Received Date : 23-Sep-2016

Revised Date : 31-Oct-2016

Accepted Date : 09-Nov-2016

Article type : Original Article

Author Manuscript

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

Xia Sun[1, 2*], Min Zhang[2], Mohamad El-Zataari[2], Gray B. Huffnagle[3], John Y. Kao[2*]

[1]Department of Pharmacology, School of Medicine, Shandong University, Jinan, Shandong, 250012, China

[2]Department of Internal Medicine, Division of Gastroenterology, University of Michigan Health System, Ann Arbor, Michigan, 48109, United States

[3]Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, University of Michigan Health System, Ann Arbor, Michigan, 48109, United States

***Corresponding authors:**

John Y. Kao, M.D, 6520A MSRB I, SPC 5682, 1150 West Medical Center Drive, Department of Internal Medicine, Division of Gastroenterology, University of Michigan Health System, Ann Arbor, Michigan, 48109, United States. Tel: (734) 647-2964, Fax: (734)-763-2535, E-mail: jykao@med.umich.edu.

Xia Sun, Ph.D., Room 8308, Building 8, School of Medicine, Shandong University, 44 Wen Hua Xi Road, Jinan, Shandong, 250012, China. Tel: +86 (531) 88382605, Fax: +86 (531)

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/hel.12366

This article is protected by copyright. All rights reserved

88382502, E-mail: sunxia@sdu.edu.cn.

Running title

CCR2 is Essential for *H. pylori*-induced Immune Tolerance in Mice

Keywords

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

Abstract

Background: We previously demonstrated that *H. pylori* infection leads to increased induction of regulatory T cells (Treg) in local and systemic immune compartments. Here we investigate the role of CCR2 in the tolerogenic programing of dendritic cells (**DC**) 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 (BMDC) on day 6 from CCR2KO and Wt mice co-cultured with or without *H. pylori* were examined to determine the impact of CCR2 signaling on DC function by qPCR, ELISA, and FACS analyses.

Results: Acute *H. pylori* infection was associated with a 3-fold increase in CCR2 mRNA expression in the gastric mucosa. *H. pylori*-infected CCR2KO mice exhibited a higher degree of mucosal inflammation, i.e. increased gastritis scores and pro-inflammatory cytokines 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 BMDC was less mature and shown a lower Treg/Th17 ratio. Moreover, blockade of CCR2 signaling by MCP-1 neutralizing antibody inhibited *H. pylori*-stimulated BMDC 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.

Introduction

Author Manuscript

This article is protected by copyright. All rights reserved

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 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) or 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) was less mature and had

This article is protected by copyright. All rights reserved

Author Manuscript

defective T cell priming function characterized by a lower Treg/Th17 response ratio, similar to that observed in 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. pylor*i-specific Treg response and may represent a **major** target in the modulation of the host response to *H. pylor*i.

Materials and Methods

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 were used between 8-10 weeks of age. 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).

Bacterial Strains and Culture Condition

H. pylori SS1 strain was cultured on *Campylobacter*-selective agar (BD Diagnostics, Bedford, MA) 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 10^9 bacteria suspended in 100µl of Brucella broth.

Generation and Stimulation of bone marrow-derived dendritic cells (BMDC)

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 (10^6 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 10^7

This article is protected by copyright. All rights reserved

Author Manuscript

CFU/ml of *H. pylori or* PBS for 18h and then co-culture with syngeneic splenocytes for 72h in a mixed leukocyte reaction (splenocyte: BMDC ratio of 10:1) as previously described (19).

Animal Studies

For acute infection, Wt and CCR2KO mice were infected for 24h with 10^9 CFU/ml *H. pylori* SS1 via oral gavage. For chronic infection, mice were gavaged with *H. pylori* SS1 (3 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 was cut along the greater curvature, and 2mm 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 co-cultured for 72h with BMDC from Wt mouse. The splenocyte-to-BMDC ratio was 10 to 1. After 72h, splenocytes were collected, and the percentages and mRNA expression of IFN-γ, IL-17A and Foxp3 were measured by FACS and qPCR, respectively.

Gastritis Score Determination

After 2-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 histological analysis (**100x** 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 histological slide** (20).

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, IL-10, IFN-γ, IL-17A, Foxp3, and GAPDH, were measured using iQ™ SYBR Green Supermix Kit obtained from BIO-RAD. Primers are shown in **Table 1**. Finally,

Author Manuscript

This article is protected by copyright. All rights reserved

quantitation of relative differences in expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method (21).

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 pre-incubated 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% NaN_3) for 30 min **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).

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). Significant values were indicated as follows: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Results

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 24h after *H. pylori* infection (**Fig. 1A**). A significant increase in CCR2 expression was found in the stomach 24h after *H. pylori* infection (**Fig. 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

Author Manuscript

This article is protected by copyright. All rights reserved

of *H. pylori*-infected mice (**Fig. 1D**). In CCR2KO mice (the lack of CCR2 was verified as shown in the right panels of **Fig. 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 that CCR2 deficiency does not significantly alter DC recruitment to the gastric tissue and may play another role during *H. pylori* infection.

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

CCR2 Deficiency Skews *H. pylori* Specific Treg/Th17 Response towards 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 (**Fig. 3A**). In CCR2KO mice, *H. pylori* infection resulted in a Th17 skewed response with lower Treg and Th1 responses (**Fig. 3B**), thus lowering the Treg/Th17 balance resulting in enhanced *H. pylori*-induced inflammation (**Fig. 3C**). This finding suggests that CCR2 regulates the balance of *H. pylori*-specific Treg priming thus contributes to *H. pylori* tolerance, which in part explains the increased gastritis observed in CCR2KO mice.

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 co-stimulatory molecules, cytokine secretion, and splenic T cell differentiation) after in vitro

This article is protected by copyright. All rights reserved
Author Manuscript

H. pylori stimulation. Overnight stimulation of BMDC by *H. pylori* revealed a failure of CCR2KO BMDC to upregulate co-stimulatory molecules CD80 and CD86 and a lower production of proinflammatory cytokines IL-1β and IL-6 (**Fig. 4A**). Assessment of the function of BMDC in priming *H. pylori*-specific adaptive T cell responses was performed by co-culturing *H. pylori*-stimulated BMDC with naïve splenocytes for 72h in a mixed leukocyte reaction. As shown **in** **Fig. 4B** and **4C**, CCR2 deficient *H. pylori*-stimulated BMDC have reduced priming of *H. pylori*-specific adaptive T cell responses most pronounce in Th1 (IFN-γ) and Treg (Foxp3) responses. The Treg/Th17 balance was significantly lowered in CCR2KO BMDC compared to Wt BMDC (**Fig. 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 a 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 non-treated group (**Supplementary Fig. 1**). 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.

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 3-fold 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 2-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

This article is protected by copyright. All rights reserved

Author Manuscript

signaling on *H. pylori*-induced DC Treg induction and found that CCR2KO BMDC had lower surface expression of co-stimulatory 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 DC upregulate co-stimulatory and adhesion molecules (e.g., 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 BMDCs 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 post-pathogen 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

This article is protected by copyright. All rights reserved

Author Manuscript

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.*

Author contributions

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

Acknowledgments and Disclosures

This work was primarily supported by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health under award number R01 DK087708-01 (JYK). Additional funding obtained by XS includes the National Natural Science Foundation, China (81302770), the Postdoctoral Science Foundation, China (2014M551917), and Postdoctoral Innovation Project, Shandong, China (201402029).

Competing interests: The authors declare that they have no competing interests.

References

1. Perez-Perez GI, Dworkin BM, Chodos JE, Blaser MJ. Campylobacter pylori antibodies in humans. Ann Intern Med. 1988 Jul 1;109(1):11-7.

This article is protected by copyright. All rights reserved

Author Manuscript

2. Suerbaum S, Michetti P. Helicobacter pylori infection. N Engl J Med. 2002 Oct;347(15):1175-86.

3. Lundgren A, Suri-Payer E, Enarsson K, Svennerholm AM, Lundin BS. Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to H. pylori in infected individuals. Infection and immunity. 2003 Apr;71(4):1755-62.

4. Raghavan S, Suri-Payer E, Holmgren J. Antigen-specific in vitro suppression of murine Helicobacter pylori-reactive immunopathological T cells by CD4CD25 regulatory T cells. Scandinavian journal of immunology. 2004 Jul-Aug;60(1-2):82-8.

5. Lundgren A, Stromberg E, Sjoling A, Lindholm C, Enarsson K, Edebo A, et al. Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in Helicobacter pylori-infected patients. Infection and immunity. 2005 Jan;73(1):523-31.

6. Rad R, Brenner L, Bauer S, Schwendy S, Layland L, da Costa CP, et al. CD25+/Foxp3+ T cells regulate gastric inflammation and Helicobacter pylori colonization in vivo. Gastroenterology. 2006 Aug;131(2):525-37.

7. Kandulski A, Wex T, Kuester D, Peitz U, Gebert I, Roessner A, et al. Naturally occurring regulatory T cells (CD4+, CD25high, FOXP3+) in the antrum and cardia are associated with higher H. pylori colonization and increased gene expression of TGF-beta1. Helicobacter. 2008 Aug;13(4):295-303.

8. Harris PR, Wright SW, Serrano C, Riera F, Duarte I, Torres J, et al. Helicobacter pylori gastritis in children is associated with a regulatory T-cell response. Gastroenterology. 2008 Feb;134(2):491-9.

9. Kao JY, Zhang M, Miller MJ, Mills JC, Wang B, Liu M, et al. Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. Gastroenterology. 2010 Mar;138(3):1046-54.

10. Kao JY, Rathinavelu S, Eaton KA, Bai L, Zavros Y, Takami M, et al. Helicobacter pylori-secreted factors inhibit dendritic cell IL-12 secretion: a mechanism of ineffective host defense. Am J Physiol Gastrointest Liver Physiol. 2006 Jul;291(1):G73-81.

Author Manuscript

This article is protected by copyright. All rights reserved

11. Fiorina P, Jurewicz M, Vergani A, Augello A, Paez J, Ricchiuti V, et al. Phenotypic and functional differences between wild-type and CCR2-/- dendritic cells: implications for islet transplantation. Transplantation. 2008 Apr;85(7):1030-8.

12. Bruhl H, Cihak J, Schneider MA, Plachy J, Rupp T, Wenzel I, et al. Dual role of CCR2 during initiation and progression of collagen-induced arthritis: evidence for regulatory activity of CCR2+ T cells. Journal of immunology. 2004 Jan 15;172(2):890-8.

13. Quinones MP, Estrada CA, Kalkonde Y, Ahuja SK, Kuziel WA, Mack M, et al. The complex role of the chemokine receptor CCR2 in collagen-induced arthritis: implications for therapeutic targeting of CCR2 in rheumatoid arthritis. Journal of molecular medicine. 2005 Sep;83(9):672-81.

14. Okamoto M, Fuchigami M, Suzuki T, Watanabe N. A novel C-C chemokine receptor 2 antagonist prevents progression of albuminuria and atherosclerosis in mouse models. Biol Pharm Bull. 2012;35(11):2069-74.

15. D'Ambrosio D, Panina-Bordignon P, Sinigaglia F. Chemokine receptors in inflammation: an overview. J Immunol Methods. 2003 Feb;273(1-2):3-13.

16. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med. 2006 Feb;354(6):610-21.

17. Kurihara T, Warr G, Loy J, Bravo R. Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. J Exp Med. 1997 Nov;186(10):1757-62.

18. Sato N, Ahuja SK, Quinones M, Kostecki V, Reddick RL, Melby PC, et al. CC chemokine receptor (CCR)2 is required for langerhans cell migration and localization of T helper cell type 1 (Th1)-inducing dendritic cells. Absence of CCR2 shifts the Leishmania major-resistant phenotype to a susceptible state dominated by Th2 cytokines, b cell outgrowth, and sustained neutrophilic inflammation. J Exp Med. 2000 Jul;192(2):205-18.

19. Zhang M, Berndt BE, Eaton KA, Rathinavelu S, Pierzchala A, Kao JY. Helicobacter pylori-pulsed dendritic cells induce H. pylori-specific immunity in mice. Helicobacter. 2008 Jun;13(3):200-8.

Author Manuscript

This article is protected by copyright. All rights reserved

20. Eaton KA, Danon SJ, Krakowka S, Weisbrode SE. A reproducible scoring system for quantification of histologic lesions of inflammatory disease in mouse gastric epithelium. Comparative medicine. 2007 Feb;57(1):57-65.

21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.

22. Dillon S, Agrawal S, Banerjee K, Letterio J, Denning TL, Oswald-Richter K, et al. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. J Clin Invest. 2006 Apr;116(4):916-28.

23. Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. 1991;9:271-96.

24. Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat Immunol. 2000 Oct;1(4):311-6.

25. Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. Nat Immunol. 2000 Sep;1(3):199-205.

26. Chiu BC, Freeman CM, Stolberg VR, Hu JS, Zeibecoglou K, Lu B, et al. Impaired lung dendritic cell activation in CCR2 knockout mice. Am J Pathol. 2004 Oct;165(4):1199-209.

27. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. J Exp Med. 2000 Nov 6;192(9):1213-22.

28. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. Nat Immunol. 2006 Mar;7(3):311-7.

Author Manuscript

This article is protected by copyright. All rights reserved

Author Manuscript

Figure Legends

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 24h 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 24h 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 ± SEM, n=5. $^{**}P < 0.01$ and $^{***}P < 0.001$ 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

This article is protected by copyright. All rights reserved

removed after 8 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 ± SEM, n=6-10 in duplicate. *$P < 0.05$ and **$P < 0.01$ compared with Wt mice.

Figure 3. CCR2 Deficiency Skews *H. pylori* Specific Treg/Th17 Response towards Th17 Immunity. A) Diagram of experimental procedure: splenocytes from Wt mice or CCR2KO mice after 2-month *H. pylori* infection were cultured for 72h with *H. pylori* sonicate. B) The mRNA expression of Th1 (IFN-γ^+), Th17 (IL-17A$^+$) and Treg (Foxp3$^+$) markers from co-cultured splenocytes were 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 ± SEM, n = 5 in duplicate. *$P < 0.05$ and **$P < 0.01$ 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. BMDC from Wt and CCR2KO mice (10^6 cells/ml) were stimulated with *H. pylori* for 18h. 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-1β and IL-6 production **were** diminished in CCR2KO vs. Wt mice. BMDC derived from CCR2KO or Wt mice were stimulated with *H. pylori* and then co-cultured with naïve Wt splenocytes for 72h. The expression of IFN-γ, IL-17A and Foxp3 were measured by qPCR (B) or ELISA (C). D) BMDC induced Treg response was lower in CCR2KO vs. Wt mice. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to Wt mice. Data are presented as mean ± SEM of three independent experiments.

Supplementary Figure 1. Neutralization of MCP-1 inhibited BMDC maturation. BMDC from Wt mice (10^6 cells/ml) were stimulated with *H. pylori* in the presence or absence of MCP-1 neutralization antibodies for 18h and BMDC CD80 and CD86 were

This article is protected by copyright. All rights reserved

Author Manuscript

measured by FACS and protein expression of IL-1β and IL-6 were measured by ELISA (*P < 0.05).

Author Manuscript

This article is protected by copyright. All rights reserved

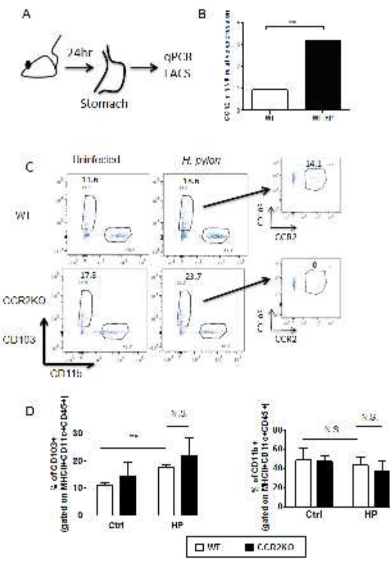
Table 1. Primers used for the amplification of each gene.

Gene	Primer (5′- 3′)
GAPDH	F:5′-TCAAGAAGGTGGGTGAAGCAGG-3′ R:5′-TATTATGGGGGTCTGGGATGG-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′-AGCTTTCCCTCCGCATTGA-3′
IL-1β	F:5′-CCAACTGGTACA TCAGCAC-3′ R:5′-TCTGCTCATTCACGAAAA GG-3′
TNF-α	F:5′-CCCACTCTGACCCCTT TACT-3′ R:5′-TTTGAGTCCTTGATGGTG GT-3′
IL-10	F:5′-AGTGGAGCAGGTGAAGAGTG-3′ R:5′-TTCGGAGAGAGGTACAAACG-3′
IL-12p35	F: 5′-ACGGCCAGGAAAAACTGAA-3′ R:5′-CTACCAAGGCACAGGGTGCAT-3′
IFN-γ	F:5′-TCAAGTGGCATAGATGTGGAAGAA-3′ R:5′-TGGCTCTGCAGGATTTTCATG-3′
Foxp3	F:5′-TCTCCAGGTTGCTCAAAGTC-3′ R:5′-GCAGAAGTTGCTGCTTTAGG-3′
HP 16s	F:5′-CAAGTCATGGCCCTTAC-3′ R:5′-TTGCGATTACTAGCGATTCC-3′

Author Manuscript

This article is protected by copyright. All rights reserved

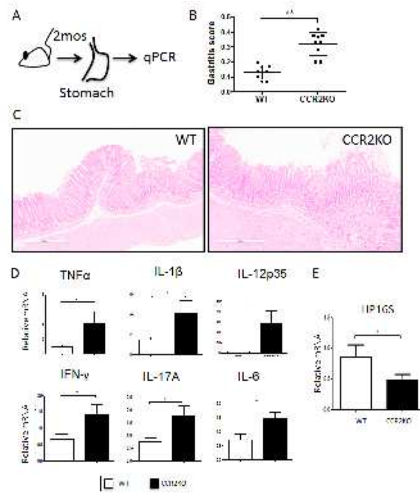
Author Manuscript



hel_12366_f1.tif

This article is protected by copyright. All rights reserved

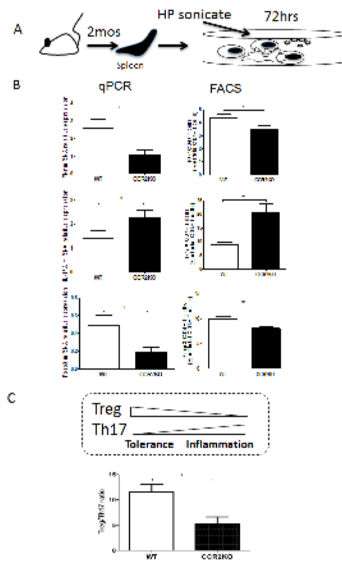
Author Manuscript



hel_12366_f2.tif

This article is protected by copyright. All rights reserved

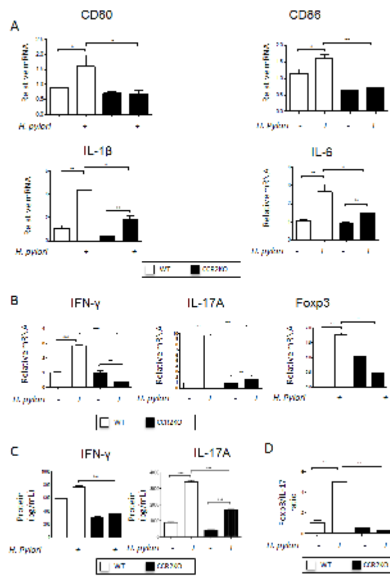
Author Manuscript



hel_12366_f3.tif

This article is protected by copyright. All rights reserved

Author Manuscript



hel_12366_f4.tif

This article is protected by copyright. All rights reserved