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IL-17 Receptor Signaling through IL-17A or IL-17F Is Sufficient to Maintain Innate Response and Control of Helicobacter pylori Immunopathogenesis

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ABSTRACT

IL-17R signaling is required for control of extracellular pathogens and is also implicated in development of chronic inflammatory processes. The response to the human pathogen Helicobacter pylori results in Th1 and Th17 cell activation and a chronic inflammatory process that can lead to adverse outcomes, such as gastric cancer. Previously, we identified IL-17RA as a requirement for the recruitment of neutrophils and control of H. pylori colonization in the gastric mucosa. Unexpectedly, H. pylori–infected Il17ra−/−− mice had significantly more chronic inflammation than H. pylori–infected wild-type mice. In this study, human epithelial cell lines and murine models were used to investigate differential roles for IL-17A, IL-17F, and IL-17A/F during H. pylori infection. Moreover, the hypothesis that IL-17RA signaling, specifically in lymphocytes, provides an autocrine feedback loop that downregulates Th17 cytokine production was investigated. The data indicate that epithelial cells exhibit a stronger response to IL-17A and IL-17A/F than IL-17F, and that IL-17A and IL-17A/F can synergize with TNF and IL-22 to induce antimicrobial genes of gastric epithelial cells. In vivo deficiencies of IL-17A or IL-17F alone did not significantly change the immunopathological response to H. pylori, but if both cytokines were absent, a hyperinflammatory lymphocytic response developed. Using a cre/flox targeting approach for IL-17RA combined with infection, our findings demonstrate that increased chronic inflammation in Il17ra−/−− mice was not attributed to a T cell–intrinsic defect. These data imply that IL-17A and IL-17F may have overlapping roles in maintenance of the gastric mucosal response to infection. ImmunoHorizons, 2022, 6:116-129.

INTRODUCTION

Chronic inflammation contributes to carcinogenesis in many tissues (1). The development of chronic inflammation can be activated by factors such as infection, smoking, obesity, high-calorie diet, genetic polymorphisms, or even dysbiosis; these triggers are also recognized risk factors for the development of cancer (2). Although numerous viruses are known to be...
associated with cancer pathogenesis, bacteria are also emerging as significant drivers of neoplasia. Global estimates suggest that infectious agents cause an estimated 2.2 million new cancer cases annually (13% of all malignancies); of these infectious agents, *Helicobacter pylori* was the most prominent, accounting for ~800,000 cases of gastric cancer annually (3–6). *H. pylori* is the single most common risk factor for gastric cancer. Infection with *H. pylori* is responsible for nearly 90% of stomach cancers worldwide and ~37% of all infection-related cancers (6). In 1994, the International Agency for Research on Cancer (a World Health Organization agency) classified *H. pylori* as a class I carcinogen. Incredibly, ~90% of noncardia gastric cancers are also attributable to *H. pylori* infection. Gastric cancer is the third most common cause of cancer-related deaths in the world, killing ~769,000 people in 2020 (7). Other risk factors that are believed to augment the development of gastric cancer can be divided into two categories: those the host can control and those the host cannot control. Host-controlled risk factors include consumption of high-salt or low-antioxidant diet and smoking. Risk factors that may be imposed on the host include colonization with a strain of *H. pylori* that expresses the cag pathogenicity island or host genetic variations that could drive a strong immune response (i.e., polymorphism in *Iltb*) (8–10).

*H. pylori* colonization is believed to result in mild inflammation in all people. However, in some individuals, this inflammation contributes to the development of peptic ulcers and gastric cancer (11). An effective adaptive immune response is essential to minimize colonization of *H. pylori*, but the immune response can also contribute to gastric inflammation (termed gastritis) (12). Wild-type (WT) mice infected with *H. pylori* also develop inflammation in response to infection. Previously, it has been shown that lymphocyte-deficient Rag1<sup>−/−</sup> mice and Prkdc<sup>scid</sup> mice colonized with *H. pylori* develop very little inflammation (13–15), despite carrying a high level of colonization with *H. pylori* compared with WT mice. These data suggested that the adaptive immune response is required for the development of gastritis and the control of bacterial proliferation. In these animals, a T cell response was sufficient to drive chronic inflammation (13, 15). In humans and in animal models, the proinflammatory CD4<sup>+</sup> Th cell response includes a mixed Th1 and Th17 response (reviewed in Refs. 16, 17). Further, regulatory T (Treg) cells and their production of IL-10 play an anti-inflammatory role that may limit gastritis (13, 18, 19). In fact, it has been demonstrated that when children are infected with *H. pylori*, they have a stronger Treg cell response with a concomitant decrease in Th17 responses (19, 20). Nevertheless, it should be noted that downregulating inflammation may contribute to disease persistence through inhibition of the antimicrobial activities of Th1 and Th17 responses.

Increased levels of Th17 cytokines, including production of IL-17A, IL-17F, IL-21, and IL-22 (and IL-26 in humans), are associated with more detrimental outcomes of *H. pylori* infection (reviewed in Ref. 16). The Th17 response contributes to recruitment of neutrophils by activating nonhematopoietic cells to produce neutrophil-recruiting chemokines, especially IL-8 (CXCL8 and its homologs) (21). IL-17A expression is commonly associated with the inflammatory response to extracellular pathogens and fungi (reviewed in Ref. 22). Further, it is well established that IL-17A and IL-22 act synergistically to activate several antimicrobials, including S100 proteins, lipocalin 2, and some β-defensins (23, 24). Collectively, the data on Th17 cells suggest that Th17 responses are important for regulating neutrophil migration (acute inflammation) and antimicrobial responses in the gastric mucosa (16, 25, 26).

Previously, we reported on the impact of *H. pylori* infection in IL-17R subunit A–deficient mice (II17ra<sup>−/−</sup>). In those studies, unlike *H. pylori*–infected WT mice, *H. pylori*–infected II17ra<sup>−/−</sup> mice did not develop a neutrophilic infiltrate. In addition, the II17ra<sup>−/−</sup> mice carry a higher *H. pylori* burden of colonization than WT mice (27, 28), suggesting that the IL-17 signaling pathway was required for control of infection. Surprisingly, the II17ra<sup>−/−</sup> mice had high levels of chronic inflammation. This inflammation was the result of the II17ra<sup>−/−</sup> mice having a significant increase in infiltrating CD4<sup>+</sup> T cells and a remarkably higher number of B cells that organized into lymphoid follicles with germinal centers. This was not observed in WT mice. Conversely, when II17a<sup>−/−</sup> mice were infected with *H. pylori* by Shiomi et al. (28), they did not develop extensive chronic inflammation or increased *H. pylori* colonization; nor did they exhibit increased mononuclear cell infiltration compared with WT mice. Comparably, they found that *H. pylori*–infected II17a<sup>−/−</sup> mice had reduced neutrophils in their tissue and reduced myeloperoxidase activity (a marker of polymorphonuclear neutrophils [PMNs]).

To create a functioning IL-17R, IL-17RA complexes with IL-17RC or IL-17RB to mediate signaling through IL-17RA/RC complexes. Although the binding affinity of IL-17F to IL-17RA is reported to be much lower than IL-17A, the homodimers have a similar affinity for the IL-17RC component of the signaling complex (31). In addition to this, IL-17A/F and IL-17F are reported to have similar immune activation profiles as IL-17A in fibroblasts, but IL-17F is reported to be less potent at similar concentrations (32). Although IL-17E (also known as IL-25) signals through IL-17RA/RB complexes (33), our previously published data on *H. pylori*–infected IL-17RB<sup>−/−</sup> mice suggest that the phenotype observed in *H. pylori*–infected II17ra<sup>−/−</sup> is not due to a lack of IL-17E signaling (34).

This study was designed to investigate the differential roles of IL-17A, IL-17F, and IL-17A/F in controlling *H. pylori* colonization and *H. pylori*–induced gastritis. This is an area of interest, because IL-17A is used by IL-17A, IL-17F, and the heterodimer IL-17A/F. In addition to this, II17a<sup>−/−</sup> and II17ra<sup>−/−</sup> exhibit very different phenotypes with chronic *H. pylori* infection (27). Therefore, this study aimed also to determine whether IL-17A may contribute to the control of Th17 cytokine production during *H. pylori* infection. The data herein suggest a role for IL-17R signaling in downregulating the inflammatory response that can be mediated by either IL-17A or IL-17F, and further that this anti-inflammatory role is not a direct effect of IL-17RA signaling in T cells.

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MATERIALS AND METHODS

Ethics statement
This study was accomplished under protocol numbers V/15/130 and V1800070-00 and was approved by the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center and the Research and Development Committee of the Veterans Affairs Tennessee Valley Healthcare System. Experiments were executed in accordance with the American Association for Accreditation of Laboratory Animal Care guidelines, the American Veterinary Medical Association Guidelines on Euthanasia, National Institutes of Health regulations (Guide for the Care and Use of Laboratory Animals), and the U.S. Animal Welfare Act. All animals were housed in an accredited research animal facility that is fully staffed with trained personnel.

Bacterial strain and growth conditions
The Premouse Sydney Strain 1 (PMSS1) of Helicobacter pylori was used in this study. PMSS1 contains a functional type 4 secretion system, which is often referred to as the major onco-genic factor of *H. pylori*. Bacteria were grown on Trypticase soy agar plates containing 5% sheep blood incubated at 37°C in 5% CO₂ and subcultured every 2 days. To infect mice, we grew PMSS1 in liquid culture made of Brucella broth with 10% heat-inactivated FBS and 10 µg/ml vancomycin. Liquid cultures were incubated in microaerophilic conditions generated by a GasPak EZ Campy Container System (BD), shaking at 150 rpm for 8 h. The *H. pylori* burden in the mice was measured by spreading stomach homogenate on tryptic soy agar plates containing sheep blood (5%), nalidixic acid (10 µg/ml), vancomycin (50 µg/ml), amphotericin (2 µg/ml), and bacitracin (100 µg/ml) in microaerophilic conditions at 37°C for 3–5 d.

Cytokine treatments and other stimulants
Recombinant human IL-17A (Invitrogen), IL-17F (Tonbo), IL-22 (PeproTech), and TNF-α (Tonbo) were reconstituted according to the manufacturers’ instructions in 0.01% BSA. These were used at a range of concentrations as described in the Results section and figure legends.

Epithelial cell culture
Gastric adenocarcinoma epithelial cells, MKN-28 cells, or AGS cells (ATCC CRL-1739) were grown and maintained in Life Technologies RPMI medium with l-glutamine, 10% FBS, 25 mM HEPES. The cells were maintained in tissue culture–treated flasks and passed every 3–4 d. During this seeding process, TrypLE Express [–] Phenol Red from Life Technologies is used according to the protocol on the manufacturer’s Web site. Cells were grown at 37°C in a 5% CO₂ humidified incubator.

For the gene expression experiments, cell lines were plated in a 24-well tissue culture–treated plate. The cells were seeded in cell media at a concentration of 2.5 × 10⁴ cells/well. Once confluent (24–48 h later), the cells were then stimulated in triplicates or quadruplicates. The PMSS1 *H. pylori* liquid culture and recombinant cytokines IL-17A, IL-17F, IL-17A/F, TNF-α, and/or IL-22 were diluted and resuspended in serum-free media. On administration of stimulant(s), the 24-well plates were placed back in the 5% CO₂ humidified incubator and incubated for 8 h before performing RNA extraction.

Animals and experimental challenge
Breeders for each species of mice were procured from multiple sources to generate the experimental groups for this project. Mice with genetic deficiency in IL-17A (Il17a<sup>-/-</sup>), IL-17F (Il17f<sup>-/-</sup>), and IL-17A/F double knockout (KO; Il17a<sup>-/-</sup>/Il17f<sup>-/-</sup>) were generated by Y. Iwakura and obtained through a material transfer agreement (MTA) with Research Institute for Biomedical Sciences Tokyo University of Science. IL-17A–deficient (Il17a<sup>-/-</sup>) mice were generated by J. Kolls and obtained from Amgen (MTA). CD4-Cre transgenic mice (Cd4<sup>cre</sup>) and WT breeders were originally from The Jackson Laboratory and have been breeding in colonies at Vanderbilt University for several years. Il17a<sup>-/-</sup>/Il17f<sup>-/-</sup> mice were procured through an MTA with M. Karin, University of California, San Diego. To generate the experimental group and littermate controls, we mated Cd4<sup>cre</sup>Il17a<sup>fl/fl</sup> with Il17f<sup>fl/fl</sup>. To sort the mice, we analyzed genomic DNA from the mouse tail using standard PCR followed by gel electrophoresis. The Cre transgene was established by a strong band present at 330 bp using 5′-GGCGTCAATCCTCGGTTCTCAACATT-3′ and 5′-GTCGAGATGGCGGCAACACCTATT-3′ forward and reverse primers, respectively. Il17a<sup>fl/fl</sup> mice were identified by a band located at 572 bp using primer set 5′-GGGGTTTTGTTTGTTTTGTTGG-3′ (P1), 5′-CGACGCTTCTACACCCCTCC-3′ (P2), and 5′-GCCAGATCTCCAAACAGCCGA-3′ (P3) (35). These mice were all on the C57BL/6J background and *Helicobacter* free prior to infection. Feces from sentinel mice housed in the same room consistently tested negative for pinworms, mouse parvovirus, and several other murine pathogens. At age 8–10 wk, mice were orogastrically inoculated with a suspension of 5 × 10⁸ or 1 × 10⁹ CFUs of PMSS1 strain of *H. pylori* in 0.5 ml of *Brucella* broth. Each dose was given twice, 2 days apart. The mice were then euthanized after 1 or 3 mo postinfection (mpi), and tissue was collected for analyses.

Harvest and stomach processing
With an excision at the duodenum and esophagus, the stomach was removed from each mouse. A cross-sectional incision was made at the gastroesophageal border separating the forestomach (nonglandular portion) from the glandular stomach (antrum and corpus). The forestomach was then discarded. Another incision was made along the lesser curvature of the glandular stomach, and its contents were removed and rinsed gently in cold PBS. The organ was then divided into three longitudinal strips that included the duodenum, pylorus, antrum, corpus, and squamocolumnar junction. The first strip was used to ascertain bacterial load and was placed into *Brucella* broth-10% FBS for immediate processing. The middle strip was used to investigate changes in

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histology and was placed in 10% normal buffered formalin for 24 h, before being embedded in paraffin and processed routinely for H&E staining. The third strip was used for genetic analysis and was stored in RNALater solution at −80°C for subsequent RNA isolation. Indices of inflammation were scored using the updated Sydney System by a single pathologist (M.B.P.) who was blind to the identity of the mice. Acute inflammation was graded based on density of neutrophils, while chronic inflammation was graded based on the density of lamina propria mononuclear cell infiltration. Both acute and chronic inflammation were graded on a 0–3 scale as follows: absent inflammation (grade 0), mild inflammation (grade 1), moderate inflammation (grade 2), and severe inflammation (grade 3) with both the antrum and the corpus scored separately. The total inflammation was calculated as a sum of acute and chronic inflammation in the corpus and the antrum, which allowed for quantification of total inflammation on a scale from 0 to 12.

Quantitative real-time RT-PCR
Tissue samples and cells were processed, and RNA was isolated using TRIzol reagent as detailed in past work (23). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and diluted 10-fold for subsequent quantitative real-time RT-PCR (qPCR). Using a StepOne Plus PCR machine (Applied Biosystems), we used TaqMan assays (Thermo Fisher Scientific) to ascertain cumulative gene expression, relative to uninfected or unstimulated control samples and normalized by Gapdh expression. Mouse primer sets were as follows: Gapdh (Mm99999915_g1), Defb14 (Mm00806979_m1), Iifng (Mm99999907_m1), Il17a (Mm00439619_m1), Il17f (Mm00521423_m1), Il21 (Mm00517640_m1), and polymeric Ig receptor (Pigr; Mm00465049_m1). Primer sets used to analyze human cell lines are as follows: GAPDH (Hs00466856_s1), CXCL8 (Hs0017403_m1), PIGR (Hs00992256_s1), NOXI (Hs00246589_m1), S100A8 (Hs00374264_g1), and S100A9 (Hs00610058_m1).

NanoString analysis
For assessing gene expression using NanoString, we isolated RNA from gastric tissue using the TRIzol method. RNA quality was assessed using the NanoDrop spectrophotometer (Nanodrop), and total RNA concentrations and size distributions were confirmed with the Bioanalyzer (Agilent Technologies) in the VANTAGE Core Facility at Vanderbilt University Medical Center. For NanoString hybridization, the input RNA was hybridized overnight (65°C) to nCounter NanoString mouse Immunology panel capture and barcoded reporter probes (CodeSet XT-CXO-MIM1). This nCounter NanoString Mouse Immunology panel analyzed the expression of 561 genes (including 14 internal reference genes). The samples were then loaded onto an nCounter SPRINT Cartridge, and the expression of the genes was determined by counting the barcodes using the nCounter SPRINT Profiler (NanoString Technologies) in the VANTAGE Core. The NanoString nSolver software (version 4.0, 2016) was used to normalize each sample based on the geometric mean of positive controls and the geometric mean of selected housekeeping genes. All NanoString counts were log2 transformed before further analyses.

Flow cytometry
To confirm that crossing the Cd4cre × Il17rafl/fl mice resulted in selective deletion of Il17ra on T cells, we stained splenocytes from Il-17RAfl/fl, Cd4creIl17rafl/fl, and Il17ra−/− mice with fluorochrome-conjugated mAbs specific for cell surface Ags. Anti–CD8a (clone 53-67), anti–CD4 (clone H129.19), anti–CD19 (clone IIJ3D1), anti–IL-17A (clone PAJ-17R), and anti–rat IgG2a (eB2R2a) mAbs were purchased from BD Biosciences (San Jose, CA). The gating strategy used for these experiments was to gate on lymphocytes by forward light scatter/side scatter (of light) and then gate on either CD19+ cells (reported in Fig. 5) or CD19−. From the CD19− gated cells, CD4+CD8a− or CD8a−CD4+ cells were gated to identify CD4+ and CD8+ cells, respectively (reported in Fig. 5).

For intracellular cytokine staining, the single-cell suspensions were prepared from spleens of Il17rafl/fl, Cd4creIl17rafl/fl, and Il17ra−/− mice. Suspensions were treated with GolgiStop protein transport inhibitor (BD Biosciences) and restimulated with PMA-ionomycin for 6 h. Subsequently, surface staining with fluorochrome-conjugated mAbs specific for cell surface Ags was performed, followed by fixation with Cytosift/Cytoperm solution (BD Biosciences). The cells were then stained with anti–IL-17 in CellPerm/Wash buffer for 30 min and washed prior to collection. Anti–CD3 (clone 145-2C11), anti–CD4 (clone GK1.5), anti–IL-17A (clone PAJ-17R), and anti–rat Ig (clone eBRG1) mAbs were purchased from BD Biosciences. Cells were acquired in a BD LSR II instrument (Vanderbilt Flow Cytometry Core), and data were analyzed using FlowJo software (FlowJo, a BD Company). The gating strategy here was to gate on the lymphocytes by forward light scatter/side scatter (of light) followed by exclusion of doublets. CD3+CD4+ cells were gated on, and the percent of those cells expressing IL-17A was then identified.

Th17 differentiation of naive murine T cells
With modifications, Th17 differentiation was carried out as described by Bedoya et al. (36) Naive CD4 (CD4+CD25−) T cells were isolated from the lymph nodes and spleens of WT

<table>
<thead>
<tr>
<th>Gene</th>
<th>PMSS1 (MOI 50)</th>
<th>PMSS1 + IL-17A</th>
<th>TNF (2 ng/ml)</th>
<th>TNF + IL-17A</th>
<th>IL-17A (50 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL17A</td>
<td>1.04 (±0.18)</td>
<td>0.60 (±0.15)</td>
<td>1.32 (±0.18)</td>
<td>0.80 (±0.20)</td>
<td>0.74 (±0.28)</td>
</tr>
<tr>
<td>IL17C</td>
<td>1.26 (±0.53)</td>
<td>0.64 (±0.13)</td>
<td>1.32 (±0.14)</td>
<td>0.91 (±0.17)</td>
<td>0.55 (±0.21)</td>
</tr>
</tbody>
</table>

Relative units (RUs 1 = 50) are presented. RUs of gene expression of IL17A and IL17C in human gastric epithelial cell line and MKN cells with different stimuli (or costimulation). RUs are relative to untreated controls.
FIGURE 1. Gastric epithelial cells, AGS cells, and response to IL-17A, IL-17F, or IL-17A/F.
Serum-starved AGS cells were stimulated with various stimuli (IL-17A at 100 μg/ml, IL-17F at 100 μg/ml, TNF-α at 2 ng/ml, IL-22 at 200 μg/ml, or PMSS1 at an MOI of 50) were added to serum-starved AGS cells for 8 h. qPCR was performed to measure expression of CXCL8, PIGR, NOX1, and S100A8 in response to these stimuli or costimuli. Expression is shown as relative units and is relative to RNA expression from cells treated with equal concentration of the carrier (BSA). Data are shown as ±SEM and are representative of three independent experiments. Statistical analysis was performed with one-way ANOVA and Tukey’s test for multiple comparisons. Error bars represent SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
and Il17ra−/− mice using MACS Naive CD4+ T cell isolation kit (Miltenyi Biotec) following the given protocol. The naive CD4 T cells were cultured in a 96-well round-bottom tissue culture plate bound with anti-CD3 (2 μg/ml) and anti-CD28 (0.5 μg/ml) and polarized in the presence of IL-6 (20 ng/ml), TGF-β1 (1 ng/ml), anti–IL-12 (1 μg/ml), anti–IFN-γ (1 μg/ml), anti–IL-4 (1 μg/ml), and at day 2 IL-23 (10 ng/ml). Cells were incubated at 37°C and 5% CO2 for 96 h, then stimulated with 50 μg/ml PMA (Sigma Aldrich) and 1 mg/ml ionomycin (Fisher Scientific). GolgiStop was added 2 h poststimulation, and the cells were incubated for another 2 h before being harvested for flow cytometric analysis or (without GolgiStop) cells were processed for RNA isolation.

RESULTS

The roles of IL-17F and the IL-17A/F heterodimer have not been investigated in the context of H. pylori infection. Although IL-17F and the IL-17A/F heterodimer have been shown to signal the IL-17R (composed of IL-17RA and IL-17RC), their contributions to the antimicrobial responses in the gastric mucosa have not be identified. In this study, MKN-28 and AGS cells, human gastric epithelial cell lines, were treated with IL-17A, IL-17F, or IL-17A/F to compare the activation of known IL-17A–induced genes, including CXCL8, S100A8, and antimicrobial genes induced in the gastric mucosa, including PIGR and NOX1. In addition, the effects of H. pylori coculture (multiplicity of infection [MOI], 50) and costimulation with TNF (2 ng/ml) or IL-22 (100 ng/ml) on cytokine stimulation were investigated because of converging of TRAF signaling and the NF-κB pathway. In the MKN cells, both IL17RA and IL17RC were expressed, and IL-17A stimulation significantly downregulated IL17RC and IL17RA expression after PMSS1 treatment, but there was no significant difference with other stimuli (Table I). When measuring CXCL8 and NOX1 expression in the cell lines, responsiveness to recombinant IL-17 cytokines was low, but there was some upregulation in expression of CXCL8 with IL-17A and IL-17A/F stimulation (at 100 μg/ml) (Fig. 1, Supplemental Fig. 1). In contrast, the polymeric Ig receptor gene, PIGR, was upregulated to a significant level with IL-17A or IL-17A/F stimulation in AGS cells, but not MKN cells. Despite this, expression of S100A8 was not induced in AGS

FIGURE 2. During Helicobacter pylori infection, both IL-17A and IL-17F can independently mediate acute inflammation, but the presence of either regulates chronic inflammation.

(A) Bacterial burden was measured in WT, Il17a−/−, Il17f−/−, Il17a/f−/−, and Il17ra−/− mice that were infected with PMSS1 for 3 mo (8–18 mice per genotype). CFUs per gram of stomach tissue was calculated and is presented in the graph. Statistical analysis was performed using Dunnett’s multiple comparisons test. (B and C) Levels of acute and chronic inflammation were scored on stomach tissue (in the corpus and antrum) at 3 mpi with strain PMSS1. Statistical analysis was performed using Kruskal–Wallis test, and error bars represented mean ± SEM. See Materials and Methods for the scoring system (scale is 0–12). *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001. Data combine two independent experiments.
TABLE II. Lymphoid follicles and plasma cells during H. pylori infection

<table>
<thead>
<tr>
<th>Pathological Finding</th>
<th>WT</th>
<th>IL-17A–/–</th>
<th>IL-17F–/–</th>
<th>IL-17A/F–/–</th>
<th>IL-17RA–/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoid follicles (% of animals in the group)</td>
<td>10%</td>
<td>50%</td>
<td>0%</td>
<td>20%</td>
<td>75%*</td>
</tr>
<tr>
<td>Average score of plasma cells (scale is 0–3 ± SEM)</td>
<td>0.9 (±0.87)</td>
<td>1.0 (±0.26)</td>
<td>0.417 (±0.154)</td>
<td>0.955 (±0.157)</td>
<td>2.5 (±0.283)*</td>
</tr>
</tbody>
</table>

A Fisher’s exact test was used to determine whether lymphoid follicles formed in the different mouse genotypes more frequently than in WT mice at 3 mpi. There was a significant increase in lymphoid follicle formation in IL-17RA–/– mice compared to WT mice.

*An asterisk indicates that the pathology score for plasma cells is significantly different when data are analyzed by one-way ANOVA followed by a Dunn multiple comparison test, WT versus IL-17RA–/–.

cultures with IL-17A, IL-17F, or IL-17A/F alone and only minimally induced in MKN cells (Supplemental Fig. 1); this is consistent with previous studies that reported that a costimulus may be necessary for activation of the IL-17R pathway and subsequent antimicrobial expression (23).

To explore how the IL-17 cytokines impact epithelial cell activation in response to a costimulus, we stimulated gastric epithelial cells with TNF, PMSI1, or IL-17 in concert with the IL-17 cytokines. These data indicate that TNF is a potent, synergizing stimulus for CXCL8 and PIGR expression. Although TNF activated low expression of NOXI and S100A8, the synergistic response was not as pronounced compared with CXCL8 or PIGR. Using H. pylori (strain PMSI1) as a costimulus resulted in greater synergy with IL-17A than the other IL-17 cytokines (i.e., CXCL8, PIGR, NOXI, S100A8). In general, it appears that IL-17A/F provides the strongest stimulus alone, but when a costimulus is used, IL-17A has similar or more potency. For example, in the case of PIGR expression, although PMSS1 and IL-17A still induced PIGR expression, the combination of PMSS1 and IL-17A/F reduced PIGR expression compared with IL-17A or IL-17F. Further, CXCL8 gene expression is upregulated more with IL-17A or IL-17A/F than IL-17F when TNF or IL-22 provides a costimulus. Further, expression of S100A8 and CXCL8 in the gastric epithelial cells were activated on stimulation by PMSS1 alone, IL-17A alone, and on costimulation by TNF and IL-17A. IL-17F did not induce these genes in MKN cells (Fig. 1).

In vivo, the potential for differential roles of these cytokines was assessed by infecting IL-17A–/–, IL-17F–/–, IL-17A/F–/–, and IL-17RA–/–deficient mice with H. pylori strain PMSS1. Because H. pylori infection chronically persists and chronic gastritis is commonly assessed at 3 mpi, we have focused these studies on the 3-mpi time point. The ability of these genotypes of mice to control H. pylori colonization was measured by serial plating of stomach homogenates. At 3 mpi, there was no significant difference in the ability of the KO mice to control H. pylori infection compared with WT mice.

In our previous findings, we reported that III7ra–/– mice develop significantly lower levels of PMNs and their acute inflammation scores are significantly reduced compared with WT mice (27). To determine whether deficiency of IL-17A or IL-17F alone led to a change in acute inflammation (or the presence of extravascular PMNs on a scale of 0 to 3), the levels were scored by a blinded pathologist at 3 mpi in the antrum and the corpus as previously described (37). Deficiency in IL-17A, IL-17F, or both IL-17A/F led to a significant decrease in acute inflammation scores compared with WT mice (Fig. 2B).

Chronic inflammation associated with H. pylori was scored using the Sydney system and to assess the ability of these different strains of mice to develop chronic gastritis as a marker of T and B cell infiltration. Deficiencies in IL-17A or IL-17F alone (III7a–/– or III7f–/–) did not significantly impact H. pylori–induced chronic inflammation during infection, but a deficiency in both IL-17A and IL-17F (III7a/f–/–) or a deficiency in IL-17R signaling (III7ra–/–) led to significantly increased chronic inflammation during H. pylori infection compared with WT infected mice (Fig. 2C). Lymphoid follicles and plasma cells are significantly increased in abundance in the III7ra–/– mice compared with other genotypes (Table II), and although there was a trend toward increased lymphoid follicles in III7a–/– and III7a/f–/– mice, the differences were not significant.

Although control of H. pylori colonization has often been inversely correlated to acute inflammation, that is not consistent in these experiments. This led us to investigate whether host factors involved in defense of the mucosal barrier were
Impact of IL-17A and IL-17F signaling on Th17 responses in the gastric mucosa by 3 mpi (27). To investigate whether T cell cytokine responses in the H. pylori–infected Il17a−/−, Il17f−/−, Il17a/f−/−, and Il17ra−/− mice were elevated compared with H. pylori–infected WT mice, we performed qPCR on gastric tissue at 3 mpi. There are no significant differences in Ifng expression. Gene expression of Il21 is significantly higher only in the Il17ra−/− mice compared with WT mice (Fig. 4A, 4C). Due to the variability in Il21 levels in these mice at this chronic time point, the correlation between Il21 expression and chronic inflammation was tested. There is a significant correlation between chronic inflammation and relative units of Il21 expression in the gastric tissue (Fig. 4B). Further, H. pylori–infected Il17ra−/− mice had significantly increased expression of Il17a and Il17f compared with H. pylori–infected WT mice, which is similar to what had been observed previously when Il17ra−/− mice were infected with SS1 (a type 4 secretion system–deficient H. pylori strain) (27) (Fig. 4D).

To investigate the immune activation in the gastric mucosa and to consider other pathways contributing to the exacerbated immunopathological outcome in the Il17ra−/− mice, we performed a broader gene expression analysis on gastric tissue from Il17ra−/− mice and WT mice using NanoString (Ms_Immunology Gene Panel of >500 genes; Table III, Supplemental Table I). Many of these findings are consistent with previous observations (27); for example, there were significant decreases in expression of neutrophil markers/antimicrobials (Cxcr5, Maf, Pdcd1), Th2 cells (Gata3, Irf4), and Th17 (Rorc). There is no evidence of increased innate lymphocytes in
It was reported previously that IL-17A controls the expansion of IL-17A–producing naive T cell populations through IL-17R signaling on nonhematopoietic cells. To confirm KO of IL-17RA on T cells, we measured IL-17RA expression by flow cytometry, and expression was down on all T cells (CD4+ and CD8+), but not down on the CD19+ B cell subset (Fig. 5A). Further, IL-17A expression was measured in splenocytes from the Cd4creIl17ra/fl and Il17ra/fl mice using intracellular cytokine staining. There was a 2-fold increase in IL-17A expression in the CD4+ cells deficient for IL-17RA signaling (Fig. 5B).

Next, Cd4creIl17ra/fl and Il17ra/fl mice were infected with *H. pylori*, and their ability to control *H. pylori* infection and inflammation was assessed. Interestingly, many of the Cd4creIl17ra/fl mice had evidence of yeast outgrowth in their stomachs independent of *H. pylori* infection (Supplemental Fig. 3A). This was evident in the histological examination of the tissue (Supplemental Fig. 3A). In this cohort of mice when many had yeast colonization in their stomachs, the data indicate that CFU levels were higher in the gastric tissue of Il17ra/fl mice (Supplemental Table I). These data suggest that in the absence of IL-17RA, *H. pylori*–infected mice develop lymphoid follicles with germinal centers activating Thf cells, IL-21 expression, and Ab production (27).

**TABLE III. NanoString (Ms_Immunology Panel) was used to determine the ratio and Log2 Fold change of abundance of transcripts of genes associated with T and B cell responses in Il17ra/fl versus WT (C57BL/6J) gastric tissue at 3 mpi with PMSS1 strain of *H. pylori***

<table>
<thead>
<tr>
<th>T Cell Subset</th>
<th>Gene</th>
<th>% Samples above Threshold</th>
<th>Ratio</th>
<th>Log2 Fold Change</th>
<th>p</th>
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<tr>
<td>T</td>
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<td>3.16</td>
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<tr>
<td>T cytotoxic</td>
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<td>2.4</td>
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<tr>
<td>Th</td>
<td>Cd4</td>
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<tr>
<td>Tfh</td>
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<td>1.22</td>
<td>0.29</td>
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<tr>
<td>Th1</td>
<td>Tbx21</td>
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<td>0.12</td>
<td>0.75548154</td>
</tr>
<tr>
<td>Th2</td>
<td>Gata3</td>
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<td>1.5</td>
<td>0.58</td>
<td>0.02776563</td>
</tr>
<tr>
<td>Th17</td>
<td>Rorc</td>
<td>100%</td>
<td>1.23</td>
<td>0.3</td>
<td>0.04490001</td>
</tr>
<tr>
<td>Treg</td>
<td>Foxp3</td>
<td>41.67%</td>
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<th>% Samples above Threshold</th>
<th>Ratio</th>
<th>Log2 Ratio 2 Fold Change</th>
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<td>Tbx21</td>
<td>Induces Ifng (Th1)</td>
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<td>1.09</td>
<td>0.12</td>
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<td>1.76</td>
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<td>0.0477938</td>
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<td>Eomes</td>
<td>Induces Ifng</td>
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<td>1.18</td>
<td>0.24</td>
<td>0.45388737</td>
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<tr>
<td>Gata3</td>
<td>Regulates Th2</td>
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<td>1.5</td>
<td>0.58</td>
<td>0.02776563</td>
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<td>Stat5b</td>
<td>Th2 differentiation</td>
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<td>0.16</td>
<td>0.12631282</td>
</tr>
<tr>
<td>Stat6</td>
<td>Induces Gata3</td>
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<td>1.14</td>
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<td>Rorc</td>
<td>Promotes Th17</td>
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<td>Induces Treg cell differentiation</td>
<td>41.67%</td>
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<td>Maf</td>
<td>Tfh</td>
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<td>Pdcd1</td>
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<td>2.73</td>
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<tr>
<td>Il21</td>
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<td>3.04</td>
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<td></td>
<td>Pax5</td>
<td>100%</td>
<td>9.45</td>
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<td>100%</td>
<td>4.66</td>
<td>2.22</td>
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<tr>
<td>Chemokines</td>
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<td>100%</td>
<td>3.82</td>
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<td>Polymeric Ig Receptor</td>
<td>Pigfr</td>
<td>100%</td>
<td>−5.22</td>
<td>−2.38</td>
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Values in bold were significantly different between genotypes.
response to IL-17 is attributable to the antimicrobial response. Although infection of these mice with *H. pylori* did induce inflammation and T cell cytokine gene expression, the T cell-specific deficiency in IL-17RA did not lead to increased chronic inflammation, nor did it lead to any increased *Il17a* expression compared with *Il17a<sup>−/−</sup>* mice (Supplemental Fig. 3B, 3C). It cannot be ruled out that the yeast did not affect the host's immune response, but it is unlikely that the yeast would have decreased the inflammation or inhibited the T cell response.

After treating breeders with fluconazole and successfully removing this yeast from the colony, more cohorts of *Cd4<sup>Cre</sup>*/*Il17ra<sup>fl/fl</sup>* and *Il17ra<sup>fl/fl</sup>* mice were infected with *H. pylori*. There was no significant difference in mice with T cells deficient for IL-17RA to control *H. pylori* colonization (Fig. 6A), no change in total inflammation scores (Fig. 6B), and no significant differences in *Ifng*, *Il17a*, or *Il21* expression in the gastric mucosa (Fig. 6C). These data suggest that IL-17RA deficiency in T cells is not sufficient to induce exacerbated chronic inflammation and hyper-Th17 responses during *H. pylori* infection.

**DISCUSSION**

A critical role for IL-17A in host immunity has been well defined. IL-17A signals through IL-17RA/RC multimeric receptors in epithelial cells to activate several transcription factors, including AP-1, C/EBP, and NF-κB. These transcription factors then upregulate mRNA expression of *cxcl8* and several antimicrobial products, especially in the presence of a costimulator such as IL-22. The role for IL-17F or the heterodimer IL-17A/F in this process has been less clear despite their use of the same multimeric receptor. Moreover, in several models, including *H. pylori* infection models, *Il17a<sup>−/−</sup>* mice do not respond similarly to *Il17ra<sup>−/−</sup>* mice, suggesting that there is either a differential role for IL-17F or compensatory impact of having one of the cytokines.

Working with gastric epithelial cell cultures, it was determined that MKN and AGS cells can be used to measure IL-17A responsiveness, but they do not respond similarly to the different costimuli tested. For example, AGS cells respond by producing *NOX1* and *PIGR* when costimuli are present, especially in response to IL-17A. The binding affinity of IL-17F to IL-17RA is reported to be much lower than IL-17A, yet the homodimers have similar affinity for the IL-17RC component of the signaling complex (39). Recent studies have suggested that the IL-17RA signaling pathway may also play a role in downregulating the inflammatory response; it is possible that this process is mediated by IL-17A and/or IL-17F. Interestingly, our data suggest that it is not as strong of a synergistic activator of either MKN or AGS cells.

The S100A9 protein forms a heterodimer with S100A8, known as calprotectin (40). Calprotectin can affect the growth and virulence of bacteria, such as *H. pylori*, by sequestering nutrient metals, such as zinc, making it harder for the bacteria to survive in the gastric mucosa (23, 40). Although S100A8 and S100A9 proteins have been characterized as antimicrobial, their role during bacterial infection may be multidimensional (40). This is because S100A9 also plays a prominent role in the regulation of inflammatory processes and immune response; S100A9 can induce neutrophil chemotaxis and adhesion, acting as a potent amplifier of inflammation. In addition to this, ligation of calprotectin to TLR4 can activate MAPK and NF-κB signaling pathways, which can also drive transcription of...
proinflammatory genes (41, 42). This activity leads to the classification of calprotectin as a damage-associated molecular pattern. Therefore, understanding the effects of calprotectin, an antimicrobial protein and a proinflammatory damage-associated molecular pattern, in the context of *H. pylori* can be challenging (43–45). Consistent with the role for IL-17A or IL-17F in mediating acute inflammation and neutrophilic responses, acute inflammation was significantly reduced in *Il17a*Δ/Δ, *Il17f*Δ/Δ, and *Il17a/f*Δ/Δ mice in comparison with WT mice. It is evident that the IL-17A/F double KO and the *Il17ra*Δ/Δ exhibit high levels of chronic inflammation, suggesting that IL-17A or IL-17F play a role in modulating a protective immune response during the control of chronic inflammation. Levels of chronic inflammation correlate to increases in *Il21* and *Ifng*, suggesting that exacerbated T cell responses are downregulated by IL-17A or IL-17F signaling through IL-17RA.

The hypothesis that IL-17A or IL-17F modulates T cell cytokine production directly leading to downregulation of proinflammatory cytokines, such as IL-17A and IL-21, was addressed. Naive T cells from both WT and *Il17ra*Δ/Δ mice were differentiated into Th17 cells, and gene expression was assessed by qPCR. We were unable to see an impact of IL-17A on naïve T cells differentiated into Th17 cells, and gene expression was assessed by qPCR. We were unable to see an impact of IL-17A on naïve T cells differentiated into Th17 cells, and there was no inherited difference in *Il17a* or *Il21* expression in the *Il17ra*Δ/Δ T cells in these in vitro models (Supplemental Fig. 2). The rationale for moving in vivo with these models was that the microenvironment of

**FIGURE 6.** IL-17RA expression on T cells is required to control bacterial burden but is not required to control gastric inflammation in the mouse model. (A) Bacterial burden was measured in *Il17ra*Δ/Δ (WT) and *Cd4creIl17ra*Δ/Δ mice that were infected with PMSS1 for 3 mo (8–11 per genotype). Colony-forming units (CFU) per gram of stomach tissue was calculated and is presented in the graph ± SEM. Statistical analysis was performed using Mann–Whitney U unpaired t test on log-transformed CFUs per gram data. These data are representative of two independent experiments. (B) Levels of acute and chronic inflammation were scored on stomach tissue (in the corpus and antrum) at 3 mpi with strain PMSS1. Total inflammation as presented is the sum of acute and chronic inflammation (8–10 per genotype). Statistical analysis was performed using Kruskal–Wallis tests and the Dunn’s multiple comparisons test, which resulted in no significant difference between genotypes. See Materials and Methods for the scoring system (scale is 0–12). Error bars represent mean ± SEM and are representative of two independent experiments. (C) qPCR was used to measure *Il21*, *Il17a*, and *Ifng* transcripts in the gastric tissue of *H. pylori*-infected *Il17ra*Δ/Δ and −infected *Cd4creIl17ra*Δ/Δ mice (5–7 per genotype). Relative units are calculated as described in the Materials and Methods, relative to Gapdh and calibrated to uninfected WT mice. qPCR data are representative of two independent experiments. Statistical analysis was performed using an ANOVA analysis and Tukey’s multiple comparisons test. Error bars represent ± SEM. *p < 0.05, **p < 0.01, compared with the uninfected group.
in vitro T cell cultures is very isolated and cannot recapitulate the chronic time course in which this pathological response is observed in the H. pylori–infected Il17ra−/− and Il17a/f−/− mice. Using the Cre/lox recombination system to develop a T cell–specific deficiency in the IL-17R, it is evident that the absence of IL-17RA on T cells does not lead to an exacerbated T cell response compared with WT mice or control models. These data suggest that the impact of congenic deficiency of IL-17RA may have an indirect impact on the T cell response or requires two hits. In the case of a two-hit hypothesis, the first hit or primary driver of the hyper-Th17 response in the Il17ra−/− mice may be driven by a shift in the gastric microbiome and/or an impact on gastric permeability or wound healing, while the second hit may be activation of an aberrant T cell response against the shifted microbiome or microbes gaining access to the submucosa. IL-17 impacting the expression of CXCL8, PIGR, and NOX1 supports the potential for IL-17R signaling being a requirement for preventing dysbiosis. These hypotheses are the focus of future investigations.

The role of the polymeric Ig receptor has only minimally been investigated in the gastric mucosa in response to H. pylori infection. Expression of PIGR is low in healthy adults and increases with gastric inflammation and development of intestinal metaplasia (46–48). This is the first report that we are aware of that links IL-17 cytokines to induction of PIGR expression in human gastric epithelial cells. Correll et al. (49) describe the course of H. pylori infection in pIgR KO mice infected with the SS1 strain. In the study, there was no differences in the gastric bacterial loads of WT and pIgR KO mice up to 3 mpi. Their experiments were carried out to 12 mpi, and by 6 mpi, they report a reduction in bacterial load in the WT mice compared with that in the pIgR KO mice. Because gene expression of Pigr is reduced in the Il17ra−/− mice and not completely absent, a different outcome is possible.

Noteworthy, the T cell–specific deficiency in IL-17RA did result in at least one known shift in the gastric microbiome colonization with a yeast (Supplemental Fig. 3A). Evidence of a yeast, likely Kazachstan pintolopesii, was observed in many of the Cds4creIl17rafl/fl mice. Our pathologist noted that the colonization was more abundant in the corpus. This yeast has been reported in the literature occasionally in immunocompromised animals (50, 51). Increased susceptibility to commensal fungi, mainly manifesting as mucocutaneous candidiasis, has been reported in individuals with mutations in IL-17F and IL-17RA (52), and anti–IL-17 treatment in Crohn’s disease patients led to more fungal infections compared with patients who did not receive anti–IL-17 (53). Although these connections between IL-17 signaling and fungal infections have been noted, there has not been any indication that this would be because of a T cell–specific response.

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REFERENCES


DISCLOSURES

The authors have no financial conflicts of interest.


