

CCR2 mediates *Helicobacter pylori*-induced immune tolerance and contributes to mucosal homeostasis

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Abstract

Background: We previously demonstrated that *H. pylori* infection leads to increased induction of regulatory T cells in local and systemic immune compartments. Here, we investigate the role of CCR2 in the tolerogenic programming of dendritic cells in a mouse model of *H. pylori* infection.

Materials and Methods: CCR2 deficient (CCR2KO) mice and wild-type (Wt) mice infected with *H. pylori* SS1 strain were analyzed by qPCR and FACS analysis. In vitro, bone marrow-derived DC on day 6 from CCR2KO and Wt mice cocultured with or without *H. pylori* were examined to determine the impact of CCR2 signaling on dendritic cells function by qPCR, ELISA, and FACS analyses.

Results: Acute *H. pylori* infection was associated with a threefold increase in CCR2 mRNA expression in the gastric mucosa. *H. pylori*-infected CCR2KO mice exhibited a higher degree of mucosal inflammation, that is, increased gastritis scores and pro-inflammatory cytokine mRNA levels, but lower degree of *H. pylori* gastric colonization compared to infected Wt mice. Peripheral *H. pylori*-specific immune response measured in the CCR2KO spleen was characterized by a higher Th17 response and a lower Treg response. In vitro, CCR2KO bone marrow-derived DC was less mature and shown a lower Treg/Th17 ratio. Moreover, blockade of CCR2 signaling by MCP-1 neutralizing antibody inhibited *H. pylori*-stimulated bone marrow-derived DC maturation.

Conclusions: Our results indicate that CCR2 plays an essential role in *H. pylori*-induced immune tolerance and shed light on a novel mechanism of CCR2-dependent DC Treg induction, which appears to be important in maintaining mucosal homeostasis during *H. pylori* infection.

KEYWORDS

adaptive immunity, Chemokine receptor 2, dendritic cells, immature, immune tolerance

1 | INTRODUCTION

Helicobacter pylori (*H. pylori*) colonizes the human stomach and contributes to diseases such as gastric ulcers and cancers. It is a highly adaptive Gram-negative bacterium, capable of evading immune surveillance despite systemic and mucosal humoral immunity.^{1,2} There is growing evidence that the failure of the host to eradicate *H. pylori*

may be due to the ability of *H. pylori* to induce a regulatory T-cell (Treg) response against helper T-cell immunity. *H. pylori*-specific Tregs were recently shown to suppress memory T-cell responses to *H. pylori* infection in individuals.^{3,4} In patients, the mRNA expression of Foxp3, a specific Treg surface marker, is higher in the gastric tissue of *H. pylori*-infected persons compared with uninfected controls.⁵⁻⁷ Also, Harris et al. reported an inverse correlation between gastric

Foxp3 expression and gastric pathology in *H. pylori*-infected children compared to adults.⁸ Our laboratory has also shown that *H. pylori* can induce Treg development by DC that lead to the inhibition of the host immune response against *H. pylori*.^{9,10}

The maturation and activation state of DC are regarded as a control point for the induction of either peripheral tolerance or autoimmunity. DC maturation has been shown to be associated with changes in the expression of chemokine (C-C motif) receptor 2 (CCR2), the receptor for monocyte chemoattractant protein-1,¹¹ which is crucial for mucosal recruitment during inflammation.¹²⁻¹⁶ CCR2 has been shown to contribute to leukocyte trafficking and the control of intracellular pathogens as CCR2 knockout (CCR2KO) mice were unable to clear infection by *Listeria monocytogenes*.¹⁷ Deficiency of CCR2 was shown to impair DC trafficking to draining lymph nodes (LNs) in mice and resulting in a defective Th1 response and an increased susceptibility to *L. major*.¹⁸ Thus, we speculate that CCR2 signaling may be critical in DC-mediated *H. pylori* tolerogenic response.

In this study, we examined the role of CCR2 in the recruitment and function of DC during *H. pylori* infection. In vivo analyses following acute *H. pylori* infection revealed no significant difference in gastric mucosal DC between wild-type (Wt) and CCR2KO mice, suggesting CCR2 is not required for DC recruitment. However, chronic *H. pylori* infection showed more severe gastritis and a lower degree of gastric *H. pylori* colonization in CCR2KO mice compared to their Wt mice. Splenic *H. pylori* antigen-specific helper T-cell cytokine analyses revealed a lower Treg/Th17 response ratio in chronically infected CCR2KO mice compared to Wt littermates. In vitro, CCR2KO bone marrow-derived DC (BMDC) were less mature and had defective T-cell priming function characterized by a lower Treg/Th17 response ratio, similar to that observed in vivo. Neutralization of MCP-1, a CCR2 ligand, resulted in decreased DC maturation. Thus, our study demonstrates that CCR2 signaling is critical for induction of *H. pylori*-specific Treg response and may represent a major target in the modulation of the host response to *H. pylori*.

2 | MATERIALS AND METHODS

2.1 | Mice

Male Wt C57BL/6 mice were purchased from Charles River and C57BL/6 CCR2KO mice were provided by Dr. Bethany Moore (Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, University of Michigan Health System, Ann Arbor, MI). Mice used were 8-10 weeks old. All animals were housed in the animal maintenance facility at the University of Michigan Health System. This research was undertaken with the approval of the University Committee on Use and Care of Animals at the University of Michigan (PRO00005890).

2.2 | Bacterial strains and culture condition

H. pylori SS1 strain was cultured on *Campylobacter*-selective agar (BD Diagnostics, Bedford, MA, USA) for 3 days in a humidified

microaerophilic chamber at 37°C (BBL Gas System, with CampyPak Plus packs, BD Biosciences San Jose, CA) as previously described.⁹ *H. pylori* infection of mice was achieved by oral gavage of 10⁹ bacteria suspended in 100 µL of Brucella broth.

2.3 | Generation and stimulation of bone marrow-derived dendritic cells (BMDCs)

BMDC from Wt or CCR2KO mice were derived using mouse GM-CSF (10 ng/mL) and IL-4 (10 ng/mL) and cultured with RPMI containing 10% fetal bovine serum (FBS) as previously described.¹⁹ BMDC were harvested and enriched (10⁶ cells/mL) by gradient centrifugation using OptiPrep density solution (Sigma, St. Louis, MO) according to manufacturer's instruction. For *H. pylori*-stimulated BMDC experiments, BMDC were stimulated with 10⁷ CFU/mL of *H. pylori* or PBS for 18 hours and then cocultured with syngeneic splenocytes for 72 hours in a mixed leukocyte reaction (splenocyte: BMDC ratio of 10:1) as previously described.¹⁹

2.4 | Animal studies

For acute infection, Wt and CCR2KO mice were infected for 24 hours with 10⁹ CFU/mL *H. pylori* SS1 via oral gavage. For chronic infection, mice were gavaged with *H. pylori* SS1 (three times over 1 week) and analyzed after 2 months. Six to ten animals per group (Wt or CCR2KO group) were used in each experiment. The stomachs from Wt and CCR2KO mice were cut along the greater curvature, and 2-mm strips of fundus and antrum were embedded in Tissue-Tek optimum cutting temperature compound (Sakura Finetek, Torrance, CA). Splenocytes from Wt and CCR2KO mice were cocultured for 72 hours with BMDC from Wt mouse. The splenocyte-to-BMDC ratio was 10-1. After 72 hours, splenocytes were collected, and the percentages and mRNA expression of IFN-γ, IL-17A, and Foxp3 were measured by FACS and qPCR, respectively.

2.5 | Gastritis score determination

After two-month infection with *H. pylori*, the mice were euthanized. The stomachs of Wt and CCR2-deficient mice were removed and prepared for later analysis. Two adjacent full-thickness longitudinal strips were removed from the greater curvature of the stomach and fixed in formalin for histologic analysis (100 × magnification). The specimens were scored separately for the presence or absence of gastritis. The results were reported as the percentage of the number of gastritis-containing fields per total number of fields examined on each histologic slide.²⁰

2.6 | qRT-PCR

Total RNA from stomach, DC, or splenocytes was prepared using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Samples were reverse-transcribed using iScript™ cDNA Synthesis Kit (BIO-RAD, Hercules, California). Expression of CCR2, *H. pylori* 16S, IL-6, IL-1β, IL-12p35,

TABLE 1 Primers used for the amplification of each gene

Gene	Primer (5' - 3')
GAPDH	F:5'-TCAAGAAGGTGGGTGAAGCAGG-3' R:5'-TATTATGGGGTCTGGGATGG-3'
CCR2	F: 5'-GGAGAA AAGCCA ACT CCT TC-3' R: 5'-AGG CAG TTGCAA AGG TACTG-3'
IL-6	F:5'-CTACCCCAATTTCCAATGCT-3' R:5'-ACCACAGTGAGGAATGTCCA-3'
IL-17A	F:5'-GCTCCAGAAGGCCCTCAGA-3' R:5'-AGCTTTCCCTCCGATTGA-3'
IL-1β	F:5'-CCAAGTGGTACA TCAGCAC-3' R:5'-TCTGCTCATTACGAAAA GG-3'
TNF-α	F:5'-CCCACTCTGACCCCTT TACT-3' R:5'-TTTGAGTCTTGATGGTG GT-3'
IL-10	F:5'-AGTGGAGCAGGTGAAGAGTG-3' R:5'-TTCGGAGAGAGGTACAAACG-3'
IL-12p35	F: 5'-ACGCCAGGAAAACTGAA-3' R:5'-CTACCAAGGCACAGGGTGCAT-3'
IFN-γ	F:5'-TCAAGTGGCATAGATGTGGAAGAA-3' R:5'-TGGCTCTGCAGGATTTTCATG-3'
Foxp3	F:5'-TCTCCAGTTGCTCAAAGTC-3' R:5'-GCAGAAGTTGCTGCTTTAGG-3'
HP 16s	F:5'-CAAGTCATGGCCCTTAC-3' R:5'-TTGCGATTACTAGCGATTCC-3'

IL-10, IFN-γ, IL-17A, Foxp3, and GAPDH was measured using iQ™ SYBR Green Supermix Kit obtained from BIO-RAD. Primers are shown in Table 1. Finally, quantitation of relative differences in expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method.²¹

2.7 | Generation of single-cell suspensions and flow cytometry

Stomachs and spleens from Wt and CCR2KO mice were digested using collagenase as described.²² Single-cell suspensions were preincubated with FcBlock (2.4G2) (BD Biosciences) to minimize nonspecific protein binding. Cells were stained with fluorochrome-conjugated Abs against surface markers MHCII, CD45, CD11b, CD11c, CD103, CCR2, CD80, and CD86 (eBioscience, San Diego, CA, USA) in staining buffer (PBS containing 2% FCS and 0.1% NaN₃) for 30 minutes at room temperature and then washed. For staining of CD4, Foxp3, IFN-γ, and IL-17A, cells were permeabilized and subsequently stained. Samples were measured on a Coulter XL Flow Cytometer (Beckman Coulter, Miami, FL) and analyzed with FlowJo software (C6).

2.8 | Statistical analyses

Experiments were performed in triplicate, and all data were expressed as mean ± SEM. Continuous ratio scale data were evaluated by unpaired Student *t* test (for comparison between two samples) or by ANOVA (for multiple comparisons) or Turkey post hoc analysis, which compares treatment groups to a specific control group by GraphPad Prism software version 5.0c (GraphPad Software, La Jolla, CA, USA).

Significant values were indicated as follows: **P*<.05, ***P*<.01, and ****P*<.001.

3 | RESULTS

3.1 | Acute *H. pylori* infection induced upregulation of CCR2 expression in mouse stomach but deficiency in CCR2 did not prevent DC accumulation in *H. pylori*-infected stomach

To determine whether CCR2 is significantly upregulated after *H. pylori* infection, we measured gastric mucosal mRNA level of CCR2 24 hours after *H. pylori* infection (Figure 1A). A significant increase in CCR2 expression was found in the stomach 24 hours after *H. pylori* infection (Figure 1B). To examine the role of CCR2 on DC recruitment, we measured gastric mucosal CD11c⁺CD103⁺ DC and CD11c⁺CD11b⁺ macrophages during *H. pylori* infection. Acute *H. pylori* infection increased the percentage of DC but not macrophages in the stomach of *H. pylori*-infected mice (Figure 1D). In CCR2KO mice (the lack of CCR2 was verified as shown in the right panels of Figure 1C), there was no significant difference in the percentage of DC after *H. pylori* infection between infected Wt and CCR2KO mice. These data indicate that CCR2 deficiency does not significantly alter DC recruitment to the gastric tissue and may play another role during *H. pylori* infection.

3.2 | CCR2 deficiency increased *H. pylori*-induced gastritis and decreased *H. pylori* colonization

To further explore the role of CCR2 signaling during *H. pylori* infection, Wt and CCR2KO mice were chronically infected for two months with *H. pylori* (Figure 2A). We measured a higher gastritis score (Figure 2B,C) and increased expression of proinflammatory cytokines (eg, TNF-α, IL-1β, IL-12p35, IFN-γ, IL-17A, IL-6) in *H. pylori*-infected CCR2KO mice compared to infected Wt mice (Figure 2D). Next, we examined whether CCR2 signaling during chronic *H. pylori* infection impacts bacterial colonization using quantitative PCR. After two-month infection, we found a lower gastric level of *H. pylori* colonization in CCR2KO mice compared to Wt mice (Figure 2E). These data indicate that CCR2 signaling contributes to *H. pylori* colonization in mouse stomach.

3.3 | CCR2 deficiency skews *H. pylori*-specific Treg/Th17 response toward Th17 Immunity

To further study the mechanisms of how CCR2 deficiency leads to increased *H. pylori* gastritis, we examined the systemic *H. pylori* antigen-specific T-cell responses by ex vivo stimulation of splenic T cells with *H. pylori* antigen and measured T-cell cytokine expression profiles (Figure 3A). In CCR2KO mice, *H. pylori* infection resulted in a Th17 skewed response with lower Treg and Th1 responses (Figure 3B), thus lowering the Treg/Th17 balance resulting in enhanced *H. pylori*-induced inflammation (Figure 3C). This finding suggests that CCR2 regulates the balance of *H. pylori*-specific Treg priming and thus contributes to *H. pylori* tolerance, which in part explains the increased gastritis observed in CCR2KO mice.

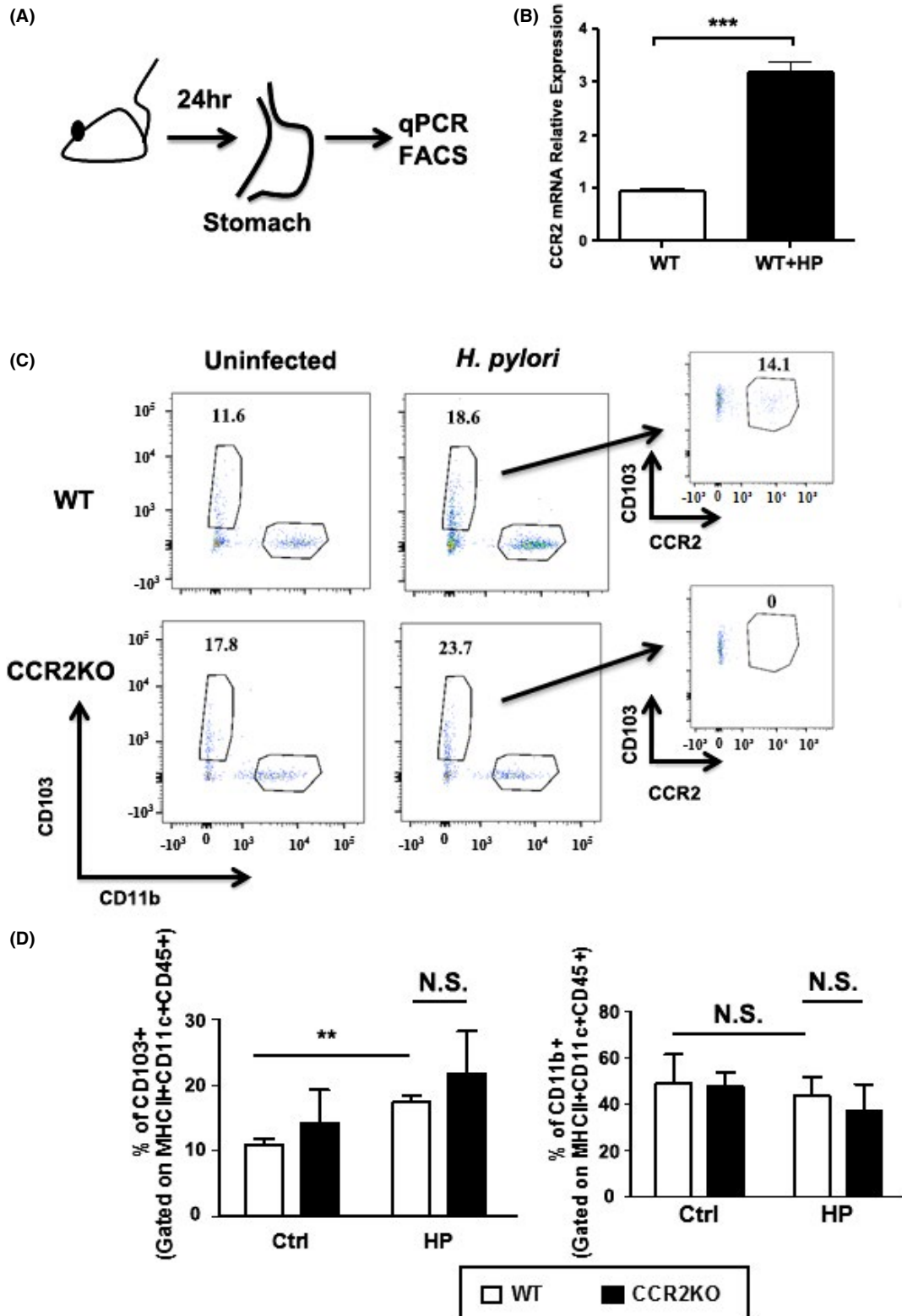


FIGURE 1 Acute *H. pylori* Infection Induced Upregulation of CCR2 Expression in Mouse Stomach but Deficiency in CCR2 Did not Prevent DC Accumulation in *H. pylori*-infected Stomach. A, Diagram of the experimental procedures: Stomachs from uninfected or 24-h infected *H. pylori* C57BL/6 mice were removed and qPCR and FACS analyses were performed to measure the expression of CCR2. B, Total gastric CCR2 relative mRNA expression was increased 24 hours post-*H. pylori* infection. C, Single-cell prep of stomach from uninfected or *H. pylori*-infected C57BL/6 mice were analyzed by FACS using fluorochrome-conjugated anti-mouse antibodies (MHCII, CD11c, and CD45 triple-labeled cells were gated and analyzed for CD103 and CD11b expression). CCR2 expression of CD103-labeled cells was also shown (right panels). D, DC and macrophage recruitment to the gastric tissue during *H. pylori* infection by FACS analysis (gated on CD45-labeled cells). Data are presented as mean \pm SEM, $n=5$. ** $P<.01$ and *** $P<.001$ compared with uninfected mice

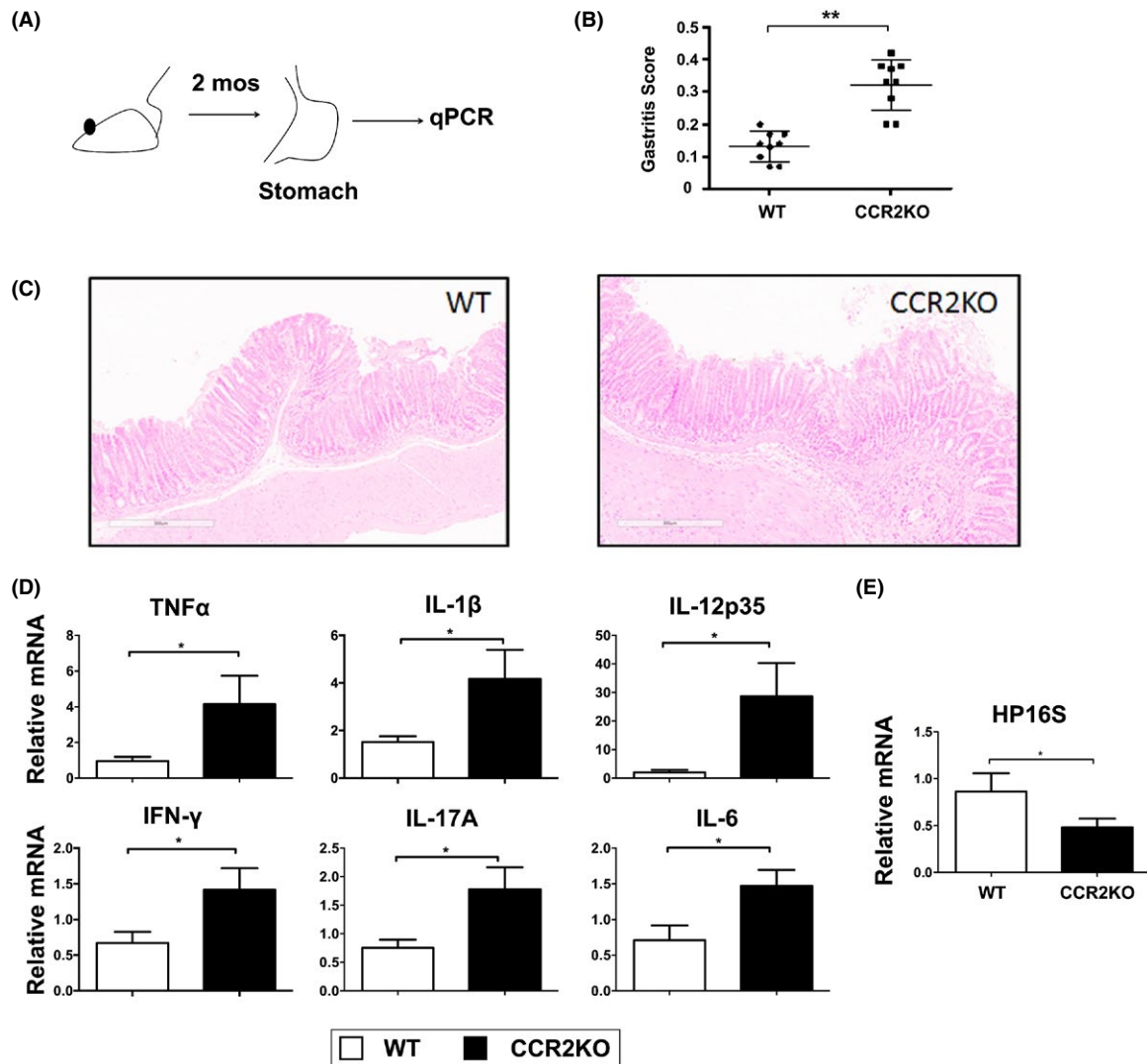


FIGURE 2 CCR2 Deficiency Increased *H. pylori*-Induced Gastritis and Decreased *H. pylori* Colonization. A, Diagram of the experimental procedures: C57BL/6 mice were orally challenged thrice with *H. pylori* SS1 and stomachs from Wt mice and CCR2KO mice were removed after eight weeks. B, Gastritis score was determined in a blinded fashion. C, Micrographs of gastric histology. D, mRNA expression of proinflammatory cytokines relative to GAPDH was measured by qPCR. D, *H. pylori* colonization was determined by quantitative PCR of *H. pylori* 16S. Results are shown as mean \pm SEM, n=6-10 in duplicate. * P <.05 and ** P <.01 compared with Wt mice

3.4 | BMDC derived from CCR2KO mice remained immature and failed to induce *H. pylori*-stimulated Treg differentiation in vitro

Because CCR2 deficiency did not prevent DC recruitment, we hypothesize that CCR2 signaling on DC may impact its function and assessed BMDC function (upregulation of costimulatory molecules, cytokine secretion, and splenic T-cell differentiation) after in vitro *H. pylori* stimulation. Overnight stimulation of BMDC by *H. pylori* revealed a failure of CCR2KO BMDC to upregulate costimulatory molecules CD80 and CD86 and a lower production of proinflammatory cytokines IL-1 β and IL-6 (Figure 4A). Assessment of the function of BMDC in priming *H. pylori*-specific adaptive T-cell responses was performed by coculturing *H. pylori*-stimulated BMDC with naïve splenocytes for 72 hours in a mixed leukocyte reaction. As shown

in Figure 4B,C, CCR2 deficient *H. pylori*-stimulated BMDC have reduced priming of *H. pylori*-specific adaptive T-cell responses most pronounced in Th1 (IFN- γ) and Treg (Foxp3) responses. The Treg/Th17 balance was significantly lowered in CCR2KO BMDC compared to Wt BMDC (Figure 4D). To further investigate whether the observed BMDC dysfunction is a result of defective DC differentiation or DC maturation, neutralizing antibodies to MCP-1, a ligand of CCR2, were added to BMDC culture during *H. pylori* stimulation to disrupt CCR2 signaling. MCP-1 depletion interfered with BMDC maturation indicated by lower surface maturation markers CD80 and CD86 as well as a lower IL-6 production of *H. pylori*-stimulated BMDC in anti-MCP-1 treated group vs nontreated group (Fig. S1). These results bring to light the importance of CCR2 signaling in mediating DC maturation and skewing the Treg/Th17 balance toward Treg dominance favoring *H. pylori* tolerance.

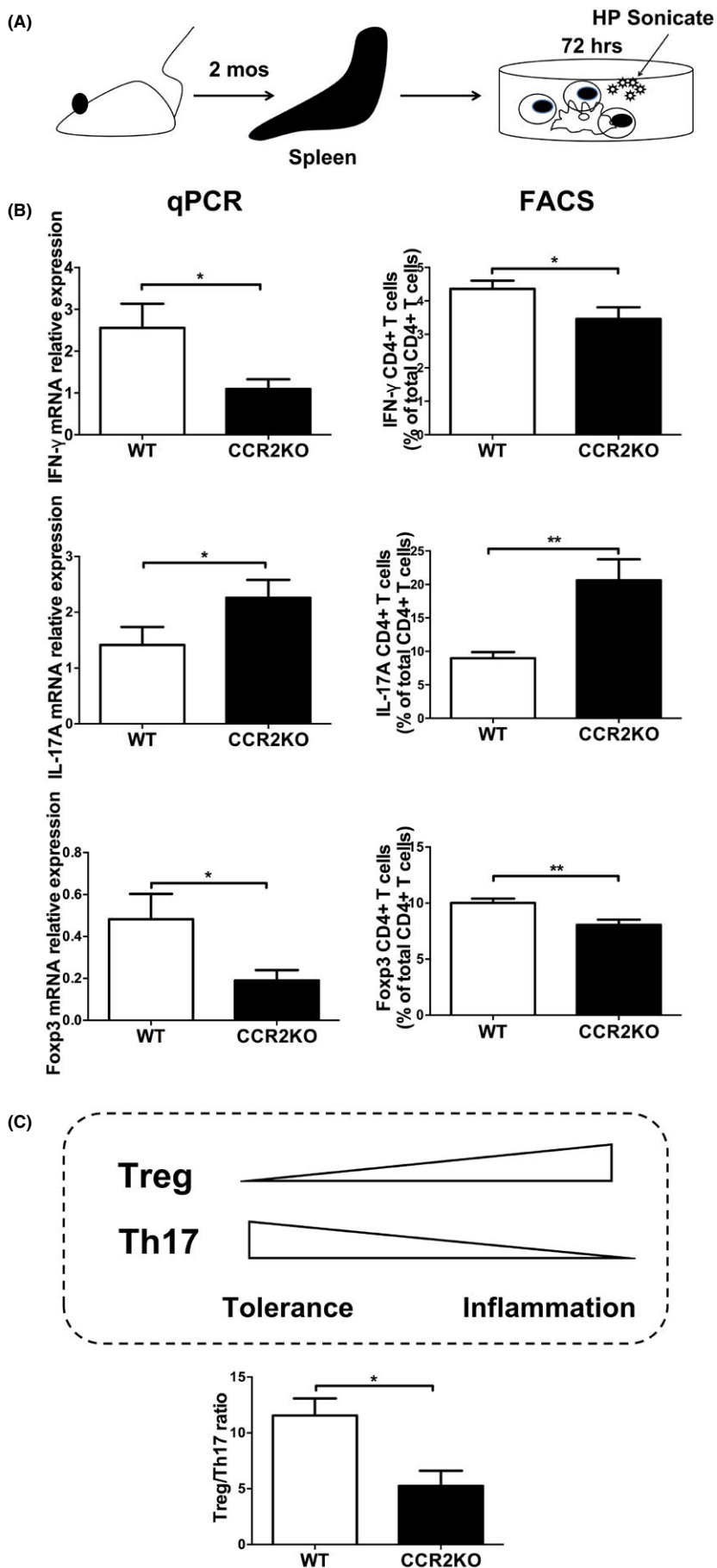


FIGURE 3 CCR2 Deficiency Skews *H. pylori*-Specific Treg/Th17 Response toward Th17 Immunity. A, Diagram of experimental procedure: Splenocytes from Wt mice or CCR2KO mice after two-month *H. pylori* infection were cultured for 72 hours with *H. pylori* sonicate. B, The mRNA expression of Th1 (IFN- γ ⁺), Th17 (IL-17A⁺), and Treg (Foxp3⁺) markers from cocultured splenocytes was determined using qPCR and FACS analysis showing increased IL-17A and decreased IFN- γ and Foxp3 expressions in CCR2KO splenocytes compared to Wt splenocytes. C, The ratio of Treg to Th17 response was calculated using data obtained from FACS (multiplied by 10). Dotted insert illustrates high Treg to Th17 ratio favors *H. pylori* survival and low Treg to Th17 favors *H. pylori* eradication. Data are expressed as mean \pm SEM, n=5 in duplicate. * P <.05 and ** P <.01 in comparison with Wt group

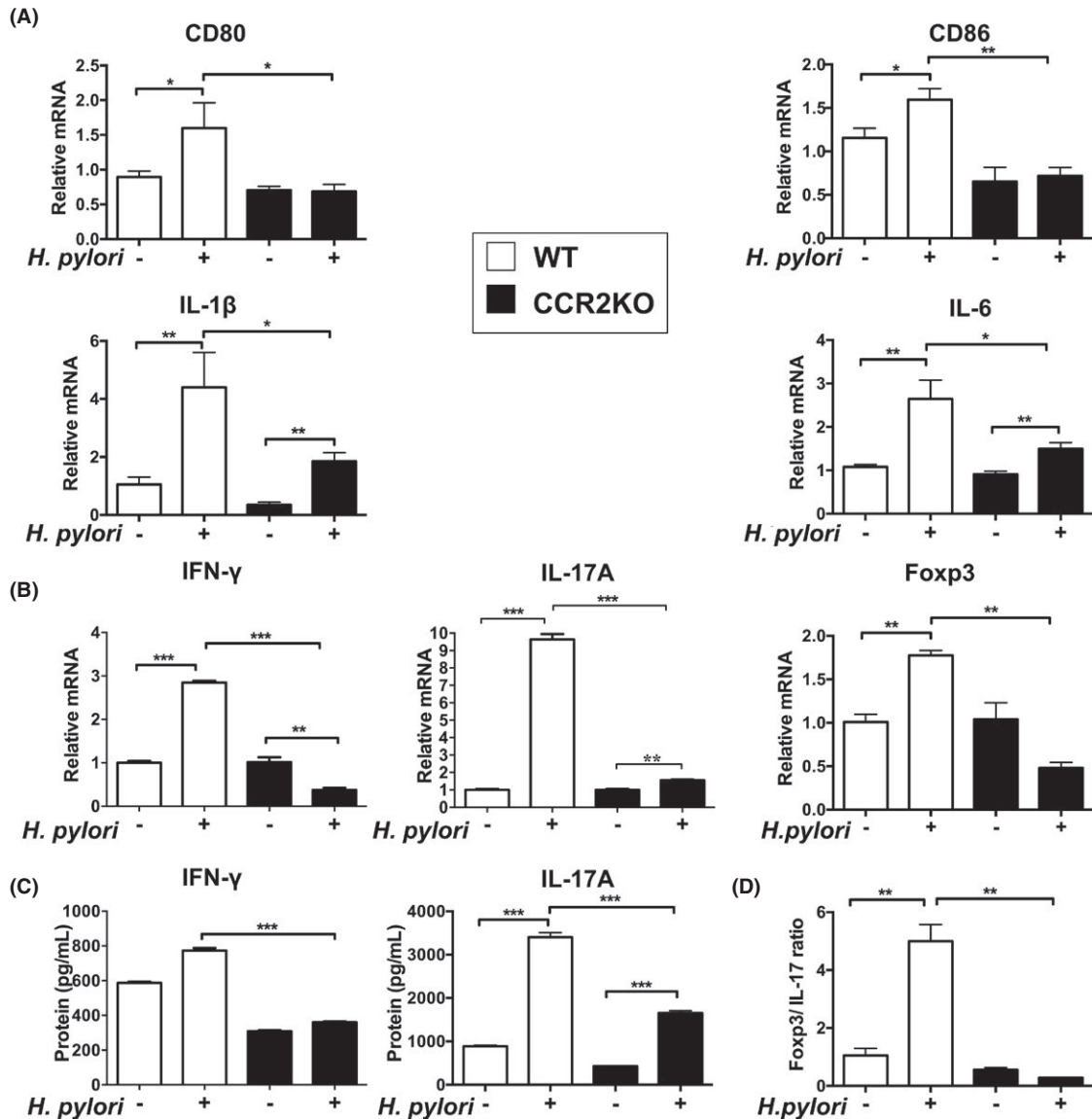


FIGURE 4 BMDC Derived from CCR2KO Mice Remained Immature and Failed to Induce *H. pylori*-stimulated Treg Differentiation in vitro. BMDCs from Wt and CCR2KO mice (10^6 cells/mL) were stimulated with *H. pylori* for 18 hours. Cells and Supernatants were collected, and qPCR and ELISA measured mRNA and protein expression. A, BMDC levels of CD80 and CD86 were not increased, and IL-1 β production and IL-6 production were diminished in CCR2KO vs Wt mice. BMDCs derived from CCR2KO or Wt mice were stimulated with *H. pylori* and then cocultured with naïve Wt splenocytes for 72 hours. The expression of IFN- γ , IL-17A, and Foxp3 was measured by qPCR B, or ELISA C. D, BMDC-induced Treg response was lower in CCR2KO vs Wt mice. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared to Wt mice. Data are presented as mean \pm SEM of three independent experiments

4 | DISCUSSION

The current study investigates the role of CCR2 in *H. pylori*-induced tolerogenic response. We showed that acute *H. pylori* infection was associated with a threefold increase in CCR2 mRNA expression in the gastric mucosa compared to uninfected control mice. CCR2KO mice developed a higher degree of mucosal inflammatory responses and had a lower degree of gastric *H. pylori* colonization with two-month infection compared to infected Wt mice. *H. pylori*-specific immune response in the CCR2KO spleen was characterized by a significant

increase in Th17 response and a decrease in Treg response. We detected recruitment of monocytes in CCR2KO mice and speculated that the decrease in Treg skewing is likely not the result of a lack of dendritic cell trafficking. We then investigated the role of CCR2 signaling on *H. pylori*-induced DC Treg induction and found that CCR2KO BMDC had lower surface expression of costimulatory molecules (CD80 and CD86) expression and a loss of Treg priming function. These findings indicate that CCR2 signaling in DC supports the induction of Treg and innately maintains mucosal homeostasis which may explain the increased severity of gastritis observed in *H. pylori*-infected

CCR2KO mice. Our results indicate that CCR2 plays an essential role in *H. pylori*-induced tolerance and shed light on a novel mechanism of CCR2-dependent DC Treg induction.

The phenotype of DC plays a major role in the initiation of immune responses in that immature DC are believed to induce tolerance to self-antigens, whereas mature DC promote immunity to foreign and self-antigens.²³ Fully mature DCs upregulate costimulatory and adhesion molecules (eg, CD40, CD80, and CD86) and MHC class II antigens, and a higher production of cytokines such as IL-12p70, but decrease antigen uptake and processing,^{24,25} which modulates T-cell responses promoting either Th1-cell or cytotoxic T-cell development. Compared to Wt BMDC, we observed decreased surface expression of CCR2KO BMDC maturation markers CD80 and CD86 after *H. pylori* stimulation, suggesting that CCR2 signaling may be required for DC maturation upon stimulation by bacterial antigens. Moreover, *H. pylori*-stimulated CCR2KO BMDC are incapable of inducing adaptive T-cell responses including Treg. Similar observations have been reported by Chiu BC et al. that DC-derived CCR2 deficient mice had a lower expression of CD40 and MHCII and a lower level of IL-12 with impaired T-cell responses postpathogen challenge in the lungs.²⁶ However, unlike immature DC which induce Treg responses,²⁷ CCR2KO DC are unable to produce a robust Treg response possibly contributing to the lower Treg/Th17 balance observed in CCR2KO mice with increased gastritis.

Although CCR2 has been shown to play a role in CD11c⁺ myeloid cell recruitment to the mucosal tissue sites in prior studies,¹²⁻¹⁶ it was recently found that CCR2 actively recruits monocytes out of bone marrow compartment and does not affect tissue recruitment.²⁸ In our study, we also did not observe a significant difference in DC or monocyte recruitment in *H. pylori*-infected CCR2KO and Wt mice. Our in vitro studies did reveal a novel role of CCR2 signaling in DC maturation and Treg priming which are necessary for maintenance of homeostasis.

In conclusion, our findings show that *H. pylori* infection in CCR2KO mice exhibits a lower *H. pylori*-specific Treg response resulting in increased gastritis and reduced *H. pylori* colonization. We also found that CCR2 signaling is essential for DC maturation and function, which are critical for *H. pylori* host immune escape. Our study demonstrates a novel mechanism of the pathogenesis of *H. pylori* infection in which CCR2 signaling represents an important target for the modulation of the host response to *H. pylori*.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

JYK involved in study concept and design; analysis and interpretation of data; study supervision; and obtained funding; XS involved in drafting of the manuscript; performed experiments; and analysis and interpretation of data; and MZ, ME and GBH assisted with experimental design and data interpretation.

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SUPPORTING INFORMATION

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