μ L
DNA Template (1000 ng/ μ L)	1000 ng	1 μ L
Total volume	–	50 μ L

Table 3. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	1 min	1
Denaturation	95°C	30 s	25–35
Annealing	68°C	1 min	
Final extension	68°C	1 min	1
Hold	4°C	Forever	

staining, use EtBr Destroyer, which effectively decomposes and destroys ethidium bromide, making it non-mutagenic. Ethidium bromide was used in this study; however, as an alternative, nucleic acid staining reagents (GelRed™ & GelGreen™ (Biotium, Inc.)) with low toxicity, high sensitivity, and excellent thermal stability could be used.

30. Photograph the gel under a UV transilluminator (Figure 1D).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Polyclonal rabbit anti- <i>Helicobacter pylori</i> (1:20)	DAKO	Cat#B047129-1; RRID: AB_2313773
Purified rat anti-mouse CD45R (1:20)	BD Biosciences	Cat#553086; RRID: AB_394616
Purified rat anti-mouse CD4 (1:20)	BD Biosciences	Cat#550280; RRID: AB_393575
Purified rat anti-mouse F4/80 (1:20)	BD Biosciences	Cat#565409; RRID: AB_2739222
Purified anti-mouse NK-1.1 (1:20)	BioLegend	Cat#108702; RRID: AB_313389
FITC hamster anti-mouse CD11c (1:20)	BD Biosciences	Cat#553801; RRID: AB_395060
Purified rat anti-mouse follicular dendritic cell (1:20)	BD Biosciences	Cat#551320; RRID: AB_394151
Monoclonal rat anti-mouse Ep-CAM (G8.8) (1:20)	BioLegend	Cat#118202; RRID: AB_1089026
Biotin-conjugated anti-mouse TLR4 (1:20-50)	Abcam	Cat#ab6788; RRID: AB_954885
DyLight 488-conjugated streptavidin (1:200)	Jackson Immuno Research Laboratories, Inc	Cat#016-480-084; RRID: AB_2313773
Goat anti-rat IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (1:200)	Thermo Fisher Scientific	Cat#A-11006; RRID: AB_2534074
Goat anti-rat IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 546 (1:200)	Thermo Fisher Scientific	Cat#A-11081; RRID: AB_141738
Goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (1:200)	Thermo Fisher Scientific	Cat#A-11008; RRID: AB_143165
Alexa Fluor 647 phalloidin (1:200)	Thermo Fisher Scientific	Cat#A22287
Bacterial and virus strains		
<i>Helicobacter suis</i>	Isolated from pig	N/A
Chemicals, peptides, and recombinant proteins		
100 bp DNA Ladder (Dye Plus)	TaKaRa Bio	Cat#3422A
Acetone	Wako	Cat#016-00346
NORMAL GOAT SERUM	VECTOR	Cat#S-1000; RRID: AB_2336615
Tissue-Tek O.C.T. Compound	Sakura Finetek	Cat#4583
50x TAE	NIPPON GENE	Cat#313-90035
Agarose S	Wako	Cat#312-01193
Ethidium Bromide Solution	Invitrogen	Cat#15585-011
Prolong Diamond (fluorescent mounting medium)	Invitrogen	Cat#P36961
Ethanol (99.5%)	Wako	Cat#057-00456
TE (pH 8.0)	NIPPON GENE	Cat#316-90025
1M Tris-HCl (pH7.5)	NIPPON GENE	Cat#318-90225

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
10% SDS Solution	NIPPON GENE	Cat#311-90271
3M Sodium Acetate (pH 5.2)	NIPPON GENE	Cat#316-90081
Phenol/Chloroform/Isoamyl alcohol (25:24:1)	NIPPON GENE	Cat#311-90151
Proteinase K	AmBion	Cat#AM2546
Advantage 2 PCR Kit	TaKaRa Bio	Cat#639206
PBS	Sigma-Aldrich	Cat#P3813
Experimental models: Organisms/strains		
Mouse: WT: C57BL/6J	CLEA Japan Inc.	C57BL/6JJcl
Mouse: WT: C57BL/6J	Japan SLC	C57BL/6JJmsSlc
Mouse: <i>Myd88</i> KO: <i>Myd88</i> deficient	Oriental Yeast Co., Ltd.	B6.129-Myd88 ^{tm1Aki} /Obs RRID: IMSR_OBS:1
Mouse: <i>Trif</i> KO: <i>Trif</i> deficient	Oriental Yeast Co., Ltd.	B6.129P2-Trif ^{tm1Aki} /Obs IMSR_OBS:16
Mouse: <i>Myd88/Trif</i> DKO: <i>Myd88/Trif</i> deficient	Oriental Yeast Co., Ltd.	B6.129P2-MyD88/Trif ^{tm1Aki} / Obs RRID: IMSR_OBS:22
Oligonucleotides		
<i>H. suis</i> 16S rRNA primer Forward; 5'-TTGGGAGGCTTTGTCTTCCA-3'	Yamamoto et al., 2011	https://pubmed.ncbi.nlm.nih.gov/21397035/
<i>H. suis</i> 16S rRNA primer Reverse; 5'-GATTAGCTGCTCGCGGCT-3'	Yamamoto et al., 2011	https://pubmed.ncbi.nlm.nih.gov/21397035/
Software and algorithms		
LSM Image Browser	Carl Zeiss Co. Ltd	https://www.embl.de/eamnet/html/body_image_browser.html
Other		
Confocal laser-scanning microscope	Carl Zeiss Co. Ltd	Cat#LSM710
Thermal cycler	Applied Biosystems	Cat#2720
SLIDE GRASS DRYER SGD-F	ASONE	Cat#1-4178-01
Cryostat	Leica Biosystems	Cat# CM1850
Incubation Chamber	COSMO BIO CO., LTD	N/A
Tissue-Tek Cryomold (15 mm x 15 mm x 5 mm)	Sakura Finetek	Cat#4566
Mupid-2plus	ASONE	N/A
FUSION SOLO S (UV trans-illuminator)	VILBER	N/A
Animal Feeding Needles (20G) (Oral sonde)	Natsume Seisakusho Co., Ltd.	Cat#KN-348-20G-50
Matsunami Cover Glass (No.1) 24 x 32mm	MATSUNAMI GLASS IND., LTD	Cat#C024321
Spectrophotometer	DeNovix	Cat#DS-11

MATERIALS AND EQUIPMENT

Materials

Preparation of 75% ethanol (Use immediately after dilution)		
Reagent	Final concentration	Amount
99.5% ethanol	75%	75.4 mL
Water	–	24.6 mL
Total	–	100 mL

Preparation of 1xTAE buffer (Use immediately after dilution)		
Reagent	Final concentration	Amount
50x TAE (Store at room temperature)	1x	1 mL
Water	–	49 mL
Total	–	50 mL

Preparation of 2% agarose mini-gels (Use immediately after preparation)

Reagent	Final concentration	Amount
1× TAE	–	25 mL
Agarose S	2%	0.5 g
Total	–	25 mL

Preparation of ethidium bromide buffer (Use immediately after dilution)

Reagent	Final concentration	Amount
Ethidium bromide (Store at room temperature)	–	20 µL
Water	–	200 mL
Total	–	200 mL

Preparation of 10% goat serum (After dilution, use immediately without storing)

Reagent	Final concentration	Amount
Goat serum	10%	10 µL
PBS	–	90 µL
Total	–	100 µL

Preparation of Anti-mouse primary antibody

Reagent	Final concentration	Amount
Anti-mouse CD45R antibody (1: 20)	1: 20	5 µL
PBS	–	95 µL
Total	–	100 µL

△ **CRITICAL:** If a sample requires staining with multiple antibodies targeting different proteins, it is essential to use antibodies raised in different hosts.

Preparation of secondary antibody

Reagent	Final concentration	Amount
Anti-rat IgG Alexa488 (1: 200)	1: 200	1 µL
Alexa Fluor 647 phalloidin (1:200)	1: 200	1 µL
PBS	–	198 µL
Total	–	200 µL

△ **CRITICAL:** Multiple secondary antibodies from different hosts could be added. In that case, the amount of PBS is adjusted according to the amount of primary antibody added.

Equipment:

- Confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany)

Alternatives: Any other confocal laser-scanning microscope could be used.

- CM1850 Cryostat (Leica, Germany)
- Incubation chamber (Cosmo Bio Co. Ltd., Tokyo, Japan)

Alternatives: Any other incubation chamber could be used.

- Slide glass dryer (As one Corporation)

Alternatives: Any other slide glass dryer could be used.

- Oral sonde (Natsume Seisakusho Co., Ltd. Tokyo, JAPAN)

Alternatives: Any other oral sonde could be used.

- Thermal cycler (Applied Biosystems)

Alternatives: This protocol is based on a PCR procedure performed with a thermal cycler (Applied Biosystems). Gradient TurboCycler 2 (Blue-Ray Biotech) was also used.

- UV trans-illuminator (VILBER)

Alternatives: ImageQuant LAS 4000 (GE Healthcare Life Science) could be used.

- Mupid-2plus (Takara Bio Inc.)

Alternatives: WSE-1710Submerge-Mini (ATTO Corporation) could be used.

STEP-BY-STEP METHOD DETAILS

Preparing a frozen block of mouse stomach

⌚ Timing: 6 months

1. Six months after *H. suis* infection, sacrifice all mice through cervical dislocation under anesthesia.
2. Resect the stomachs, opening at the outer curvature (Figure 2).
3. Free half of the stomach in cryo-embedding compound (O.C.T) to be cut into sections (Sakura Finetek, Tokyo, Japan).

⚠ **CRITICAL:** The OCT compound is added according to the size of the Cryomold (15 mm × 15 mm × 5 mm).

⚠ **CRITICAL:** The cryomolds are stored at -80°C until sectioning.

Preparing a frozen sliced sample

⌚ Timing: ~1 h

The objective of this step was to create a sliced sample of 10- μm thickness.

4. Set the inside temperature of the device between -15°C and -20°C .

⚠ **CRITICAL:** When preparing a sliced sample, ensure that the tissue does not crack or break after sectioning

5. After slicing, immediately place the tissue sections in a staining vat containing 200 mL of acetone for about 1 s, dry the sections using an air dryer, and store at -30°C until staining.

⚠ **CRITICAL:** If the sliced sample is not stored at -30°C , start the staining immediately using the following procedure.

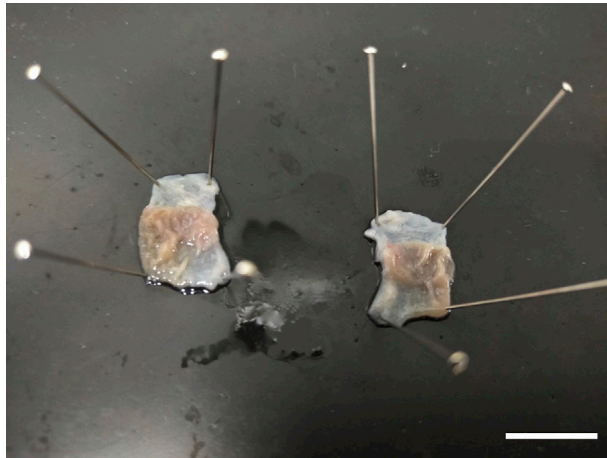


Figure 2. Preparing frozen samples of mouse stomach

Photograph after resecting the stomach, opened at the outer curvature. Scale bar; 1.0 cm

Immunostaining protocol

⌚ Timing: ~24 h

- Air dry the tissue sections for 1–2 h at room temperature.
- Use a staining vat containing 200 mL of acetone to fix tissue sections. Fix the sections in acetone for 5–10 min.

⚠ **CRITICAL:** This fixation step should be done inside the chemical hood.

- Allow the sections to stand in a staining vat containing 200 mL of 1× PBS for 3 min, for each wash. Repeat the wash thrice.
- Remove the excess 1× PBS from the section using a wipe.
- Add 100 mL of 10% goat serum drop-wise onto the section and incubate for 30 min for blocking, in a humid chamber.
- Wash the sections thrice with PBS, incubating for 3 min each time.
- After removing excess 1× PBS from the section, add 100 μL of primary antibody (diluted 1:20–50 with 1× PBS) drop-wise and incubate at room temperature for 2 h (or at 4°C for 12–24 h in a humid chamber).

⚠ **CRITICAL:** Be careful not to let the sample dry out, because this could affect the staining. After removing excess PBS from the section, it is highly recommended to stain the samples immediately with the respective antibody.

- Wash the sections thrice with 1× PBS, incubating for 3 min each time.
- Remove the excess 1× PBS from the section. Add 100 μL of secondary antibody (diluted in 1× PBS) drop-wise and incubate at room temperature for 1 h in a dark room in a humid chamber.

⚠ **CRITICAL:** Be careful not to let the sample dry out, because this could affect the staining.

- Wash the sections thrice with 1× PBS incubating for 3 min each time.
- After removing the excess 1× PBS from the section, add 100 μL of fluorescent mounting medium drop-wise, and cover the tissue section with a coverslip (Size; 24 × 32 mm).

△ **CRITICAL:** Be careful not to let the sample dry out, because this could cause the fading of the fluorescence.

△ **CRITICAL:** After adding the mounting medium, ensure there are no bubbles in the sample. If there are air bubbles, use a PIPETMAN® pipetman to completely remove them by aspiration.

△ **CRITICAL:** After encapsulating the mounting medium, the sample can be stored at 4°C for 2–3 days.

17. Observe fluorescence signals under a confocal laser-scanning microscope.
18. Use a suitable software, such as LSM Image Browser, to analyze the images. Set the pixels in the x-y plane to 1024 × 1024 pixels at a pixel size of 0.83 μm/pixel.

EXPECTED OUTCOMES

Myd88 knockout (KO), *Trif* KO, and *Myd88/Trif* double knockout (DKO) mice were infected with *H. suis* for 6 months, to investigate the roles of these proteins in the formation of MALT lymphoma. We detected the formation of the gastric MALT lymphoma mainly in the stomachs of *H. suis*-infected WT and *Myd88* KO mice but not in the stomachs of *Trif* KO and *Myd88/Trif* DKO mice (Figure 3A) (Yamamoto et al., 2021). The incidence of gastric MALT lymphoma is similar between WT and *Myd88* KO mice. The immunocompetent cells that infiltrate follicles after *H. suis* infection were visualized using immunofluorescence staining with antibodies of interest. Consistent with our recent reports (Yamamoto et al., 2014; Yang et al., 2015), B cells, CD4⁺ T cells, Dendritic Cells (DCs), and Follicular dendritic Cells (FDCs), but not natural killer cells and monocytes/macrophages, were identified in the gastric lymphoid follicles of *H. suis*-infected WT and *Myd88*-KO mice. In contrast, the infiltration of these cells was less in the *H. suis*-infected *Trif*-KO and *Myd88/Trif*-DKO mice, with the detection of fewer follicles (Figure 3B) (Yamamoto et al., 2021). Immunostaining showed that *H. suis* was mainly located in the gastric mucosa of infected WT and *Myd88*-KO mice, but not in that of *Trif*-KO and *Myd88/Trif*-DKO mice (Figure 3C) (Yamamoto et al., 2021). A round structure infiltrated with lymphocytes is observed in the stomach of mice infected with *H. suis*; this is not found in normal gastric epithelial tissue. We refer to this as a follicle. *H. suis* was not localized in the lymphoid follicles. (Figure 3C) (Yamamoto et al., 2021). In addition, immunostaining results indicated that TLR4 was mainly localized in the gastric mucosa of *H. suis*-infected WT and *Myd88* KO mice but not in the gastric MALT lymphoma (Figure 3D) (Yamamoto et al., 2021). Moreover, epithelial cell adhesion molecule (EpCAM), a marker of gastric epithelial tissues, was detected in the gastric epithelium of mice after *Helicobacter* infection (Figure 3E) (Yamamoto et al., 2021; Shibata et al., 2010; El-Zaatari et al., 2013; Lina et al., 2013; Shigematsu et al., 2013).

LIMITATIONS

The limitation of our study was the failure to identify cells that infiltrated the stomach by performing similar immunostaining of human gastric MALT lymphoma patient specimens. *H. suis*, similar to *Helicobacter pylori*, is a gram-negative bacterium that colonizes the stomach of humans and various animals, such as pigs, dogs, and cats (O'Rourke et al., 2004; Priestnall et al., 2004; Yamamoto et al., 2011). *H. suis* is strongly associated with gastric MALT lymphoma (Nakamura et al., 2007; Okamura et al., 2013), and it has been detected in human patients. TLR4–TRIF is activated in the gastric epithelial cells following *H. suis* infection; this induces the production of type 1 Interferon (IFN), which interacts with the IFNAR expressed on gastric B cells. Gastric MALT lymphoma is formed in response to the IFN-γ production from gastric B cells. In addition, the IFN-γ produced from B cells promotes the formation of gastric MALT lymphoma by stimulating IFNGR on B cells to further increase IFN-γ production through feedback regulation. (Yamamoto et al., 2021). Human MALT-type outer marginal zone B-cell lymphoma expresses only TLR4 (Adam et al., 2008). Therefore, the activation of the TLR4 signaling pathway is associated with gastric MALT lymphoma formation

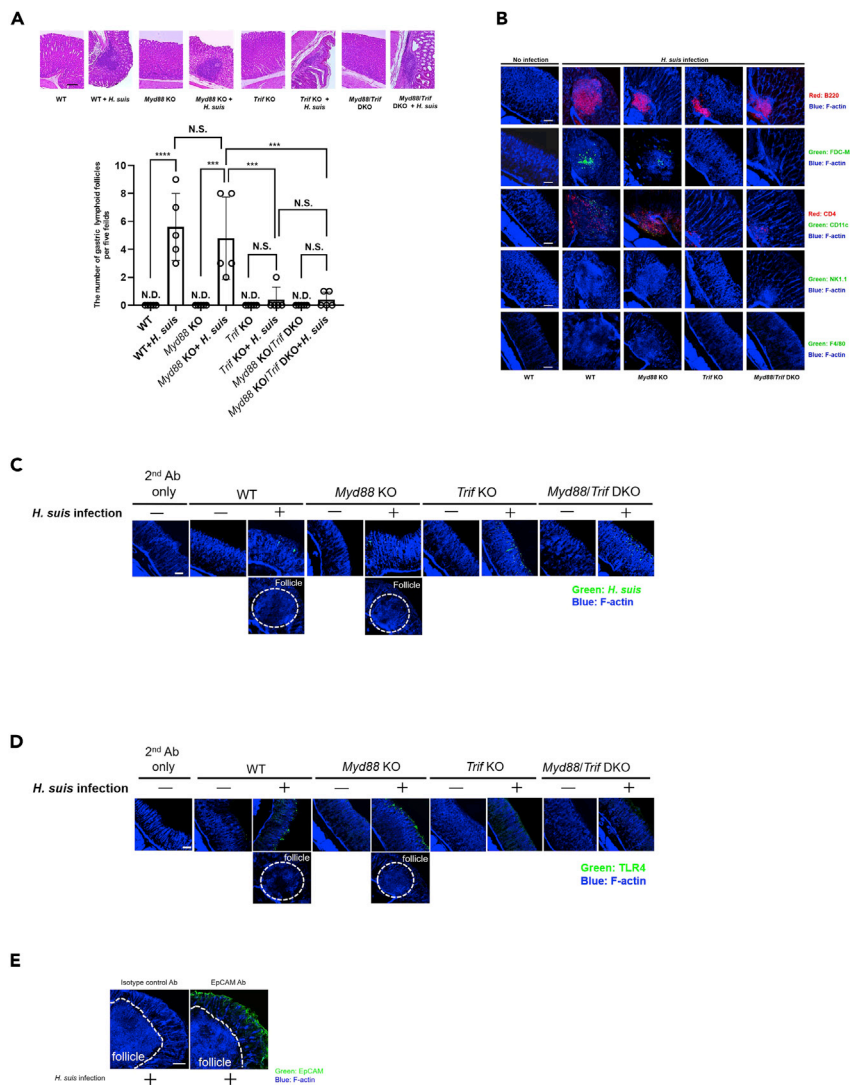


Figure 3. Immunofluorescence staining of mouse stomach

C57BL/6J WT, Myd88-KO, Trif-KO, and Myd88/Trif-DKO mice (n = 5/group) were analyzed 6 months after *H. suis* infection.

(A) Histological examination and mean number of gastric lymphoid follicles. Original magnification, 200 \times ; Scale bar; 100 μ m, (Yamamoto et al., 2021).

(B) Immunofluorescence of immunocompetent cells in the stomach (n = 5 per group). B220-positive cells (B cells), CD4-positive cells (helper T cells), CD11c-positive cells (dendritic cells), follicular dendritic cells, NK1.1-positive cells (natural killer cells), and phalloidin (F-actin) positive cells were analyzed using confocal microscopy. Magnification, 100 \times ; Scale bar; 100 μ m, (Yamamoto et al., 2021).

(C) Immunofluorescence of *H. suis* distribution (n = 5/group) after confocal microscopy. Magnification, 100 \times ; Scale bar; 100 μ m, (Yamamoto et al., 2021).

(D) Immunofluorescence of TLR4-expressing cells analyzed using confocal microscopy. Magnification, 100 \times ; Scale bar; 100 μ m, (Yamamoto et al., 2021).

(E) Analysis of EpCAM-positive cells in the stomachs of *H. suis*-infected WT mice. Magnification, 100 \times . The formation of gastric lymphoid follicles is indicated using the dotted lines. Representative images are shown. Magnification, 100 \times ; Scale bar; 100 μ m, (Yamamoto et al., 2021).

in both humans and in this animal model. However, further investigation is needed to determine whether the gastric MALT lymphoma development process shown in the *H. suis*-infected mouse model mimics the pathogenic process of human gastric MALT lymphoma development. In addition,

when this bacterium infects mice orally, it forms lymphomas that cause lymphocyte infiltration. Therefore, infiltrating cells could be easily identified using an actual human gastric MALT lymphoma patient sample. Further studies using human samples are warranted.

TROUBLESHOOTING

Problem 1

When preparing a mouse gastric homogenate solution, it is possible the gastric mucosa is not sufficiently peeled off and a proper gastric homogenate is not obtained.

Potential solution

As shown in [Figure 1C](#), sufficient stripping of the gastric mucosa with a cover glass creates enough homogenate until all epithelial tissue is stripped and only the muscularis remained (before beginning). If the homogenate is not sufficient, gastric mucosa of two or more mice can be used for stripping.

Problem 2

The tissue sample dried out, and the subsequent antibody staining was significantly reduced.

Potential solution

After washing with 1 × PBS, remove the excess liquid, and quickly wipe the area around the tissue sample with a paper towel. Take special care to not dry the surface of the sample ([step-by-step method details](#). 9, 11, 13, 15).

Problem 3

Air bubbles are formed when adding the fluorescent mounting medium to the stained sample, hindering the capture of high-quality images with a confocal microscope.

Potential solution

If there are fluorescent encapsulant bubbles on the stained tissue sample, use a pipetman to thoroughly remove them before placing the coverslip and observing with a confocal microscope. This will help obtain high-quality pictures without the influence of air bubbles ([step-by-step method details](#) 15).

Problem 4

No positive signals are observed after staining the tissue section with antibody ([step-by-step method details](#) 11).

Potential solution

If positive signals are not observed after antibody staining, increase the concentration of the antibody.

Problem 5

PCI mixture is not always finely separated at 25:24:1 (Preparation 2, step 16)

Potential solution

This product has two layers. The upper layer is the TE layer, which acts as an antioxidant. The lower layer is the PCI mixture. However, if it is not in two layers, allow it to stand until the layers separate and use the lower layer.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Koji Yamamoto (kyama@pop.med.hokudai.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

This study was supported in part by grants from the Japan Agency for Medical Research and Development (AMED; grant number 18ck0106267h0002) [T.S.], JSPS KAKENHI (grant numbers 15K19092, 17K15684, 19K07553 [K.Y.]), and Health Labour Sciences Research Grant (grant number 19HA1007 [T.S.]).

AUTHOR CONTRIBUTIONS

K.Y. and Y.K. designed the study. K.Y. and Y.K. performed the experiments. K.Y. performed the animal experiments. Y.K. and T.S. provided essential materials and contributed to data-related discussions. K.Y., Y.K., T.S., and N.S. interpreted the experimental data. K.Y., Y.K., and T.S. wrote the manuscript. N.S. critically revised the manuscript and supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Adam, P., Schmausser, B., Göbeler-Kolve, M., Müller-Hermelink, H.K., and Eck, M. (2008). Gastric extranodal marginal zone B-cell lymphomas of MALT type exclusively express Toll-like receptor 4 in contrast to other lymphomas infiltrating the stomach. *Ann. Oncol.* *19*, 566–569.
- Ben Suleiman, Y., Yoshida, M., Nishiumi, S., Tanaka, H., Mimura, T., Nobutani, K., Yamamoto, K., Takenaka, M., Aoganghua, A., Miki, I., et al. (2012). Neonatal Fc receptor for IgG (FcRn) expressed in the gastric epithelium regulates bacterial infection in mice. *Mucosal Immunol.* *5*, 87–98.
- El-Zaatari, M., Kao, J.Y., Tessier, A., Bai, L., Hayes, M.M., Fontaine, C., Eaton, K.A., and Merchant, J.L. (2013). *Gli1* deletion prevents *Helicobacter*-induced gastric metaplasia and expansion of myeloid cell subsets. *PLoS One* *8*, e58935.
- Lina, T.T., Pinchuk, I.V., House, J., Yamaoka, Y., Graham, D.Y., Beswick, E.J., and Reyes, V.E. (2013). CagA-dependent downregulation of B7-H2 expression on gastric mucosa and inhibition of Th17 responses during *Helicobacter pylori* infection. *J. Immunol.* *191*, 3838–3846.
- Mimura, T., Yoshida, M., Nishiumi, S., Tanaka, H., Nobutani, K., Takenaka, M., Suleiman, Y.B., Yamamoto, K., Ota, H., Takahashi, S., et al. (2011). IFN-gamma plays an essential role in the pathogenesis of gastric lymphoid follicles formation caused by *Helicobacter suis* infection. *FEMS Immunol. Med. Microbiol.* *63*, 25–34.
- Nakamura, M., Murayama, S.Y., Serizawa, H., Sekiya, Y., Eguchi, M., Takahashi, S., Nishikawa, K., Takahashi, T., Matsumoto, T., Yamada, H., et al. (2007). *Candidatus Helicobacter heilmannii* from a cynomolgus monkey induces gastric mucosa-associated lymphoid tissue lymphomas in C57BL/6 mice. *Infect. Immun.* *75*, 1214–1222.
- Okamura, T., Iwaya, Y., Yokosawa, S., Suga, T., Arakura, N., Matsumoto, T., Ogiwara, N., Higuchi, K., Ota, H., and Tanaka, E. (2013). A case of *Helicobacter heilmannii*-associated primary gastric mucosa-associated lymphoid tissue lymphoma achieving complete remission after eradication. *Clin. J. Gastroenterol.* *6*, 38–45.
- O'Rourke, J.L., Solnick, J.V., Neilan, B.A., Seidel, K., Hayter, R., Hansen, L.M., and Lee, A. (2004). Description of '*Candidatus Helicobacter heilmannii*' based on DNA sequence analysis of 16S rRNA and urease genes. *Int. J. Syst. Evol. Microbiol.* *54*, 2203–2211.
- Priestnall, S.L., Wiinberg, B., Spohr, A., Neuhaus, B., Kuffer, M., Wiedmann, M., and Simpson, K.W. (2004). Evaluation of "*Helicobacter heilmannii*" subtypes in the gastric mucosae of cats and dogs. *J. Clin. Microbiol.* *42*, 2144–2151.
- Shibata, W., Takaishi, S., Muthupalani, S., Pritchard, D.M., Whary, M.T., Rogers, A.B., Fox, J.G., Betz, K.S., Kaestner, K.H., Karin, M., et al. (2010). Conditional deletion of I κ B-kinase-beta accelerates *Helicobacter*-dependent gastric apoptosis, proliferation, and preneoplasia. *Gastroenterology* *138*, 1022–1034.e1.
- Shigematsu, Y., Niwa, T., Rehnberg, E., Toyoda, T., Yoshida, S., Mori, A., Wakabayashi, M., Iwakura, Y., Ichinose, M., Kim, Y.J., et al. (2013). Interleukin-1beta induced by *Helicobacter pylori* infection enhances mouse gastric carcinogenesis. *Cancer Lett.* *340*, 141–147.
- Yamamoto, K., Nishiumi, S., Yang, L., Klimatcheva, E., Pandina, T., Takahashi, S., Matsui, H., Nakamura, M., Zauderer, M., Yoshida, M., et al. (2014). Anti-CXCL13 antibody can inhibit the formation of gastric lymphoid follicles induced by *Helicobacter* infection. *Mucosal Immunol.* *7*, 1244–1254.
- Yamamoto, K., Tanaka, H., Nishitani, Y., Nishiumi, S., Miki, I., Takenaka, M., Nobutani, K., Mimura, T., Ben Suleiman, Y., Mizuno, S., et al. (2011). *Helicobacter suis* KB1 derived from pig gastric lymphoid follicles induces the formation of gastric lymphoid follicles in mice through the activation of B cells and CD4 positive cells. *Microbes Infect.* *13*, 697–708.
- Yamamoto, K., Kondo, Y., Ohnishi, S., Yoshida, M., Sugiyama, T., and Sakamoto, N. (2021). The TLR4–TRIF–type 1 IFN–IFN- γ pathway is crucial for gastric MALT lymphoma formation after *Helicobacter suis* infection. *iScience* *24*, 103064.
- Yang, L., Yamamoto, K., Nishiumi, S., Nakamura, M., Matsui, H., Takahashi, S., Dohi, T., Okada, T., Kakimoto, K., Hoshi, N., et al. (2015). Interferon-gamma-producing B cells induce the formation of gastric lymphoid follicles after *Helicobacter suis* infection. *Mucosal Immunol.* *8*, 279–295.