Myeloid-Derived Suppressor Cells and γδT17 Cells Contribute to the Development of Gastric MALT Lymphoma in *H. felis*-Infected Mice

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INTRODUCTION

Extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue (MALT) accounts for 7–8% of all newly diagnosed lymphomas, of which gastric MALT lymphoma is the most common (1). Furthermore, 90% of the gastric MALT lymphoma cases are associated with *Helicobacter pylori* infection (2–4). Since, gastric MALT lymphoma is a typical model of chronic inflammation-induced gastric tumor development (5), it is essential to dissect its underlying immune mechanisms.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature cells derived from myeloid progenitors with strong immunosuppressive functions (6). Human MDSCs are commonly defined as CD11b+CD33+ and lack mature myeloid and lymphoid markers, as well as major histocompatibility complex class II molecule human leukocyte antigen DR isotype (7, 8). Murine MDSCs are characterized by the myeloid-cell lineage differentiation antigens Gr-1

Helicobacter*-induced chronic inflammation and immune disorders are closely associated with the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Myeloid-derived suppressor cells (MDSCs) exhibit strong immunosuppressive properties and promote the growth of various solid tumors. However, the role of MDSCs in the development of MALT lymphoma has not been elucidated so far. We detected significant infiltration and enrichment of MDSCs in patients with MALT lymphoma, as well in *Helicobacter felis*-infected mouse model of gastric MALT lymphoma. In addition, the expression of arginase-1 and inducible nitric oxide synthase was significantly elevated both in gastric MALT lymphoma tissues and *H. felis*-infected stomach. Persistent *H. felis* infection closely reproduced the development of gastric MALT lymphoma and was accompanied by increased numbers of γδT17 cells. Accumulation of γδT17 cells was also validated in the human gastric MALT lymphoma tissues. Furthermore, the elevated cytokines interleukin-23 and interleukin-1β, as well as chemokines CCL20/CCR6, may be involved in the accumulation of γδT17 cells and the subsequent immunosuppression. These findings highlight the role of MDSCs and γδT17 cells in immune dysregulation during gastric MALT lymphoma development and their potential as therapeutic targets.

Keywords: MDSCs, γδT17, immunosuppression, MALT lymphoma, murine model
and CD11b (6, 9). MDSCs suppress immune responses in pathological conditions by upregulating arginase 1 (Arg-1), inducible nitric oxide synthase (iNOS) and regulatory T cells (Tregs) (10, 11). Studies show that MDSCs accumulate during inflammation, infection, and in tumors such as multiple myeloma, chronic lymphocytic leukemia, and lymphoma (10, 12). However, the function of MDSCs in MALT lymphoma development remains to be elucidated.

The γδT cells are abundant in the gastrointestinal mucosa, wherein they maintain immune homeostasis. In addition, these cells are activated during H. pylori-associated chronic inflammation and drive the immune imbalance in gastric mucosa (13). The γδT17 cell subset in particular modulates immune response in the colorectal cancer and hepatocellular carcinoma microenvironment by recruiting and activating MDSCs (14, 15). Furthermore, the MDSCs may promote γδT17 polarization by secreting IL-23 and IL-1β, which further mediate tumor immune tolerance (15). Therefore, we hypothesized that γδT17 and MDSCs are involved in the development of gastric MALT lymphoma. To this end, we established a mouse model of chronic Helicobacter-induced lymphomagenesis and detected MDSCs and γδT17 accumulation in the murine stomach tissues as well as in human MALT lymphoma tissues. Our findings provide new insights into the pathogenesis of gastric MALT lymphoma and identify novel therapeutic targets.

**MATERIALS AND METHODS**

**Patients and Controls**

Eight newly diagnosed MALT lymphoma patients (two male and six female; median age, 57 years; age range, 27–76 years) presenting at the Qilu Hospital, Shandong University from May 2017 to March 2018 were enrolled in this study. In addition, 12 healthy donors (6 male and female each; median age, 51 years; age range, 30–68 years) were enrolled as the control group. The study was approved by the Medical Ethical Committee of Qilu Hospital, Shandong University. Detailed clinical information of the patients is summarized in Table 1.

**Table 1 | Clinical characteristics of patients and healthy donors.**

| Clinical characteristics | MALT lymphoma | Healthy donor |
|--------------------------|---------------|---------------|
| Median age (range), years | 57 (27–76)    | 51 (30–68)    |
| Male, n                  | 2             | 6             |
| Female, n                | 6             | 6             |
| IPI, n                   |               |               |
| 0–1                      | 5             | Null          |
| 2–3                      | 2             | Null          |
| 4–6                      | 1             | Null          |
| Ann Arbor stage          |               |               |
| I                         | 3             | Null          |
| II                       | 1             | Null          |
| III                      | 1             | Null          |
| IV                       | 3             | Null          |

**H. felis Culture and Infection**

*H. felis* (American Type Culture Collection 49179) was obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured on tryp绀ate soy agar containing 5% defibrated sheep blood in a microaerophilic atmosphere for 48 h at 37°C. The bacterial colonies were harvested and resuspended in phosphate-buffered saline (PBS), and analyzed by Gram staining. In addition, bacterial DNA was extracted using TIANamp Bacteria DNA Kit (TIANGEN, China) and sequenced. The density of the bacterial suspension was adjusted to 10⁸ CFU/ml for infecting animals.

**H. felis Infection Model**

Female 6- to 8-week-old BALB/c mice were purchased from Nanjing Biomedical Research Institute of Nanjing University, and housed in specific pathogen-free animal care facility and closely monitored. The animal experiments were reviewed and approved by the Medical Ethical Committee of Qilu Hospital, Shandong University. The mice were divided into the *H. felis*-infected and control groups and accordingly inoculated with 100 µl of the bacterial suspension (10⁸ CFU) or PBS three times every other day via the intragastric route, as previously described (16). The animals were killed 8, 11, 14, and 19 months after infection.

**Urease Test**

Bacterial colonization was assessed from 4 weeks after *H. felis* inoculation. Stomach tissues were excised, cut along the greater curvature, rinsed with saline, and cut into small pieces. Rapid urease test was conducted using a commercial kit (Begen, China) according to the manufacturer’s instructions. Appearance of a red color indicated positive result.

**PCR**

Bacterial DNA was extracted from the gastric tissues or stool samples using QIAamp cador Pathogen mini kit (Cat. no. 54104) or QIAamp DNA Stool Mini Kit (Cat. no. 51505) as appropriate. The *H. felis* FlaB gene was amplified as previously described (17) using the following primers: FlaB, 5′-TTCGATTGGTCCTACAGGCTCAGA-3′ and 5′-TTCTTCDGTTATGACATTGACCAACCGCA-3′; glyceraldehyde 3-phosphate dehydrogenase, 5′-GCTAACGAGTTGGGTGGAAGGA-3′ and 5′-TCACCACCAGGGAAGGCG-3′.

**Histology and Immunohistochemistry**

Longitudinal stomach tissue strips from *H. felis*-infected or uninfected mice were fixed with 10% formaldehyde, embedded in paraffin, and cut into 4-µm-thick sections. Hematoxylin and eosin staining was performed as per standard protocols, and the presence of lymphoid follicles and lymphoepithelial lesions (LELs) were recorded. Sections of murine stomach or human MALT lymphoma tissues were subjected to heat-induced epitope retrieval and then incubated overnight with anti-TCRγδ (Abcam, Cat. no. ab118864), anti-IL-17 (Abcam, Cat. no. ab79056), anti-IL-1β (Abcam, Cat. no. ab9722), anti-IL-23 (Santa Cruz, Cat. no. sc-50303), anti-Arg-1 (Proteintech, Cat. no. 16001-1-AP), anti-iNOS (Proteintech, Cat. no. 18985-1-AP), anti-Gr-1 (R&D systems, Cat. no. MAB1037-100), anti-CD11b (Abcam, Cat. no. ab118864).
no. ab133357), anti-CD33 (Abcam, Cat. no. ab11032), and anti-CD11b (Abcam, Cat. no. ab133357) primary antibodies at 4°C. The following day, the sections were probed with biotin-streptavidin horseradish peroxidase-conjugated secondary antibody, and stained using 3,3′-diaminobenzidine reagent. For immunofluorescence, fluorochrome-conjugated conjugated secondary antibodies were used. Positively stained cells were counted in five random non-overlapping fields (400× magnification; Nikon, Ni-U) using ImageJ software. The histoscore of each biomarker was evaluated according to Table 2.

Flow Cytometry
Peripheral blood samples were treated with red blood cell lysis buffer (eBioscience, USA), and the spleens were gently crushed and filtered through a 40-µm nylon mesh strainer to obtain single-cell suspension. The cells were washed and resuspended in PBS and stained with Brilliant Violet 421 anti-mouse Ly-6G/Ly-6C (Gr-1) (Biolegend, Cat. no. 108434), APC/Cy7 antihuman CD45 (Biolegend, Cat. no. 368516), APC antihuman Lineage Cocktail (CD3, CD19, CD20, CD56; Biolegend, Cat. no. 363601), PE antihuman CD33 (Biolegend, Cat. no. 303404), PE-CF594 mouse antihuman leukocyte antigen DR isotype (BD Horizon, Cat. no. ab138483), anti-IL-1β (Abcam, Cat. no. ab138483), anti-IL-17A (eBio17B7) (eBioscience, Cat. no. 17-7177-81) and APC antihuman IL-17A (Biolegend, Cat. no. 512334) antibodies. For intracellular cytokine staining, the cells were stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (ThermoFisher Scientific) and Brefeldin A (3 µg/ml, Invitrogen) at 37°C for 4 h. After staining with APC/Cy7 antihuman CD45 (Biolegend, Cat. no. 103116), APC/Cy7 antihuman CD45 (Biolegend, Cat. no. 304014), PE/Cy7 antihistone CD3 (Biolegend, Cat. no. 100220), PE/Cy7 antihistone CD3 (Biolegend, Cat. no. 300420), fluorescein isothiocyanate antihistone CD4 (Biolegend, Cat. no. 100406), fluorescein isothiocyanate antihistone CD4 (Biolegend, Cat. no. 300506), PE-CF594 rat antihistone CD8a (BD Horizon, Cat. no. 562283), PE-CF594 mouse antihistone CD8 (BD Horizon, Cat. no. 562282), PE antihistone TCR γ/δ (Biolegend, Cat. no. 118108), and PE antihistone TCR γ/δ (Biolegend, Cat. no. 331210) antibodies, the cells were fixed, permeabilized, and stained with APC anti-IL-17A (eBio17B7) (eBioscience, Cat. no. 17-7177-81) and APC antihuman IL-17A (Biolegend, Cat. no. 512334) antibodies. For staining the Treg marker, the cells were permeabilized as per the manufacturer’s instructions (eBioscience, San Diego, CA, USA) before incubating with the anti-Foxp3 antibody. Data were acquired using BD FACSaria III flow cytometer (BD Biosciences, USA) and analyzed with the FlowJo V10 software (Tree Star).

Real-Time Quantitative Polymerase Chain Reaction
Total RNA from the stomach tissues and spleens were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using Prime script RT reagent kit (Takara Bio Inc., Japan). Real-time quantitative PCR was performed using 5 µl 2× SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan), 1 µl complementary DNA, 0.8 µl forward and reverse primers, and 3.2 µl ddH2O in a LightCycler 480II PCR machine (Roche, Switzerland) according to the manufacturer’s instruction. The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The primer sequences are listed in Table 3.

Western Blotting
Stomach tissues were immersed in NP40 lysis buffer (Solarbio, China) supplemented with protease and phosphatase inhibitors, and sonicated on ice. The homogenates were centrifuged at 15,000×g for 15 min, and the supernatants were aspirated. The protein concentration was assessed using bicinchoninic acid protein assay kit (Beyotime, China), and equal amounts of protein per sample were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 10% gel. The protein bands were transferred onto nitrocellulose filter membranes, blocked with 5% non-fat milk for 1 h at room temperature, and then incubated overnight with anticleaved caspase-1 (Asp296; CST, Cat. no. 67314), antitumorase-1 (Abcam, Cat. no. ab138483), anti-IL-1β (3A6; CST, Cat. no. 12242).

### Table 2: Criteria for immunohistochemistry (IHC) score.

| Percentage of positive cells (a) | Staining intensity grading (b) | Percentage (%) | Score | Intensity | Score |
|---------------------------------|---------------------------------|----------------|-------|-----------|-------|
| <5                              | None                            | <5             | 0     | None      | 0     |
| 6–25                            | Light brown yellow              | 6–25           | 1     | Light brown yellow | 1   |
| 26–50                           | Brown yellow                    | 26–50          | 2     | Brown yellow | 2   |
| 51–75                           | Dark brown yellow               | 51–75          | 3     | Dark brown yellow | 3   |
| More than 76                    |                                 |                | 4     |            |       |

Histoscore = a + b.

### Table 3: Primer sets and genes included in real-time quantitative PCR analysis.

| Name                | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---------------------|------------------------|------------------------|
| NF-kB (mus)         | ATGTAGTTGCCACGCA       | CAGA                   |
| IL-17 (mus)         | TTTAATCCTCCTTGGGCG    | CAAA                   |
| IL-23 (mus)         | ATGCTGATTTGCAGAG       | CAGTA                  |
| IL-1β (mus)         | TTCAAGGCAAGCAGATAT     | CACTC                  |
| CCL20 (mus)         | GCCCTCTGCTACATA        | GACGC                  |
| CCR6 (mus)          | CCGGCGCAACATTAG        | GTGCT                  |
| CCR7 (mus)          | GCAAACGCGGTAT          | GTGCT                  |
| TLR2 (mus)          | GCACAGCGCTGT           | GTGCT                  |
| TLR7 (mus)          | ATGTGGCAGACGCCG        | AAGGACAGA             |
| TLR9 (mus)          | ATGGTTCCTCGTGA         | GQQCT                 |
| GAPDH (mus)         | CTCGCCACTTCCTACCAC     | TCGG                  |

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| NF-kB (mus)         | ATGTAGTTGCCACGCA       | CAGA                   |
| IL-17 (mus)         | TTTAATCCTCCTTGGGCG    | CAAA                   |
| IL-23 (mus)         | ATGCTGATTTGCAGAG       | CAGTA                  |
| IL-1β (mus)         | TTCAAGGCAAGCAGATAT     | CACTC                  |
| CCL20 (mus)         | GCCCTCTGCTACATA        | GACGC                  |
| CCR6 (mus)          | CCGGCGCAACATTAG        | GTGCT                  |
| CCR7 (mus)          | GCAAACGCGGTAT          | GTGCT                  |
| TLR2 (mus)          | GCACAGCGCTGT           | GTGCT                  |
| TLR7 (mus)          | ATGTGGCAGACGCCG        | AAGGACAGA             |
| TLR9 (mus)          | ATGGTTCCTCGTGA         | GQQCT                 |
| GAPDH (mus)         | CTCGCCACTTCCTACCAC     | TCGG                  |
anticleaved-IL-1β (Asp117; CST, Cat. no. 52718), antinuclear
factor kappa B (anti-NF-κB) p65 (Abcam, Cat. no. ab32536),
and anti-NLRP3 (Abcam, Cat. no. ab210491) primary antibodies
at 4°C. The membranes were then probed with horseradish
peroxidase-conjugated secondary antibodies for 1 h at
room temperature and visualized using chemiluminescent
reagents (Millipore, USA). β-Actin was used as the internal
control.

ELISA
The gastric homogenates were processed as above, and the levels
of IL-17, IL-23, and IL-1β were analyzed using commercially
available sandwich ELISA kits (eBioscience, USA) in accordance
with the manufacturer's instructions. Absorbance was measured
at 450 nm using Synergy H1 Hybrid Microplate Reader (BioTek,
USA), and the cytokine concentrations were calculated from a
standard curve.

Statistical Analysis
Data were expressed as mean ± standard deviation (SD)
and evaluated by the Fisher's exact test, Student's t-test, and
Mann-Whitney test as appropriate. All tests were performed
using GraphPad Prism 6.0 system. Two-tailed P < 0.05 were
considered statistically significant.

RESULTS
MDSCs Infiltrate in Human MALT
Lymphoma Tissues
The proportion of circulating MDSCs (CD45+lin−CD33+HLA−DR−CD11b+) was significantly
higher in the MALT lymphoma patients compared to that in
healthy donors (Figures 1A,B). The gating strategy of MDSCs
is shown in Figure S1A. Furthermore, the CD33+CD11b+ MDSCs were also enriched in the gastric MALT lymphoma
biopsies (Figures 1C,D), which correlated with significant upregulation of Arg-1 and iNOS compared to paired normal
gastric tissues (Figures 1E,F). Since Arg-1/iNOS overexpression
is a key mechanism through which MDSCs mediate local
immune suppression (9–11), it is likely involved in creating an
immunosuppressive MALT lymphoma microenvironment as
well. In addition, the proportion of circulating Tregs was also
significantly elevated in the MALT lymphoma patients compared
to healthy donors (Figure S1B). Taken together, MDSCs are
enriched in MALT lymphoma and are responsible for the
immunosuppressive conditions.

Persistent H. felis Infection Induces
Lymphoepithelial Lesions in Murine
Stomach
To determine the possible pathogenic role of MDSCs in
MALT lymphomagenesis, we infected mice with H. felis to
mimic gastric MALT lymphoma development. The presence of
Helicobacter strain-specific gene FlaB in the gastric tissues of
these mice indicated successful infection (Figure 2A).
Furthermore, the urea test showed that the infection rates of
H. felis were, respectively, 75, 100, 90, and 100% at 8, 11,
14, and 19 months postinfection (Figure S2A). Furthermore,
the infected mice developed lymphoid follicles with classical
features like germinall center and mantle zone from 8 months
after bacterial gavage, whereas no pathological changes were
detected in the uninfected mice (Figure S2B). The pathological
damage gradually increased in a time-dependent manner, and
after 11 months of infection, the marginal zone expanded
with infiltrating centrocye-like cells that destroyed the gastric
glands. LELs, a characteristic feature of MALT lymphoma,
were observed 14 months postinfection (Figure 2B). These
changes simulated the histopathological characteristics of MALT
lymphomagenesis. Furthermore, the lymphoid aggregates
consisted predominantly of B cells (Figure 2C), along with
some T cells (CD3+), and very few macrophages (F4/80+) (Figure S2C). The NF-κB pathway, a critical mediator
of the inflammatory response, was also activated in the
infected mice (Figures 2D–F), indicating an important role in
MALT lymphomagenesis.

MDSCs Are Enriched in the Gastric LELs of
H. felis-Infected Mice
To determine whether the histological alterations in H. felis-infected mice were also associated with MDSCs enrichment
as observed in the MALT lymphoma tissues, we analyzed the
distribution and percentage of MDSCs at various time
points postinfection. At the early-stage of infection (8–11 months), only mild lesions were seen in the stomach, and
the number of MDSCs in peripheral blood was unaffected.
However, at the later stages (14–19 months), the circulating
MDSCs increased significantly and was accompanied by gastric
accumulation of Gr-1+CD11b+ MDSCs (Figures S3A,B)
and severe LELs (Figures 3A,B). Furthermore, Arg-1 was
significantly upregulated in the stomach of H. felis-infected
mice, indicating MDSCs activation in response to gastric
MALT lymphoma development (Figures 3C,D), whereas
iNOS levels were not altered. This suggested that Arg-
1 was the primary immunosuppressive factor employed
by MDSCs.

The γδ T17 Cells Are Enriched in
H. felis-Infected Mice
IL-17, an important proinflammatory cytokine, exerts crucial
functions in carcinogenesis and tumor growth (18) by recruiting
MDSCs at tumor sites (14, 15, 19). We detected a significant
upregulation in IL-17 protein and messenger RNA (mRNA)
levels in the gastric homogenates of infected mice at 8
months postinfection (Figures 4A,B). Furthermore, IL-17 was
enriched in the gastric lymphoid aggregates of the infected
mice (Figures 4C,D). Since IL-17 could be secreted by several
cell types, including CD4+ T cells (Th17), CD8+ T cells
(Tc17), and γδ T cells (γδT17), we assessed the changes of
CD4+, CD8+, and γδ T cells following H. felis infection. The
results showed a significant increase in γδT cells population,
while the percentages of CD4+ and CD8+ T cells were
similar to that in the control group (Figure 4E). We next
compared the proportion of CD4+, CD8+, and γδ T cells
among the splenic IL-17-producing T cells and found that,
although the Th17 cells were the predominant type, the
difference was not significant between the *H. felis*-infected and control mice. However, the number of splenic γδT17 cells increased significantly after *H. felis* infection (Figure 4F) and is possibly the main immune regulatory cell population in *H. felis*-induced pathologies. We subsequently compared the proportion of splenic Th17, Tc17, and γδT17 cells at different time points and detected increased numbers of γδT17 cells at 8, 11, and 14 months postinfection (Figure 4G). The proportion of splenic γδT17 cells increased in a time-dependent manner during chronic *H. felis* infection (Figure 4H), indicating a possible relationship between systemic γδT17 cells and the development of gastric MALT lymphoma.

**IL-1β Is Activated in Gastric Mucosa After Long-Term *H. felis* Infection**

IL-1β and IL-23 stimulate γδT cells to secrete IL-17 in multiple experimental models (15, 20). We found that both were
significantly elevated in the gastric homogenates of *H. felis*-infected mice at 8 months postinfection (Figures 5A,B). Studies show that the cytokine response to *Helicobacter* infections is mediated by Toll-like receptor 2 (TLR2) (21). Consistent with this, TLR2, TLR7, and TLR9 were upregulated in the gastric mucosa after *H. felis* infection (Figure 5C). In respiratory
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**FIGURE 3** | H. felis infection induced myeloid-derived suppressor cells (MDSCs) expansion and upregulated arginase 1 (Arg-1) and inducible nitric oxide synthase (iNOS). (A) Percentage of MDSCs in the peripheral blood of control and H. felis-infected mice at 8 months (control, n = 4; H. felis, n = 4), 11 months (control, n = 4; H. felis, n = 4), 14 months (control, n = 4; H. felis, n = 6), and 19 months (control, n = 6; H. felis, n = 8) postinfection; *P < 0.05. (B) Representative flow cytometry dot plots showing MDSCs in the H. felis-infected and control mice at 14 months postinfection. (C) Representative IHC images showing Arg-1 and iNOS expression in the stomach of H. felis-infected and control mice. Original magnification, 200×. (D) Comparison of Arg-1 and iNOS levels in both groups, *P < 0.05.

**The γδT17 Cells Infiltrate Into Human MALT Lymphoma Tissues**

Consistent with the observations in the murine model, there was an obvious infiltration of γδT17 cells in the MALT lymphoma tissues (Figures 6A,B). Furthermore, IL-1β and IL-23 levels were also higher in the MALT lymphoma compared to the paired normal tissues (Figures 6C,D). However, there was no significant difference in the number of peripheral Th17, Tc17, and γδT17 cells between the patients and controls (Figure S4), suggesting that the tumor-infiltrating rather than peripheral γδT17 cells regulate the development of MALT lymphoma.

**DISCUSSION**

Chronic inflammation is the pathological basis of multiple solid tumors, wherein immune dysfunction triggers malignant transformation of cells. Mounting evidence indicates that MDSCs are enriched both locally and systemically during cancer growth in mice and humans (7, 9–12, 26), and their activation in pathological conditions upregulates Arg-1 and iNOS. Arg-1 consumes L-arginine, an intermediate for TCR biosynthesis, resulting in failure of T-cell proliferation and activation (26). Furthermore, the nitric oxide produced by iNOS induces CD8+ T-cell apoptosis by downregulating CD44 and CD62L, upregulating CD95, and inhibiting JAK3 and STAT5 signaling (27). In an A20 lymphoma murine model, MDSCs induced an immunosuppressive microenvironment through the activation of Tregs (10). In the present study as well, MDSCs accumulated in large numbers in MALT lymphoma tissues and expressed high levels of Arg-1 and iNOS, suggesting an important role in the development of MALT lymphoma.

bacterial infections, NLRP3 inflammasome-dependent IL-1β regulates the γδT17 cell response (22). In the mice with persistent H. felis infection as well, we observed a marked increase in the levels of caspase-1 and NLRP3 (Figure 5D), indicating activation of the NLRP3 inflammasome and subsequent IL-1β activation. Since the γδT17 cells are CD27−CCR6+ (23) and are recruited by the CCL20-CCR6 axis (24, 25), we also evaluated the expression levels of these chemokines in the gastric mucosa. As shown in Figure 5E, CCR6 and CCL20 levels were significantly higher in the H. felis-infected gastric tissue at 19 months postinfection, suggesting that γδT17 is recruited to the gastric tissues by these chemokines.
FIGURE 4 | γδT17 cells were elevated in H. felifs-infected mice. (A) Interleukin-17 (IL-17) levels in the gastric homogenates at 8 months postinfection. (B) IL-17 messenger RNA (mRNA) level in the stomach at 8 months postinfection. (C) Representative immunohistochemistry (IHC) image showing in situ IL-17 in the stomach.
FIGURE 4 | of H. felis-infected and control mice and (D) corresponding histoscores. Original magnification, 400×. (E) Percentage of CD4+, CD8+, and γδT cells among CD45+ cells in the spleen of H. felis-infected mice and control mice at 8 months postinfection. (F) Percentage of CD4+ IL-17+ (Th17), CD8+ IL-17+ (Tc17), and TCRγδ+ IL-17+ γδT17 cells in the spleen from H. felis-infected and control mice at 8 months left, and representative flow cytometric plots of the same gated with IL-17+CD3+ γδT cells right. (G) The percentage of Th17, Tc17, and γδT17 cells among CD45+ cells in the spleen at 8, 11, and 14 months postinfection. H. felis infection: 8 months (control, n = 4; H. felis, n = 4), 11 months (control, n = 5; H. felis, n = 4), and 14 months (control, n = 5; H. felis, n = 10). *P < 0.05, **P < 0.01.

Gastric H. felis infection simulates the pathological changes associated with chronic gastritis, lymphoid follicle formation, and LELs during gastric MALT lymphoma development (16, 28–30). The histopathological changes in our murine model were consistent with previous studies (16, 28). Although the stomach is a priori devoid of lymphoid tissue, we observed lymphoid hyperplasia in the chronically infected mice. Lymphoid follicles appeared from 8 months postinfection, and the gastric glands were invaded by multiple lymphocytes 14 months after infection. These LELs are an important pathological feature of gastric MALT lymphoma and predominantly consisted of B cells. The lymphoid aggregates in the infected mice were also enriched with MDSCs expressing high levels of Arg-1 and iNOS. Interestingly, the significant expansion of MDSCs was observed from 14 months postinfection and coincided with severe tissue damage and LELs, suggesting a possible relationship between MDSCs increment and severity of disease.

The number of γδT17 cells also increased significantly in the gastric lesions of the H. felis-infected mice, as well as in clinical gastric MALT lymphoma specimens. Although γδT cells and IL-17 are potent antitumor effectors (31), studies also report protumor effects of the γδT17 cells (14, 15). In animal models of fibrosarcoma, skin cancer, and ovarian...
cancer, γδT17 cells infiltrate the tumors and secrete IL-17, thereby promoting tumor growth (32, 33). Furthermore, recent studies show that γδT17 mediate immune dysfunction in the tumor microenvironment by recruiting MDSCs (14, 15, 34). Furthermore, IL-23 induces γδT17 cells to secrete IL-17, IL-8, tumor necrosis factor α, and granulocyte-macrophage colony-stimulating factor, consequently recruiting and activating MDSCs in colorectal tumors (14). In addition, activated γδT17 cells in the liver tumor microenvironment produced large amounts of IL-17 and recruited MDSCs, which enabled tumor cells to escape the immune surveillance by inhibiting CD8+ T cell functions (15). In the present study, we found that the proportion of γδT17 increased as H. felis infection progressed, indicating a possible relationship between these cells and the pathological gastric lesions. As observed with the MDSCs, γδT17 numbers markedly increased with the deterioration of lymphoepithelial defects. In view of these observations, we hypothesized that γδT17 cells were involved in the malignant transformation of the inflamed gastric epithelium during persistent Helicobacter infection. However, the evidence is insufficient at present to conclude that γδT17 cells mediate immune dysregulation in MALT lymphoma by recruiting MDSCs, and further studies are warranted to validate this hypothesis.

Previous studies have shown that Helicobacter infection activates the innate immune system in a TLR-dependent manner, leading to activation of the NF-κB pathway and cytokine production (21). Consistent with this, we observed a significant increase in the levels of TLR2, TLR7, and TLR9 in the H. felis-infected gastric mucosa, along with NF-κB upregulation. The chronic inflammation and numerous genetic aberrations seen in gastric MALT lymphoma are linked with dysregulated NF-κB signaling (35). The activation of the NF-κB pathway, along with the pathological role of Helicobacter, indicate that chronic inflammation is essential for lymphomagenesis (5). In addition, the critical role of NF-κB in MDSCs accumulation and function has become apparent in recent years. Studies show that IL-1β activates MDSCs through the NF-κB pathway, and blocking IL-1 receptor signaling inhibited gastric preneoplasia and MDSC mobilization (36). Thus, NF-κB pathway likely regulates the immune responses in gastric MALT lymphoma through various mechanisms.

IL-1β and IL-23 are known to drive γδT17 responses and induce IL-17 production (20). In addition, MDSCs also polarize the γδT cells to the γδT17 phenotype by secreting IL-23 and IL-1β (15). Both cytokines were elevated in the H. felis-infected gastric mucosa, indicating γδT17 activation. In addition, NLRP3 inflammasome increases IL-18 and IL-1β production, thereby promoting tumor cell proliferation and inhibiting apoptosis during lymphoma
development (37). The NLRP3 inflammasome was activated in the gastric mucosa 8 months postinfection. Based on these observations, we hypothesize that TLR2-mediated recognition of H. felis activates the NLRP3 inflammasome and triggers IL-1β production. Chemokines such as CCL20 then recruit γδT17 cells to the gastric lesions and aggravate immunosuppression.

In conclusion, we provided evidence of the enrichment of γδT17 and MDSCs in Helicobacter-induced MALT lymphomagenesis. Our findings highlight the therapeutic potential of modulating γδT17 cells and MDSCs in gastric MALT lymphoma.

DATA AVAILABILITY STATEMENT
The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT
The studies involving human participants were reviewed and approved by Medical Ethical Committee of Qilu Hospital, Shandong University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Medical Ethical Committee of Qilu Hospital, Shandong University. The patients/participants provided informed consent to participate in the study.

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AUTHOR CONTRIBUTIONS

CJ, YZ, and MJ designed the research and analyzed the data. YZ, FL, YP, and LW performed the experiments. JY, GL, JL, TS, and DM provided scientific suggestions and supervised the project. YZ wrote the manuscript. CJ, FL, MJ, and YW contributed to the manuscript revision. All authors critically reviewed the article and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.03104/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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