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The homing receptor CD44 is involved in the progression of precancerous gastric lesions in patients infected with *Helicobacter pylori* and in development of mucous metaplasia in mice

Jone Garay¹, M. Blanca Piazuelo², Sumana Majumdar¹, Li Li¹, Jimena Trillo-Tinoco¹, Luis del Valle^{1,3}, Barbara G. Schneider², Alberto G. Delgado², Keith T. Wilson², Pelayo Correa², and Jovanny Zabaleta^{1,4,*}

¹Stanley S. Scott Cancer Center, LSUHSC, New Orleans, LA

²Division of Gastroenterology, Hepatology, and Nutrition, Department of Medicine, Vanderbilt University Medical Center

³Department of Pathology, LSUHSC, New Orleans

⁴Department of Pediatrics, LSUHSC New Orleans

Abstract

Infection with *Helicobacter pylori* (*H. pylori*) leads to inflammatory events that can promote gastric cancer development. Immune cells transition from the circulation into the infected mucosa through the interaction of their receptors and ligands in the endothelial compartment. CD44 expression is increased in advanced gastric lesions. However, the association of this molecule with the progression of these lesions over time has not been investigated. In addition, there is a lack of understanding of the CD44-dependent cellular processes that lead to gastritis, and possibly to gastric cancer. Here we studied *H. pylori*-positive subjects with gastric lesions that ranged from multifocal atrophic gastritis to dysplasia to determine gene expression changes associated with disease progression over a period of six years. We report that *CD44* expression is significantly increased in individuals whose gastric lesions progressed along the gastric precancerous cascade. We also show that *CD44*^{-/-} mice develop less severe and less extensive *H. pylori*-induced metaplasia, and show fewer infiltrating Gr1⁺ cells compared to wild type mice. We present data suggesting that CD44 is associated with disease progression. Mechanisms associated with these effects include induction of interferon gamma responses.

*To whom correspondence should be sent: Jovanny Zabaleta, M.S., Ph.D, Assistant Professor, Departments of Pediatrics and Genetics, Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, Louisiana Cancer Research Center, 1700 Tulane Ave, Room 909, New Orleans, LA, 70112, Phone 504-210 2979, Fax 504-210 2970, jzabal@lsuhsc.edu.

Conflict of interest

None

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Keywords

Inflammation; gastritis; gastric cancer; CD44

1. Introduction

Gastric cancer (GC) is one of the most common and lethal cancers worldwide. In the United States, more than 20,000 new cases of GC and more than 10,000 deaths attributable to the disease are expected in 2015 [1]. Several factors have been associated with the disease, including race/ethnicity, genetic and environmental factors, gender, and age [1–6]. However, infection with *Helicobacter pylori* (*H. pylori*) has been described as the strongest factor associated with risk of intestinal type gastric adenocarcinoma, such that the International Agency for Research in Cancer (IARC) has classified *H. pylori* as a Type I carcinogen [7]. Infection with *H. pylori* usually occurs early in life [8] and persists, in most cases, without causing any major complications to the human host [9]. However, the infection may lead to a cascade of inflammatory events that trigger the transformation of the normal gastric mucosa into non-atrophic gastritis (NAG), followed by multifocal atrophic gastritis without intestinal metaplasia (MAG), intestinal metaplasia (IM), dysplasia, and finally cancer [10;11]. Despite the high prevalence of *H. pylori* infection, it is estimated that 1% of infected people will develop non-cardia gastric cancer [12].

Studies of human gastric samples and mice infected with mouse-adapted *H. pylori* indicate a major role of the immune response in this process [13–15]. We have shown that single nucleotide polymorphisms (SNPs) and SNP haplotypes may be associated with differential risk of more advanced precancerous gastric lesions in certain populations [16–18]. The inflammatory process, in general, involves cellular activation, migration, and infiltration into the inflamed site through the interaction between cellular receptors and ligands on the endothelium (reviewed in [19]). Selectins are major endothelial ligands induced in response to inflammation [20], and of these, E-selectins seem to be especially induced by *H. pylori* infection [21]. CD44, a cell-adhesion molecule expressed on a great variety of cell types including leukocytes and epithelial cells, has been shown to participate in the migration of inflammatory cells [22–26]. CD44 is a glycoprotein that binds mainly hyaluronan in the extracellular matrix [27], but also other matrix components including collagen, fibronectin, osteopontin and growth factors [28–30]. The most common form of CD44 is of 80–100kD but several other variants generated by splicing and posttranslational modifications are also expressed in various cell types [31;32]. In the gastric epithelium, CD44 has been associated with cell progenitors within the isthmus, which actively proliferate in response to *H. pylori*- or tamoxifen-induced atrophy [33]. In gastric tissues CD44 expression is weaker in intestinal metaplasia and grows stronger in dysplasia, intramucosal carcinoma and invasive carcinomas [34]. Additionally, 92% of intestinal-type gastric cancers expressed one splice variant of the CD44 molecule (CD44v6) [35]. Interestingly, CD44v4 has been associated with increased migration in tumor cells through endothelial monolayers by interacting with E-selectin [36].

The association of epithelial CD44 in gastric disease progression in a cohort of individuals has not been previously investigated, nor have the mechanisms leading to development of

precancerous lesions in response to *H. pylori* in *CD44*⁺ or *CD44*^{-/-} cells. Here we show that *CD44* expression in the gastric mucosa increased over time in individuals who progressed to more advanced precancerous gastric lesions over time. We also show that gastritis progression over time is associated with increased expression of *CD44v4* at baseline. *In vitro* experiments show that *H. pylori* induces the expression of this marker. *In vivo* models of *H. pylori* infection indicate that *CD44* is involved in the development of mucous metaplasia, a process that, according to our data, is driven by interferon-gamma (IFN- γ) responses and differential infiltration of Gr1⁺ cells into the infected gastric mucosa.

2. Materials and methods

2.1. Patient description and microarray analysis

All patients were from an area of high incidence of gastric cancer in the southwest region of Colombia [37;38]. Inclusion criteria have been previously reported and included the presence of MAG or IM, but otherwise in good health, with no major diseases, i.e. cancer [38]. All patients signed a consent form for their participation in the study and the unrestricted use of their biological samples. The study was approved by the Institutional Review Board of Louisiana State University Health Sciences Center and the Committees on Ethics of Universidad del Valle and Hospital Departamental de Nariño in Colombia [38]. For the present study, gastric mucosa biopsy samples at baseline and at 6-year follow-up were compared. Four endoscopic biopsies (two from antrum, one from *incisura angularis* and one from corpus) were obtained at each time point, and the more advanced histological lesion observed in each set of biopsies was considered the diagnosis. Diagnoses were considered from less to more advanced in the following order: MAG, IM, and dysplasia. Subjects were assigned to three different groups based on the evolution of the gastric pathology at 6 years, as follows: the “No change”, similar diagnosis at baseline and at follow-up; “Regression”, milder gastritis at follow-up; and “Progression”, more advanced lesions over time.

A random sample of 39 subjects was selected for this study among subjects that were *H. pylori*-positive at baseline, including 11 subjects with no change in diagnosis, 12 with regression and 16 with progression. Baseline biopsy samples and their corresponding 6-year follow-up paired samples containing the most advanced lesion in each set of biopsies were used for microarray analysis. Total RNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues using the RecoverAll Total Nucleic Acid Isolation from Ambion (Austin, TX), in five 10–20 μ m-thick tissue sections, as recommended by the vendor. The RNA was resuspended in DEPC-water and quantified by NanoDrop (ThermoFisher Scientific, Waltham, MA). The suitability of the RNA samples was tested by amplifying the gene *RPL13A* using SYBR green (Life Technologies, Foster City, CA), as recommended by the vendor of the microarray kits (Illumina Inc, San Diego, CA). Briefly, 1 μ l cDNA was mixed with 5 μ l SYBR green Master Mix, and 250 nM each of forward and reverse primers (Forward 5'-GTACGCTGTGAAGGCATCAA-3'; reverse 5'-GTTGGTGTTTCATCCGCTTG-3') in a total volume of 10 μ l and subjected to amplification for 40 cycles at 95°C for 15 sec and 60°C for 2 min. This verification step is required to determine if replicates of the samples are necessary, based on their quality, as recommended

by Illumina, as follows: the best reproducibility is found in those samples with a threshold cycle (Ct) of 28 or less, and larger variability in those with Ct values greater than 28 for which technical replicates are mandatory.

For the analysis of the gene profiles, we used the focused DASL (cDNA-mediated Annealing, Selection, extension and Ligation) Cancer Panel Array (Illumina), which allows the profiling of 502 gene transcripts in RNA extracted from FFPE tissues. Briefly, 200 ng of RNA were used to make biotin-labeled cDNA which was later hybridized to bead chips. After hybridization, the chips were washed and scanned in the Illumina BeadArray Scanner to record the intensity of the fluorescence emitted. Before the analysis, the nonspecific binding was removed using the “Detection P value” algorithm that removes background signals based on the emission of negative probes (lacking targets in the human genome but thermodynamically similar to the regular probes). The signal was normalized using the “cubic spline algorithm”, assuming that the distribution of transcripts is similar among samples of the same group. Differential gene expression analysis was done separately per outcome group (No change, Regression or Progression) using the baseline values as reference and the “Illumina Custom Algorithm” that assumes that the intensity of the signals is normally distributed among all replicates under the same condition, as well as multiple testing corrections using the Benjamini and Hochberg false discovery rate (FDR). We then identified the genes that showed statistically significant differences between the baseline and the follow-up signals. The log₁₀ of the ratio between each one of the samples and one internal calibrator (the average signals in all the samples) was obtained to build heat maps, as recommended previously [39;40]. Gene microarray data reported in this paper has been uploaded to the Gene Expression Omnibus (GEO) under reference number GSE69146.

2.2. Bacterial strain and co-culture conditions

H. pylori strain 26695 was used for co-culture experiments. Bacteria were grown for 3 days in CDC anaerobic agar plates supplemented with 5% sheep blood (BD Diagnostics, Sparks, MD) under microaerobic conditions using a Campy Pouch system (BD Diagnostics). Bacteria cultures were harvested and resuspended in PBS. AGS gastric epithelial cells (ATCC CRL-1739, Rockville, MD) were cultured in F-12 medium with 10% FBS at 37°C in an atmosphere of 5% CO₂. For *H. pylori* co-culture experiments, 1 × 10⁶ AGS cells were seeded into 6-well plates containing 2 ml fresh F-12 supplemented with 3% heat-inactivated FBS and cultured for 8 h. The medium was replaced with 2 ml fresh F-12 containing 3% heat-inactivated FBS before inoculation of *H. pylori* at a multiplicity of infection (MOI) of 20:1. The infected cells were cultured for additional 16 h after which RNA was extracted for real-time PCR analysis.

2.3. Harvest of peritoneal macrophages with thioglycollate

Peritoneal macrophages (PM) from six-to-eight-week old specific pathogen-free female *CD44*^{-/-} mice (Jackson Laboratories, Bar Harbor ME, stock 005085) and PM from the corresponding wild type (WT) controls (Jackson Laboratories, stock 00664) were collected by peritoneal lavage with PBS three days after the peritoneal injection of thioglycollate (Becton Dickinson, Sparks, MD). Macrophages were seeded onto 6-well plates at 1 × 10⁶ per well in complete RPMI (containing 10% FBS, antibiotics, L-glutamine and 25mM

HEPES). After an overnight incubation, the macrophages were infected with the mouse-adapted Sydney strain *H. pylori* (SS1)[41] at MOI of 20:1 (bacteria:macrophage) for 16 h. Brucella broth was added to control PM. The RNA was extracted with Trizol reagent (Life Technologies) as recommended by the manufacturer; this RNA was later used to generate double-stranded cDNA that served as template to make biotin-labeled RNA which was then used for microarray analysis following the manufacturer's instructions (Illumina). The processing of the data was done as described above for human samples. The analysis was done separately for WT and *CD44*^{-/-} PM. We used the "differential expression" algorithm and identified the genes that were significantly different in *H. pylori*-infected vs non-infected controls (in WT and *CD44*^{-/-} separately). Metacore software (Thomson Reuters, Philadelphia, PA) was used to enrich for unique and common pathways in infected cells from WT and *CD44*^{-/-} mice, with the non-infected cells as controls, as previously described [39].

2.4. Real-time PCR analysis

Results from both human and mice microarray analyses were confirmed by real-time PCR as follows: the RNA was converted into cDNA using SuperScript III as recommended by the vendor (Life Technologies) and subjected to real-time PCR using Assays-on-Demand Taqman probes (Life Technologies; human primer-probe set # Hs00153304_m1; mouse primer-probe set # Mm01277163_m1). We compared the signal in the combined "No change" and "Regression" groups versus the "Progression" group. The fold induction of the genes was determined by the 2^{-Ct} method using *GAPDH* as the housekeeping gene and an internal control for normalization.

2.5. Mice infection and processing of stomach tissues

Six- to eight-week-old specific pathogen-free female *CD44*^{-/-} and WT mice were inoculated by gavage for three consecutive days, with 200 μ l of Brucella broth containing 1×10^8 colony forming units (c.f.u) of *H. pylori* SS1, or with broth only. Mice were fed the Teklad Global rodent diet (2019S) (Harlan Laboratories, Indianapolis, IN) and water *ad libitum* in accordance with the guidelines of LSUHSC Institutional Animal Care and Use Committee. Seven months after inoculation, mice were euthanized by CO₂ inhalation and their stomachs removed, opened along the greater curvature, cut longitudinally into strips, fixed with 10% neutral-buffered formalin, and embedded in paraffin as recommended [42;43].

2.6. Histologic evaluation of mouse gastric mucosa

Hematoxylin- and eosin-stained sections were assessed blindly for pathological changes. Acute and chronic inflammatory infiltration was graded from 0 to 3 in the antrum and corpus independently, using a scoring system based on the updated Sydney System [44]. Mucous metaplasia was scored from 0 to 3 based on the extension of the foamy mucus-containing cells observed in the corpus: the presence of moderate foci of foamy cells replacing less than 1/3 of the parietal cells was scored as 1, large foci affecting between 1/3 and 2/3 of the parietal cells was scored 2, and when the change affected more than 2/3 of the parietal cells the assigned score was 3.

2.7. Immunohistochemistry

The variant 4 of the human CD44 (CD44v4) was detected in biopsies using a 1:200 dilution of a mouse IgG1-k antibody against human CD44v4 from eBioscience (San Diego, CA) and a mach 2 mouse polymer labeled with horseradish peroxidase (HRP) from Biocare Medical Co. (Concord, CA) as secondary antibody solution (overnight at 4°C and 30 mins at room temperature, respectively). The antigen retrieval was done in citrate buffer in a pressure cooker for 20 minutes and the staining done with DAB for 5 mins at room temperature. Gr1 and pStat-1 detection in mouse tissues was performed using the Avidin-Biotin-Peroxidase complex system, according to the manufacturer's instructions (Vectastain Elite ABC Peroxidase Kit; Vector Laboratories). Antigen retrieval was performed by heating slides in 0.01 M sodium citrate buffer (pH 6.0) to 95°C under vacuum for 40 minutes and allowing them to cool for 30 min at room temperature, then incubating in MeOH/3% H₂O₂ for 20 min to quench endogenous peroxidase. Slides were incubated with the primary antibodies anti-Gr1, (eBioscience, 1:100) or anti-pStat-1, (SantaCruz, 1:100) overnight, followed by incubation with biotinylated secondary antibodies at room temperature for 1 h, and by avidin-biotin peroxidase complexes for 1 h. Finally, slides were developed using a diaminobenzidine substrate, and counterstained with hematoxylin. Images were collected at 200x and 600x magnification using an Olympus BX61 (UIS2 optical system) microscope equipped with a high resolution Olympus DP72 camera and Standard Cell Sense image capture software.

2.8. Statistical analysis

All statistical analysis was done in GraphPad Prism Software 4.0 using Mann-Whitney or Wilcoxon's tests, except for the comparison of the infiltration of Gr1 cells into the gastric mucosa for which the R Studio software, version 0.98.1062 was used.

3. Results

3.1. CD44 is associated with progression of the gastric lesions in humans

Baseline and 6-year follow-up gastric samples from 39 individuals were compared by microarray analysis to determine gene patterns associated with evolution of the gastric lesions over time. After normalizing the data, we looked for genes with at least a 50% change between the two time points and $p < 0.05$. Only one gene (*ARHA1*) in the "No change" group, 17 genes in the "Regression" group and 26 genes in the "Progression" met these criteria. However, after applying FDR correction, only the *ARHA1* gene remained significant in the "Regression" group while four genes, *ARHA1*, *NUMA1*, *LCN2*, and the canonical form of *CD44*, remained significant in the "Progression" group. Interestingly, these genes have all been associated with cancer risk. For example, mutations in *ARHA1* (*RHOA*) have been repeatedly associated with risk of diffuse gastric cancer [45;46]; *NUMA* over expression has been significantly associated with disease stage and local metastases to lymph nodes in ovarian cancer [47]; and *LCN2*, after the activation of the PI3/AKT/NFkB [48], has been shown to form complexes with MMP9 and these, in turn, being associated with reduced survival in gastric cancer [49;50]. Since progression to more advanced gastric lesions may be associated with the development of gastric cancer, and because previous studies had suggested that *CD44* may be a marker of advanced gastric lesions [34;35], we

wanted to investigate the role of this gene in development of gastric lesions. The transformed Log10 ratios between the follow-up and baseline signals were used to compare the groups. We found a non-significant reduction in the expression of *CD44* in the “Regression” group when compared to the “No change”, but a significant difference between the level of *CD44* expression between the “Regression” and “Progression” groups (Figure 1A). To confirm the results of the microarray, we performed real-time PCR in baseline gastric mucosa samples and found that individuals with progression had increased expression of *CD44*, compared to the group that combined individuals with regression and with no change (Figure 1B). Because all of the subjects studied were infected with *H. pylori*, we infected the gastric cell line AGS with *H. pylori* at a MOI of 20:1 for 16 hours and found that the infection induced a significant increase in the expression of *CD44* (Figure 1C), a finding that is in agreement with previously published data [51]. Interestingly, we found that baseline expression of *CD44v4* (both the percentage and intensity) (Figure 1D and 1E, respectively) in the progression group was significantly higher than those in the no change or regression groups. Taken together, our results suggest that infection with *H. pylori* induces the expression of *CD44* and this induction, in turn, is associated with progression to advanced gastric lesions over time. To identify the mechanism of the *CD44*-mediated progression of gastritis, we infected mice as described in the following sections.

3.2. *CD44* is necessary to induce the expression of IFN- γ -inducible genes in response to *H. pylori* infection

To explore the effect of *CD44* after *H. pylori* infection, we infected WT and *CD44*^{-/-} PM with *H. pylori* SS1 and performed microarray analysis as described in Materials and Methods. Infection of PM with *H. pylori* induces the expression of a set of genes that is different from that of non-infected PM as evidenced by the separation of the two groups in the dendrogram (Figure 2A). Interestingly, *H. pylori*-infected PM from wild type and *CD44*^{-/-} mice further separated, suggesting that the lack of *CD44* leads to the induction of different gene pathways in response to the infection. That separation was not observed in the non-infected PM, indicating that this difference was an effect of the infection. Analysis using MetaCore revealed that at least 80% of the gene networks associated with these responses were related to inflammation (Figure 2B; blue bar, genes unique to WT PM; orange bar, genes unique to *CD44*^{-/-} PM; hatched bar, common genes). As can be observed, genes associated exclusively with *CD44*^{-/-} mice had non-significant participation in the networks listed, suggesting the importance of *CD44* in mounting specific responses to the *H. pylori* infection (Figure 2B).

It has been shown that *CD44* is essential to mount IFN- γ -induced responses during infections [52], and that overexpression of IFN- γ in the gastric mucosa may lead to the development of advanced pre-cancerous lesions in the stomachs of mice [53]. We investigated the expression of IFN- γ -associated genes in the gastric mucosae of infected and non-infected wild type and *CD44*^{-/-} mice. These genes included IFN- γ , the interferon-induced protein with tetratricopeptide repeats 2 and 3 (*IFIT2* and 3), the immunity-related GTPase family member 2 (*IIGP2*), the interferon regulatory factor 7 (*IRF7*) and the signal transducer and activator of transcription 1 (*STAT1*). We found that none of these genes were up-regulated in the *CD44*^{-/-} mice in response to *H. pylori* infection to the same levels as

they were in the wild type mice (Figure 3). Thus, only wild type mice showed an infection-induced response of IFN- γ -dependent genes, suggesting that CD44 is needed for this response. It is interesting that there was also loss of induction of *Nos2*, a pro-inflammatory gene that can be activated by IFN- γ [54;55] suggesting that CD44 may be regulating this interaction. Additional confirmation of this affected pathway in *CD44*^{-/-} mice was observed when the level of phosphorylation of STAT-1 was studied by immunohistochemistry (IHC), revealing that *CD44*^{-/-} mice had lower numbers of pSTAT-1 positive cells in their gastric mucosae in response to *H. pylori* infection when compared to WT mice (Figure 4A). A representative IHC staining for pSTAT-1 is shown in Figure 4B.

3.3. CD44 is involved in the appearance of mucous metaplasia in mice

Our *in vitro* and *in vivo* results suggested that *CD44* is crucial to mount an appropriate immune response after infection with *H. pylori*. Since CD44 has been implicated in the movement of immune cells, namely macrophages and neutrophils, into the inflamed tissues [22–25], we chose to investigate the role of CD44 in the modulation of the gastric inflammatory responses to *H. pylori* infection *in vivo*. We infected wild type and *CD44*^{-/-} mice with *H. pylori* and determined the levels of mucous metaplasia and inflammatory response. As can be observed in Figure 5A gastric tissue of *CD44*^{-/-} mice had a significantly lower degree of mucous metaplasia compared to that of wild type mice. Representative staining for mucous metaplasia is shown in Figure 5B, including an inset showing a 40X magnification of a metaplastic gland. This difference between WT and *CD44*^{-/-} mice is an important finding, because this type of metaplasia has been implicated as a precancerous lesion in mice [56–59]. To verify the presence of this gastric lesion, we stained the gastric biopsies with Alcian Blue-Periodic acid-Schiff (AB-PAS), as recommended [53;60–62] (Figure 5B). AB-PAS staining confirmed the replacement of fundic parietal and chief cells by foamy cells containing abundant mucins. Even though significant differences between the inflammation scores (infiltration of mononuclear and polymorphonuclear cells) in antrum and corpus were observed, no significant differences were noted between WT and *CD44*^{-/-} mice (Figures 5C and D, respectively).

Depletion of Gr1 cells dysregulates the immune response against many pathogens including *Helicobacter* [63;64]. In mice, the myeloid differentiation antigen Gr1 is highly expressed on neutrophils and thus it has been considered a neutrophil marker. However, in addition to neutrophils other cells including dendritic cells, and myeloid suppressor monocytes also express Gr1 [63;65;66]. Recent studies have shown that myeloid-derived suppressor cells (MDSCs) contribute to *H. pylori*-related inflammation and carcinogenesis [67–69], as well as modulation of IFN- γ responses [70]. To determine whether the levels of Gr1 expression in the gastric mucosa of wild type and *CD44*^{-/-} infected mice differed, we conducted IHC for Gr1 and found that WT mice infected with *H. pylori* had a high number of Gr1⁺ cells infiltrating their gastric mucosa (>30 per high power field) both in the corpus and in the antrum (Figure 6A and B, respectively). Representative IHC staining showing Gr1⁺ positive cells in the gastric mucosae of *H. pylori*-infected wild type and *CD44*^{-/-} mice is shown in Figure 6C.

4. Discussion

Infiltration of immune cells into the sites of inflammation is a crucial step to mount specific immune responses [19]. *In vitro* and *in vivo* data show that *H. pylori* induces the migration of immune cells [71–78]. The adhesion cell molecule CD44 is one of the molecules that is involved in this immune cell recruitment [23–25;36]. CD44 has been previously shown to be up-regulated in gastric cancer compared to IM and to normal tissue, but the changes in CD44 expression following *H. pylori* infection and during the evolution of precancerous lesions have not been completely elucidated [34;35;79;80]. In this study, we compared the gene expression signatures of baseline and 6 year follow-up biopsies by microarray and found that individuals with progression of the gastric precancerous lesions over time had significant changes in their expression of *CD44*. We also found that *CD44*^{−/−} mice have reduced levels of mucous metaplasia in response to *H. pylori* infection. This response is accompanied by reduced levels of *IFN-γ* and *IFN-γ*-related genes as well as reduced infiltration of Gr1⁺ cells into the gastric mucosa.

Recent interesting publications have associated the epithelial expression of CD44 with regeneration of atrophy of parietal cells in mice after treatment with tamoxifen [33]. Other studies have shown that CD44 suppresses the production of reactive oxygen species (ROS) while cells expressing variants of this molecule (CD44v8–10) or lacking the marker (CD44^{−/−}) are associated with increased production of ROS [81]. Reduced levels of ROS in CD44⁺ cells protect CagA, a known *H. pylori* virulence factor, from degradation by autophagy [82]. These CD44⁺/CagA⁺ gastric cells have been proposed as the origin of spasmolytic polypeptide-expressing metaplasia (SPEM) in mice [83]. Our model of mouse infection uses the *H. pylori* SS1 which, as previously reported, does not translocate cagA into epithelial cells [84] suggesting that other mechanisms in addition to the interaction of CagA and CD44 are involved in gastric tissue damage in response to the infection. Although the expression of gastric epithelial CD44 is associated with the appearance of metaplasia, the role of the immune system is also important. Several publications have demonstrated that the infiltration of IFN-γ-producing Th1 cells into the gastric mucosa during infection is partially responsible for the *H. pylori*-induced pathology [85;86]. It has been shown that CD44 regulates inflammation by providing stimulatory signals that activate T lymphocytes [87], inducing the secretion of pro-inflammatory molecules by macrophages [87–89], and regulating the production of IFN-γ by CD4 T cells [53]. Our findings expand current knowledge by showing that CD44 may be associated with differential infiltration of Gr1⁺ cells into infected gastric mucosae.

Recent *in vitro* evidence shows that different subpopulations of MDSCs, Gr1⁺/CD11b⁺ myeloid cells, may induce different CD8⁺ T-cell responses, including increased IFN-γ [90]. Our results show increased levels of Gr1⁺ inflammatory cells and increased expression of mucosal *IFN-γ* and *IFN-γ*-related genes in response to *H. pylori* in WT mice but not in CD44^{−/−} mice. This difference suggests that the presence of CD44 is essential to induce an IFN-γ-mediated inflammatory response against *H. pylori*. The full identity of the cells expressing Gr1 and their role in modulating the production of IFN-γ in the *H. pylori*-infected gastric mucosa has not been totally established.

Taken together, our data suggest that after infection with *H. pylori*, CD44 is a crucial player in the evolution of the gastric lesions by recruiting cells that can either ameliorate or potentiate immune responses associated with tissue damage. Our results also suggest that modulating CD44 responses may be of benefit to reduce the inflammation induced by *H. pylori*, making it a potential target for treatment.

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Highlights

- *CD44* expression is significantly increased in individuals whose gastric lesions progressed along the gastric precancerous cascade.
- *CD44* is associated with increased Th1 responses, increased tissue damage and higher counts of Gr1+ cells in the gastric tissues of *H. pylori* infected mice.
- We show, for the first time, the association of *CD44* expression, Th1 responses, cellular infiltration and tissue damage in *H. pylori*-induced gastritis.

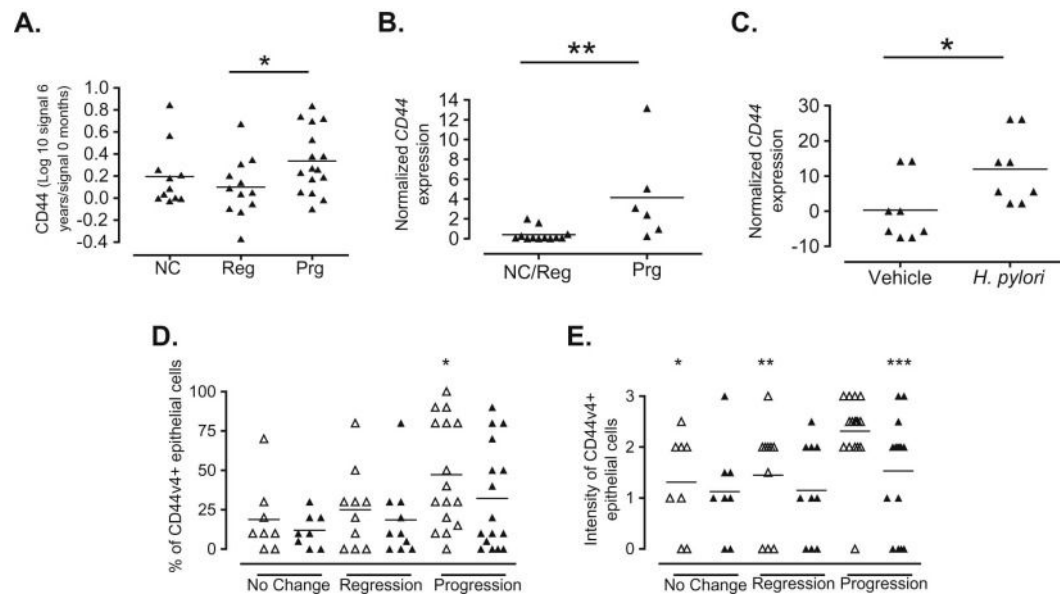


Figure 1. CD44 expression is associated with the progression of gastric premalignant lesions over time and with infection with *H. pylori*

A. CD44 levels were determined by microarray analysis in baseline and 6-year follow-up biopsies from 39 subjects that were grouped based in the evolution of their gastric lesions. CD44 levels were expressed as a log transformation of the ratio between the follow-up and baseline signals in the three comparison groups (NC, no change; Reg, regression; Prg, progression). * $p=0.039$ when comparing Prg with Reg. **B.** CD44 expression in baseline biopsies was determined by real-time PCR and normalized to *GAPDH* expression. * $p=0.0057$ when comparing Prg to NC/reg. **C.** AGS cells were infected for 16 hours with *H. pylori* (26995) at a MOI of 20 and CD44 expression was determined by real-time PCR. * $p=0.0379$ when comparing *H. pylori*-infected AGS with non-infected cells. **D.** Percentage of CD44v4 expression in gastric epithelial cells of baseline and follow-up gastric biopsies of 34 individuals with different outcomes of gastritis over time. * $p=0.0295$ when compared to no change at baseline. **E.** Intensity of the CD44v4 expression in gastric tissue of 34 individuals with different gastritis outcome over time. * $p=0.0116$, ** $p=0.0159$, *** $p=0.0188$ when compared to baseline progression.

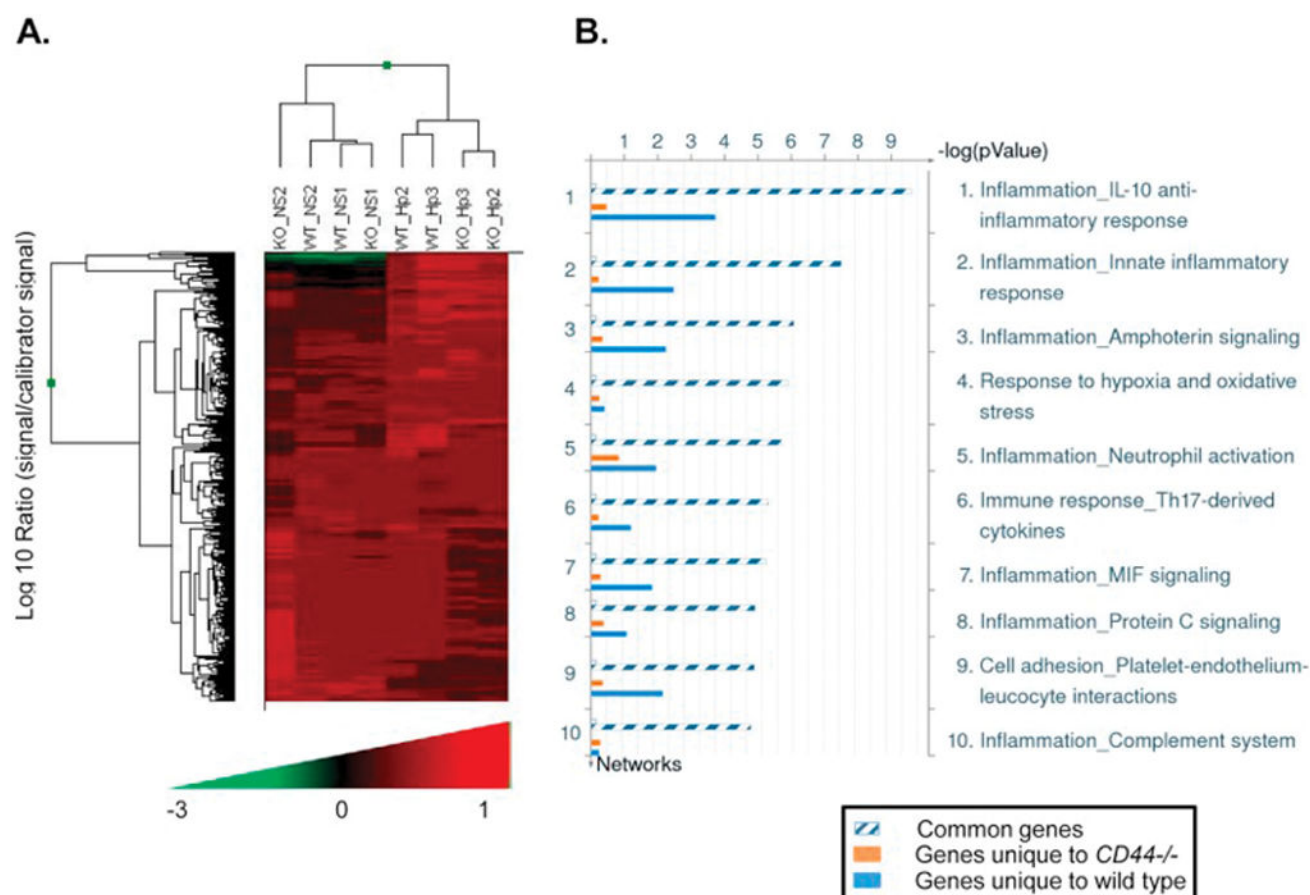


Figure 2. Differential gene profiles in wild type and *CD44*^{-/-} peritoneal macrophages infected with *H. pylori*

Thioglycollate-induced peritoneal macrophages (PM) obtained from wild type or *CD44*^{-/-} mice were infected with *H. pylori* at a MOI of 20 for 16h and gene expression profiles determined by microarray analysis. Two mice were analyzed in each group. **A.** Dendrogram of gene profiles of wild type (WT) or *CD44*^{-/-} (KO) PMs infected with *H. pylori* (Hp) or brucella broth alone (NS). **B.** Distribution of gene networks in which genes unique to WT PM (blue bar), those unique to *CD44*^{-/-} PM (orange bar) and common genes participate (hatched bar).

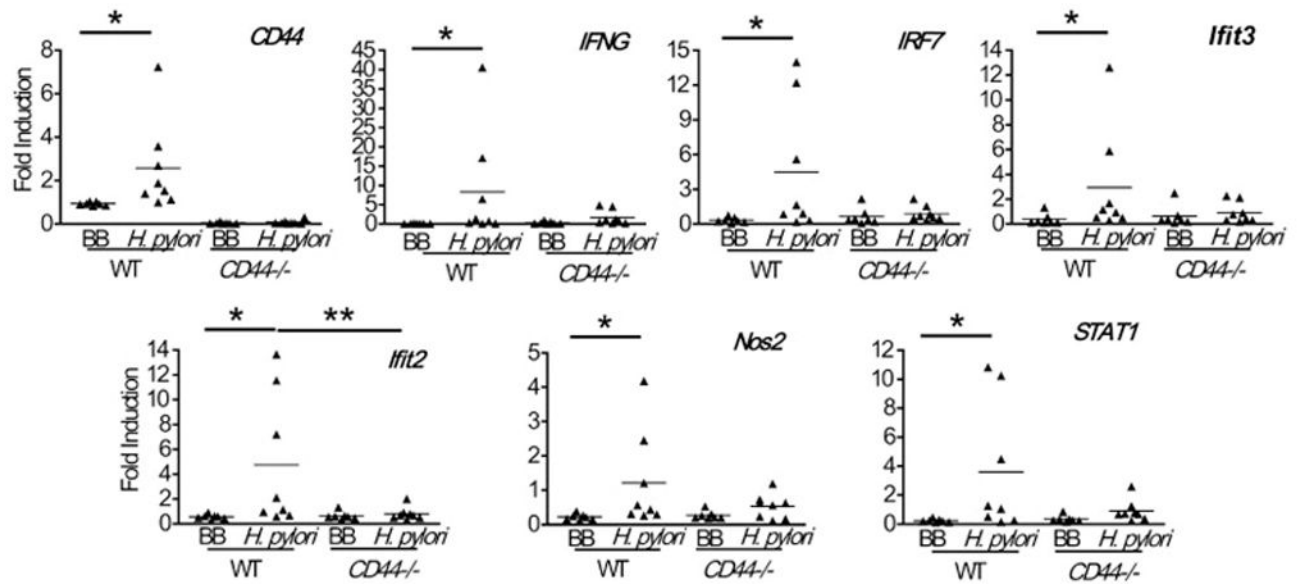


Figure 3. *CD44*- deficient mice failed to up-regulate the expression of *IFN*- γ and *IFN*- γ -induced genes following *H. pylori* infection

mRNA levels of *IFN*- γ and the indicated *IFN*- γ related genes were assessed by real-time PCR in the gastric mucosae of *CD44*^{-/-} and wild type (WT) mice following infection with *H. pylori* for 7 months. *, ** p<0.05; BB, Brucella broth.

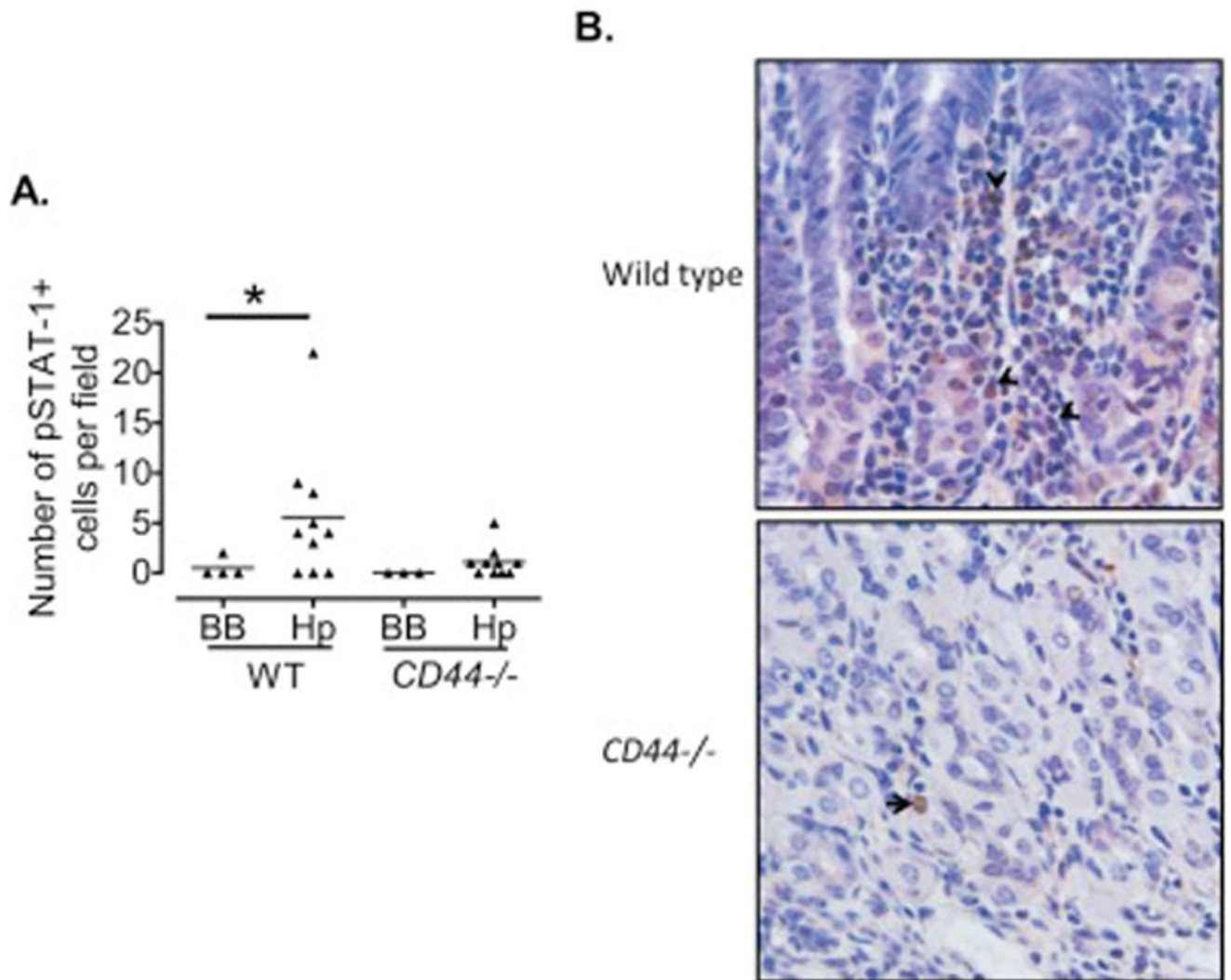


Figure 4. Reduced expression of pSTAT-1 in *CD44*^{-/-} mice following *H. pylori* infection
A. The number of pSTAT-1+ cells in 10 high power fields (200X) was assessed using immunohistochemistry in the gastric mucosae of *CD44*^{-/-} and wild type mice infected with *H. pylori* for 7 months. **B.** Representative immunohistochemistry showing pSTAT-1 positive cells in the gastric mucosa (200X magnification). **p*=0.07; BB, Brucella broth.

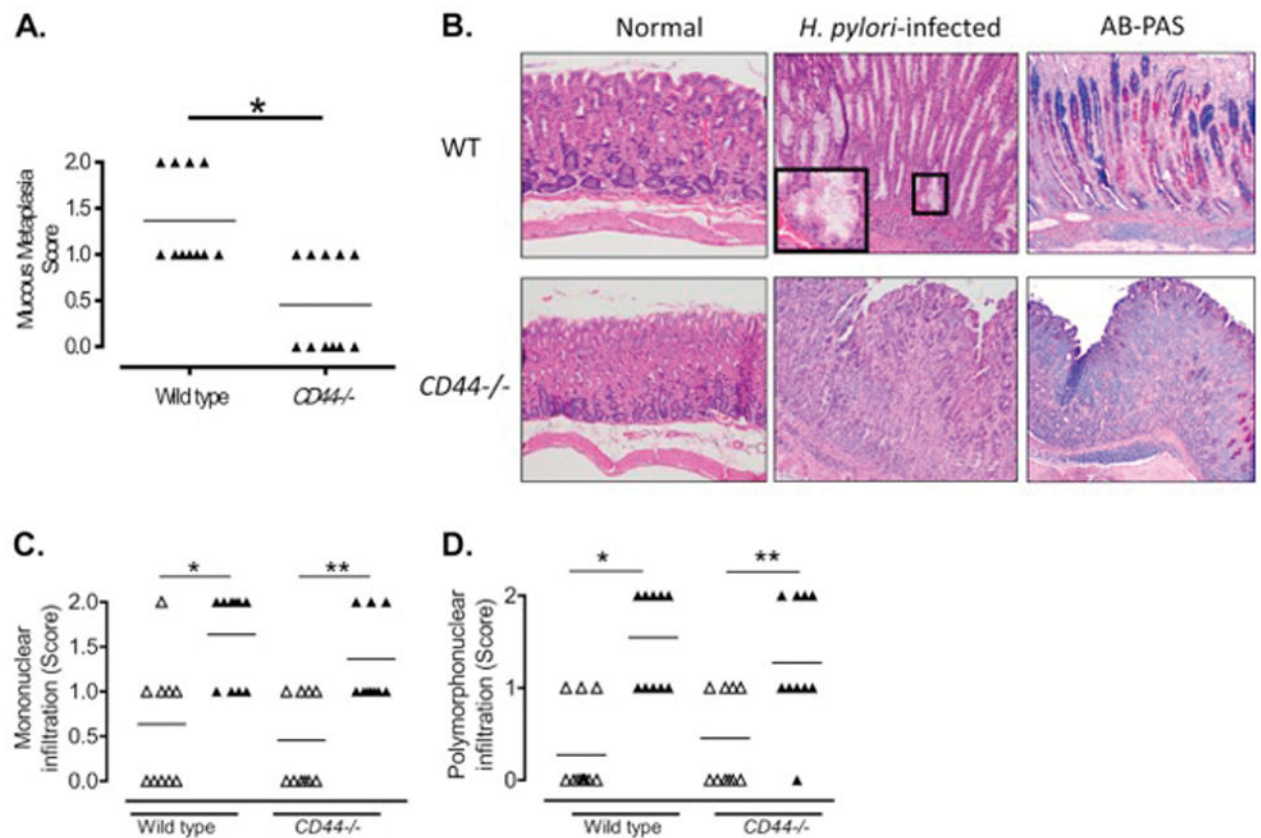


Figure 5. *CD44*^{-/-} mice infected with *H. pylori* developed less mucous metaplasia

A. Metaplastic changes in the mucosae of the corpus of *CD44*^{-/-} and WT mice 7 months after inoculation with *H. pylori* were scored from 0 to 3 as described in Material and Methods. * $p=0.016$. **B.** Representative images of H&E and AB-PAS stains showing mucous metaplasia in *H. pylori*-infected gastric corpus of wild type (WT) mice, but not in *H. pylori*-infected *CD44*^{-/-} mice (200X magnification). Inset shows higher magnification of a metaplastic gland. Semiquantitative score of the mononuclear (**C**, * $p=0.004$; ** $p=0.008$) and polymorphonuclear (**D**, * $p=0.002$; ** $p=0.027$) infiltrate in the antrum and corpus of *CD44*^{-/-} and WT following infection with *H. pylori* for 7 months; open triangles, antrum; closed triangles, corpus.

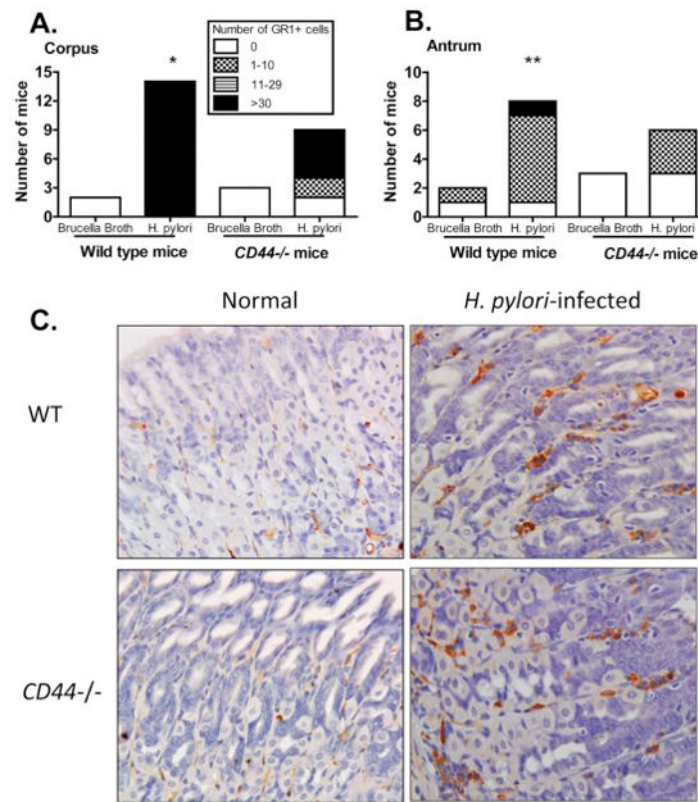


Figure 6. Decreased influx of Gr1+ myeloid cells in *CD44*^{-/-} mice upon *H. pylori* infection
 Gr1+ cells were quantified in IHC stains of the mucosae of the corpus (A) and the antrum (B) of *CD44*^{-/-} and wild type (WT) mice by counting positive cells in 10 fields in each slide (200X magnification). * $p=0.037$ and ** $p=0.019$ when comparing the number of Gr1+ cells in WT and *CD44*^{-/-} in corpus and antrum, respectively; BB, Brucella broth. (C). Representative IHC staining showing Gr1+ positive cells in sections of the gastric mucosae of WT and *CD44*^{-/-} mice infected with *H. pylori* and non-infected controls (200X magnification).