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ORIGINAL ARTICLE

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GM-CSF is key in the efficacy of vaccine-induced reduction of *Helicobacter pylori* infection

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Abstract

Background: *Helicobacter pylori* (*H. pylori*) colonizes the human gastric mucosa with a high worldwide prevalence. Currently, *H. pylori* is eradicated by the use of antibiotics. However, elevated antibiotic resistance suggests new therapeutic strategies need to be envisioned: one approach being prophylactic vaccination. Pre-clinical and clinical data show that a urease-based vaccine is efficient in decreasing *H. pylori* infection through the mobilization of T helper (Th) cells, especially Th17 cells. Th17 cells produce interleukins such as IL-22 and IL-17, among others, and are key players in vaccine efficacy. Recently, granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing Th17 cells have been identified.

Aim: This study explores the possibility that GM-CSF plays a role in the reduction of *H. pylori* infection following vaccination.

Results: We demonstrate that GM-CSF$^+$ IL-17$^+$ Th17 cells accumulate in the stomach mucosa of *H. pylori* infected mice during the vaccine-induced reduction of *H. pylori* infection. Secondly, we provide evidence that vaccinated GM-CSF deficient mice only modestly reduce *H. pylori* infection. Conversely, we observe that an increase in GM-CSF availability reduces *H. pylori* burden in chronically infected mice. Thirdly, we show that GM-CSF, by acting on gastric epithelial cells, promotes the production of βdefensin3, which exhibits *H. pylori* bactericidal activities.

Conclusion: Taken together, we demonstrate a key role of GM-CSF, most probably originating from Th17 cells, in the vaccine-induced reduction of *H. pylori* infection.

KEYWORDS
antimicrobial peptide, defensin, GM-CSF, *Helicobacter pylori*, Th17 response, vaccine

1 | INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the most common chronic bacterial infections of the human stomach mucosa.[1] This infection is acquired commonly during childhood and persists lifelong if not treated. The transmission of the infection is not fully understood but lots of evidence prone gastro-oral, oral-oral or fecal-oral contamination routes, especially in context of intra-familial clusters or mother-to-child transmission.[2] Although the majority of cases remain asymptomatic for decades, *H. pylori* infection is associated with increased occurrences of peptic ulcers, and more seriously with the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma.[3,4] Currently, a combination of antimicrobials and antisecretory drugs[5,6] is considered the best way

to eradicate *H. pylori* infection. However, its high global prevalence of approximately 50%[7] is associated with the rapid emergence of antibiotic resistance, and the lack of specificity of the available therapies suggests the eradication of this bacterial infection is an important public health concern. Because *H. pylori* displays many immune evasion strategies to persist in the mucus layer of the stomach mucosa,[8] it is of clinical interest to better characterize immune responses elicited upon infection and further improve therapeutic protocols.

Currently, alternative prophylactic and therapeutic vaccines have been conceptualized. As vaccination induces a specific protective immune response and does not trigger resistance as compared to antibiotics, this therapeutic strategy has clinical merit and as such, should be improved. To be efficient, a vaccine requires the selection of immunogenic and protective antigens mixed with appropriate adjuvants. Antigen selection requires extensive knowledge of *H. pylori* bacterium and an understanding of the immune responses involved in its clearance. First, oral administration of a bacterial lysate plus cholera toxin (CT) in mice, conferred protection against *Helicobacter felis* (a close relative of *H. pylori*).[9] Then, several protective antigens have been characterized, including urease, a protein expressed at the cell surface of all *H. pylori* strains[10] and is now considered a promising protective antigen candidate.[11–15]

In humans, a phase III clinical trial evaluated that a urease-based vaccine protects 71.8% of children from the acquisition of *H. pylori* infection.[16] Unfortunately, this protection was not stable, and its efficacy decreased to 55.8% after one year. Although this study clearly indicates that the urease-based vaccine is protective in humans, major efforts are still needed to increase its protective effect and induce long-term protective immunity.

In order to improve vaccine-induced protection, understanding the immune-protective mechanisms involved in conferring *H. pylori* immunity is essential to select the best vaccine candidate. Key studies have demonstrated that $CD4^+$ Th cells confer protection against *H. pylori*.[17–20] In particular, Th17 cells produce a number of cytokines, such as IL-17 and IL-22, which are key players in the mediation of adaptive immune responses against *H. pylori* infection, and in the vaccine-induced clearance of *H. pylori*.[20–22]

IL-22, in response to *H. pylori* infection, triggers the production of antimicrobial peptides (AMPs) such as regenerating islet-derived protein 3-beta (RegIIIβ) by gastric epithelial cells. RegIIIβ is one of the key molecules involved in vaccine-induced reduction of *H. pylori* colonization in mice.[23] Indeed, AMPs are an important part of the innate immune response against *H. pylori* by protecting the gastrointestinal mucosa from pathogen invasion. Apart from RegIIIβ, βdefensins and Lipocalin2 also play a pivotal role in *H. pylori* infection and alteration of the gut microbiota.[24–26] These small proteins are efficient gram-positive and negative bacteria killers and are inducible after exposure to lipopolysaccharides (LPS) and/or pro-inflammatory cytokines,[27] suggesting their possible role in vaccine-induced *H. pylori* clearance.

In parallel, IL-17 plays a major role in stimulating granulopoiesis, mobilization of granulocytes into sites of inflammation, and stimulating fibroblasts, endothelial cells, macrophages, and epithelial cells to produce multiple pro-inflammatory mediators, leading to the vaccine-induced reduction of *H. pylori* infection.[19,20] Recently, a new Th cell subset was identified. This subset, Th1/Th17 cells, produce IL-17, interferon gamma (IFNγ) and GM-CSF.[28] Th cell differentiation into the Th1/Th17 subset is mediated, in part, by IL-23,[29,30] a cytokine secreted by dendritic cells (DCs) and macrophages during *H. pylori* infection.[31] Since their identification, several studies demonstrated that these Th1/Th17 cells, also named pathogenic Th17 cells, are involved in the pathogenesis of inflammatory diseases including autoimmune encephalomyelitis, multiple sclerosis and colitis.[30,32] One of pathogenic Th17 cells product, GM-CSF, is a key factor known to play an important role in gut homeostasis.[33–35] Indeed, GM-CSF is secreted by many other cell types such as epithelial cells[36] and is a key factor in sustaining and promoting innate and adaptive mucosal immune responses.[37,38]

The objective of this study was to probe for a role of GM-CSF in the vaccine-induced reduction of *H. pylori* infection. We observed that the inhibition of the biological activities of GM-CSF jeopardizes the vaccine-induced reduction of *H. pylori* infection. We detected GM-CSF-producing pathogenic Th17 cells in mice stomach and show that GM-CSF stimulated gastric epithelial cells produce βdefensin3, which has the capacity to kill *H. pylori*.

2 | MATERIALS AND METHODS

2.1 | Mice

Female BALB/c OlaHsd (Balb/c) mice (6–8 weeks old) were purchased from Envigo (Ad Horst). BALB/c GM-CSFR-deficient (GM-CSFRko) mice were generously provided by Prof. Angel Lopez (Institute of Medical and Veterinary Science, Adelaide, Australia). This study was approved by the State of Vaud Veterinary Office (authorization no. 836.11/2). Mice were bred under specific-pathogen-free conditions in our animal facility.

2.2 | *H. pylori* infection

Helicobacter pylori P49 (*H. pylori*), a human clinical isolate adapted to mice expressing VacA but not CagA,[20] was grown in brain heart infusion (BHI, Becton Dickinson) supplemented with 10% fetal bovine serum (FBS, Biowest) under microaerophilic conditions for 36 h. Adult mice were infected twice with 5×10^8 *H. pylori* bacteria. Bacteria were administered by oral gavage in 200 μl of BHI at a 2-days interval. The control group received 200 μl of BHI.

2.3 | Assessment of *H. pylori* colonization

Quantification of *H. pylori* CFU was used to assess infection status.[39] CFU were determined immediately after stomach resection. The stomach is cut lengthwise from the pylorus to the fundus into three equal parts. One-third of the stomach was immersed in 200 μl of selective culture medium (10 μg/ml Vancomycin (Sigma), 20 μg/ml Bacitracin (Sigma), 5 μg/ml Amphotericin B (Sigma),

0.3 μg/ml Polymyxin B (Sigma), 1.07 μg/ml Nalidixic acid (Sigma) in BHI) and homogenized with a fitted plastic pestle in a sterile Eppendorf tube (Vaudaux-Eppendorf). Serial 10-fold dilutions of the homogenate were then plated on Helicobacter plates (Becton Dickinson). Plates were incubated for 3–4 days in microaerophilic conditions, after which the CFU were counted. Identification of *H. pylori* was based on the appearance of colonies on plates and gram staining. Results were expressed as number of CFU per one-third of stomach.

A rapid biochemical test based on the urea reaction (named Rapid Urease Test, RUT) (Cleartest Histo *HP*; servoprax GmbH) was also used to assess infection status. Briefly, stomachs were resected, and one-third of the stomach was immersed in 500 μl of the supplier's suspension and incubated at 37°C for 2 h. Specimens were centrifuged, and the supernatant was used for spectrophotometric quantification at an optical density of 550 nm. The last part of the stomach was snap-frozen and used for RNA extraction.

2.4 | GM-CSF neutralization

BALB/c mice were injected intraperitoneally on days −1 and 2 post *H. pylori* infection with 125 μg of anti-mouse GM-CSF monoclonal antibody (MP1-22E9, Biolegend). Monoclonal Rat IgG2a (BioXcell) was used as an isotype-matched control antibody.

2.5 | Vaccination

Mice were immunized intranasally four times at 1-week intervals with 30 μg of recombinant *H. pylori* urease (kindly provided by Sanofi-Pasteur) combined with 5 μg of cholera toxin (CT) (Calbiochem). Control mice were administered only with cholera toxin.[20]

2.6 | Hydrodynamic gene delivery injection

Anesthetized mice received one injection of either 20 or 50 μg of IL-22, GM-CSF, and control expression plasmid (MR225471, MC208342, and PS100001 OriGene Technologies, respectively). Injections were performed intravenously in 2 ml of Ringer "Bichsel" solution (Ringer-Lösung "Bichsel," Laboratorium Dr. G. Bichsel AG) as described by Liu F et al.[40]

2.7 | Flow cytometry

The blood and stomach were processed immediately after sacrifice. Blood was recovered just before being sacrificed in a 15 ml falcon® polystyrene conical tube (Corning) containing 20 μl heparin (Braun Medical AG) in 2 ml PBS. Red blood cells were removed by adding 5 ml of red blood cell (RBC) lysis buffer (1.5 × 10–5 M NH_4Cl [Sigma], 1 × 10–2 M $KHCO_3$, [Sigma]). The mixture was incubated for 5 min on ice, completed with PBS and centrifuged for 10 min at 300 g at 4°C. Lysis cycles were repeated until supernatant had become clear. Lastly, blood cells were resuspended in FACS buffer.

Isolation of gastric immune cells was performed as described elsewhere.[41] Isolated gastric immune cells were resuspended in FACS buffer. All cells were counted using cellometer® Auto T4 cell counter (Nexcelom Bioscience, Lawrence, MA) prior to activation.

Gastric immune cells were first activated for 5 h at 37°C in RPMI 1640 supplemented with 10% FBS, 1% Penicillin/Streptomycin (BioConcept), 1 mM sodium pyruvate (Sigma), 0.05 mM 2-Mercaptoethanol (Gibco), and 10 mM HEPES (BioConcept) in the presence of Brefeldin A (BioLegend), Phorbol 12-myristate 13-acetate (Sigma), and Ionomycin calcium salt (Sigma). The cells were then stained extracellularly as described below.

For extracellular staining, cells were incubated with anti-mouse CD16/CD32 (Clone 2.4G2; Becton Dickinson) for 20 min on ice. Then, cells were stained for viability assessment with LIVE/DEAD™ fixable Aqua Dead cell stain kit (Invitrogen, Life Technology Corporation) for 20 min on ice. Cells were then stained with anti-mouse antibodies CD4-PE/Cy7 (Clone GK1.5, BioLegend), CD3-PerCP/Cy5.5 (Clone 17A2, BioLegend), CD45-AF700 (Clone 30-F11, BioLegend), CD45-VioBlue (Clone REA737, MACS Miltenyi Biotec), MHCII-AF70 (Clone M5/114.15.2, BioLegend), Ly6G-APC (Clone 1A8, BioLegend), CD11b-PerCP/Cy5.5 (Clone M1/70, BioLegend), and Ly6C-FITC (Clone AL-21, BD Biosciences) for 20 min on ice. Cells were then fixed using BD Cytofix/Cytoperm™ solution (BD Biosciences) for 20 min on ice.

For intracellular staining, cells were stained with anti-mouse antibodies IL-17A-FITC (Clone TC11-18H10.1, BioLegend), GM-CSF-APC (Clone MP1-22E9, Biolegend) and resuspended in FACS buffer.

Cells were acquired using Attune NxT Flow Cytometer (ThermoFisher). Samples were analyzed with FlowJo V.10 software (FLOWJO LLC).

2.8 | Quantitative PCR

RNA extraction was performed on stomach tissue and AKP cells using Trizol (Invitrogen Corporation) and an RNeasy mini kit (Qiagen). RNA (300 ng) was reverse transcribed into cDNA using a PrimeScript reverse transcriptase (RT) reagent kit (TaKaRa Bio Inc.). Quantitative PCR (qPCR) amplification was performed on a QuantStudio 6 Flex Real-Time PCR Systems apparatus (ThermoFisher), using 96 or 384-well plates (ThermoFisher). The qPCR was performed in duplicate with FASTSTART SYBR GREEN MASTER (Roche). The primers used were as follows: GAPDH (5′-GCTAAGCAGTTGGTGGTGCA-3′ and 5′-TCAC CACCATGGAGAAGGC-3′, Microsynth AG), Lipocalin2 (QT00113407, Qiagen), IL-17 (5′-GCTCCAGAAGGCCCTCAGA-3′ and 5′-AGCT TTCCCTCCGCATTGA-3′, Microsynth AG), βdefensin3 (QT00265517, Qiagen), RegIIIβ (QT00239302, Qiagen), RegIIIγ (QT00147455, Qiagen), Csf2 (QT00251286, Qiagen), GM-CSFRb (5′-TGTTCCAGGATGGAGG TAAA-3′ and 5′-CCCACACTGCACATCCATAG-3′, Microsynth AG), IL-22R1 (5′-AAGCGTAGGGGTTGAAAGGT-3′ and 5′-CTACGTGTG CCGAGTGAAGA-3′, Microsynth AG), GM-CSFRa (5′-TGCGGG GCCAGTGCGGTTCCT-3′ and 5′-CAGTGCTTCATCCTCGTGTCG-3′, MicrosynthAG), Elastase (5′-GTTGGGCACAAACAGACC-3′ and 5′-CAA

ACTCAGCCACAGG-3′, Microsynth AG), Myeloperoxidase (5′-TCCC ACTCAGCAAGGTCTT-3′ and 5′-TAAGAGCAGGCAAATCCAG-3′).

Quantification of input cDNA from the unknown samples was performed by including a standard curve as described elsewhere.[39] Briefly, to construct the standard DNA curve, amplicons generated by RT-PCR using the primers described above were purified on silica columns (QiAquick PCR purification, Qiagen) and cloned into pGEM-Teasy (Promega Corp.). Ligated fragments were transformed into DH5-competent cells and plasmid DNA was prepared using silica cartridges (Qiagen). The sequence of the cloned amplicons was determined by cycle sequencing. DNA plasmid concentrations were measured by optical density spectrophotometry and the corresponding copy numbers calculated using the following equation: 1 μg 1000-bp DNA = 9.1×10^{11} molecules. Serial 10-fold dilutions of plasmids ranging from 10^7 to 10^2 DNA copies were used as a standard curve in each PCR run. The calculated number of mRNA copies for the gene of interest was then normalized per million of mRNA copies obtained for GAPDH, which was used as a housekeeping gene. For Figure S3, the fold change gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method, and the levels of gene expression were normalized with GAPDH housekeeping gene.

2.9 | Generation of AKP cells

The antral glands of the stomach of Apcfl/fl; KrasLsl-G12D; Tp53fl/fl; and villin-CreERT2 mice[42] were isolated[43] and infected with an adenovirus encoding Cre recombinase.[44] Limited dilutions were performed and an AKP cell clone was isolated. AKP cells expressing markers characteristic of gastric epithelial cells were determined by PCR, see "PCR for AKP characterization" section. In addition, AKP cells expressing mRNA encoding IL-22 and GM-CSF receptors were confirmed by qPCR, see "Quantitative PCR" section.

2.10 | PCR for AKP characterization

DNA of AKP cells was isolated using DNeasy Blood and Tissue kit (Qiagen). The primers used were as follows: Villin (5′-CAGTG GGGATGAGAGGGAGA-3′ and 5′-CCTGCTTCACCACGATGATA-3′, Microsynth AG) Gastrin (5′-TGTGGACAAGATGCCTCGAC-3′ and 5′-TGGTCCCTGGTCCAGATGAT-3′, Microsynth AG) Somastotastin (5′-CTGCGACTAGACTGACCCAC-3′ and 5′-GAA ACTGACGGAGTCTGGGG-3′, Microsynth AG) H+/K+/ATPase (5′-GTTCCAGTGGTGGCTGGT-3′ and 5′-GCTGATAGTGGAGAG ATG-3′, Microsynth AG) Lrg5 (5′-TGCCATCTGCTTACCAGT GTTGT-3′ and 5′-ATTCCGTCTTCCCACCACGC-3′, Microsynth AG) Olfm4 (5′-GCCACTTTCCAATTTCAC-3′ and 5′-GAGCCT CTTCTCATACAC-3′, Microsynth AG) Gif (5′-TGAATCCTCGGCCTT CTATG-3′ and 5′-CAGTTAAAGTTGGTGGCACTT-3′, Microsynth AG) Apc (5′-TGAGGAATTTGTCTTGGCGAG-3′ and 5′-GCACTTCCCATG GCAATCATT-3′, Microsynth AG). DNA samples were amplified by PCR using a mix containing the primers cited above, PCR Rxn buffer (inVitrogen), dNTP mix (Promega), $MgCl_2$ (inVitrogen), Taq DNA polymerase recombinant (inVitrogen) (annealing temperature 55°C, 35 cycles). PCRs were loaded on a 2% agarose gel.

2.11 | AKP cells co-culture with *H. pylori*

AKP cells were cultured in collagen type I solution from rat tail (Sigma) pre-coated T flasks (Falcon) in Minigut medium composed of DMEM/F12 (Gibco), 1% Penicillin/streptomycin, 1% N-2 supplement (Gibco), 1% B-27 supplement (Gibco), 10% FBS in a 37°C – 5% CO2 incubator. Then, 4×10^5 cells were added on a collagen pre-coated filter of a transwell plate (costar, 0.4 μm) in the presence of Minigut medium supplemented with FBS. Twenty-four hours later, cells were basolaterally stimulated with 100 ng/ml of IL-22 or GM-CSF (Peprotech). Twenty-four hours later, 4×10^5 cells of 36 h-old *Hp*49 were added apically to each transwell and incubated 12 or 24 h in a 37°C in a 5% CO2 incubator. Bacterial killing was assessed by CFU counting according to the "Assessment of *H. pylori* colonization" section.

2.12 | Immunohistochemistry

One-third sections of mouse stomachs were recovered and fixed in 10% (v/v) buffered formalin for subsequent paraffin embedding and histological analysis. Paraffin-embedded stomach tissue sections (4 mm) were decorated with rabbit anti-β-defensing 3 antibodies (Alpha Diagnostic International) followed by Dako EnVision®+, Peroxidase (Agilent Technologies) and Dako, DAB+, substrate buffer and chromogen (Agilent Technologies). Finally, Harris hematoxylin was used as a nuclear counterstain.

2.13 | Antimicrobial assay

To evaluate the antimicrobial activity of βdefensin3 on *H. pylori*, *H. pylori* was cultured as described in "*H. pylori* infection" section. Bacterial suspension at 8×10^6 CFU/ml was co-cultured in the presence of 50 μg/ml of recombinant mouse βdefensin3 protein (NBP2-35146, Novusbio) and incubated for one hour under microaerophilic conditions. Bacteria treated with PBS alone served as negative controls. At the end of the incubation period, CFU counting was performed to assess bacterial viability.

2.14 | Statistical analysis

The distribution of the data was compared using Mann–Whitney tests using GraphPad software (GraphPad Software), with a p value of .05 being considered as the limit of significance.

3 | RESULTS

3.1 | Pathogenic Th17 cells accumulate in the gastric mucosa during the vaccine-induced reduction of *H. pylori* infection

As it has been shown that the infection of vaccinated mice triggers massive urease-specific Th17 responses,[20,22] we determined whether these Th17 cells secrete GM-CSF. To this end, we immunized Balb/c mice with urease adjuvanted with cholera toxin (CT). After immunization and challenge with *H. pylori* (Figure S1A), gastric colonization was assessed by Rapid Urease Test (RUT), 7 days post infection. As expected, vaccinated mice were infected to a lesser extent than non-vaccinated mice, confirming the vaccine efficacy (Figure 1A). This decreased *H. pylori* burden is concomitant with massive immune cell infiltration into the gastric mucosa (Figure 1B.1). Among these infiltrating cells, $CD4^+$ T cells were particularly numerous (Figure 1B.2). Interestingly, the expression of mRNA encoding IL-17 and GM-CSF are, respectively, twofold and forth fold increased in vaccinated infected mice compared with non-vaccinated infected mice (Figure 1C). Remarkably, flow cytometric analysis revealed that the stomach mucosa of vaccinated and *H. pylori* infected mice is infiltrated by pathogenic Th17 cells secreting IL-17 and GM-CSF (Figure 1D and Figure S2). Taken together, our results demonstrated that the vaccine-induced *H. pylori* reduction is associated with the gastric accumulation of pathogenic Th17 cells.

3.2 | Inhibition of the biological activity of GM-CSF jeopardizes vaccination efficacy

Next, as GM-CSF is secreted by vaccine-primed Th17 cells, we evaluated whether the absence of GM-CSF is detrimental for vaccine efficacy. To assess the role of GM-CSF, we used two different approaches. As a first approach, vaccinated and non-vaccinated GM-CSF receptor knockout (GM-CSFRko) mice were challenged with *H. pylori*. In parallel, vaccinated and non-vaccinated wild type (WT) mice, challenged with *H. pylori*, were injected with neutralizing anti-GM-CSF monoclonal antibodies (αGM-CSF mAb) (Figure S1A,B). We observed that the injection of anti-GM-CSF blocking antibodies decreases the number of circulating neutrophils (Figure S3A) and abolishes the increase expression of mRNA encoding myeloperoxidase and elastase (two proteins present in the granules of neutrophils[45]) in the stomach of vaccinated and infected mice (Figure S3B). These data establish that biological activities of GM-CSF are efficiently abolished by the injection of neutralizing anti-GM-CSF monoclonal antibodies. In non-immunized mice, the absence of GM-CSF biological activity had no impact on *H. pylori* infection (Figure 2A). As expected, in GM-CSF sufficient mice, only 33% of vaccinated WT mice remained infected compared with non-vaccinated mice. However, the vaccine-induced reduction of *H. pylori* infection was jeopardized in GM-CSFRko mice and in WT mice injected with αGM-CSF mAb. Indeed, 67% and 60% of GM-CSFRko mice and αGM-CSF mAb-injected WT mice, respectively, remained colonized by *H. pylori* after vaccination (Figure 2A). Consequently, these results showed that the vaccine efficacy is partially dependent on the biological activities of GM-CSF.

Knowing that AMPs produced by gastric epithelial cells are key molecules involved in the vaccine-induced reduction of *H. pylori* colonization, we evaluated whether the absence of GM-CSF lead to a reduction of AMP production. Gastric mRNA expression levels of Lipocalin2 and βdefensin3 were significantly increased in vaccinated WT mice as compared to non-vaccinated WT mice (Figure 2B.1, B.2). The mRNA expression level of Lipocalin2 was also found to be significantly upregulated in vaccinated GM-CSFRko mice, but not in the αGM-CSF mAb treated mice, when compared against their non-vaccinated counterparts (Figure 2B.1). Remarkably, we did not detect any significant difference in the mRNA expression level of βdefensin3 among the vaccinated and non-vaccinated mice with deficient GM-CSF biological activity. This result suggests that GM-CSF directly and/or indirectly promotes an increased expression of gastric βdefensin3 during the vaccine-induced reduction of *H. pylori* infection (Figure 2B.2).

3.3 | Therapeutic injection of GM-CSF decreases *H. pylori* infection burden

As deficiency in GM-CSF dampens *H. pylori* vaccine efficacy, we determined whether therapeutic administration of GM-CSF could decrease *H. pylori* infection burden. To this end, we performed hydrodynamic gene delivery (HGD) injection of plasmid DNA coding for GM-CSF or IL-22 to chronically *H. pylori* infected mice (Figure S1C). HGD is recognized as a simple method to induce, in the short term, a massive production of cytokines into the circulatory system.[46,47] Twelve days post HGD, *H. pylori* colonization was assessed by RUT or colony forming unit (CFU) numeration. Moyat et al[23] already demonstrated that IL-22 is a key cytokine for vaccine-induced *H. pylori* clearance, as it stimulates AMP production by gastric epithelial cells. Consequently, plasmid DNA coding for IL-22 was used as a positive control in this experimental setting. As expected, IL-22 HGD induced a decrease in *H. pylori* infection burden in chronically infected mice as compared to control mice (Figure 3). Remarkably, GM-CSF HGD decreased *H. pylori* infection as observed with IL-22 HGD (Figure 3). Consequently, like IL-22, therapeutic GM-CSF administration promotes the reduction of *H. pylori* burden.

3.4 | Therapeutic GM-CSF administration induces gastric βdefensin3 expression

The absence of the biological activities of GM-CSF decreases the mRNA expression levels of gastric AMPs (Figure 2B.1, B.2).

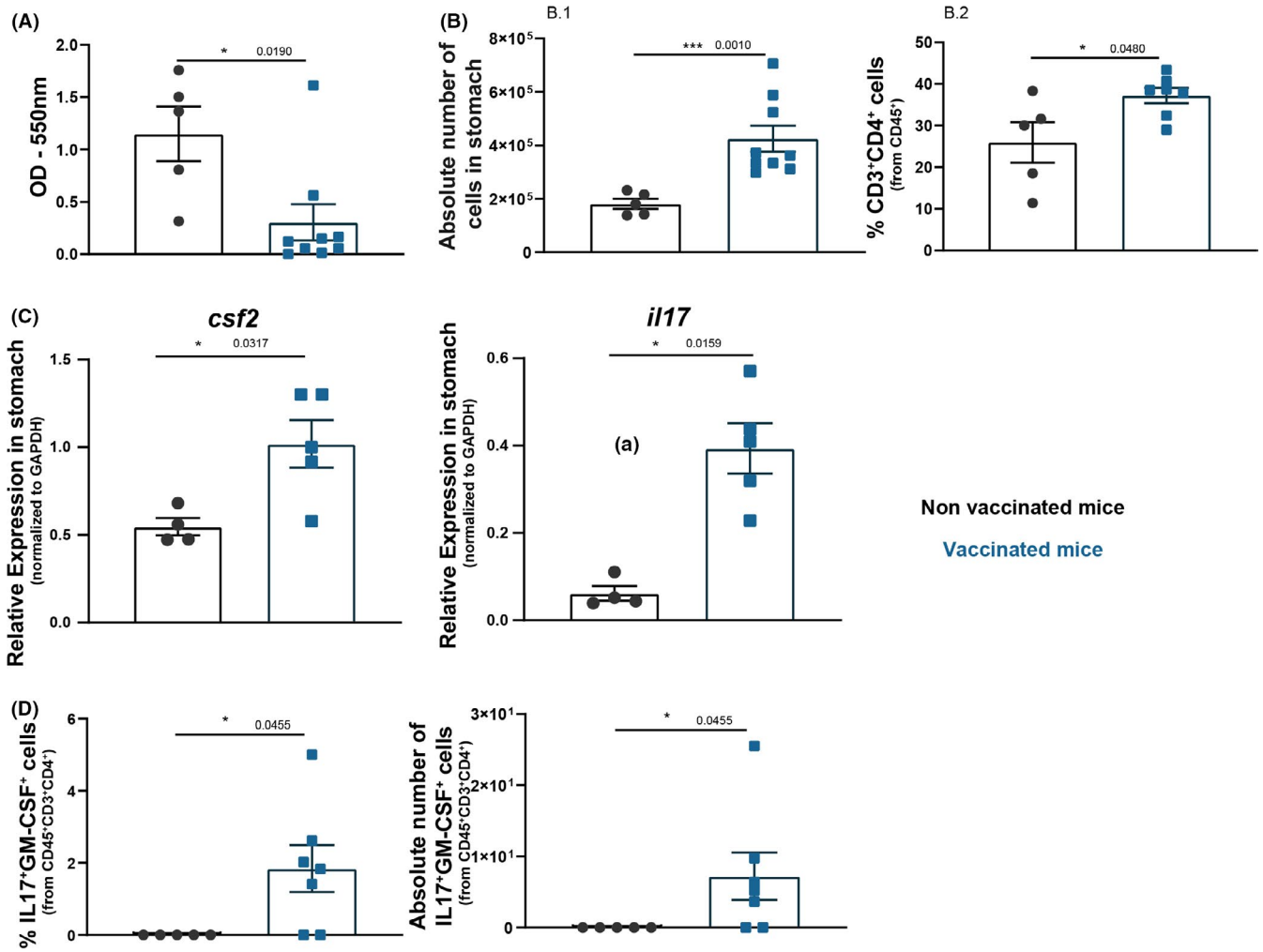

FIGURE 1 Upregulation of GM-CSF gastric expression during the vaccine-induced reduction of *Helicobacter pylori* infection. Eight-week-old Balb/c mice were either vaccinated with urease and cholera toxin or treated with cholera toxin alone as a control. Two weeks after vaccination, mice were challenged with *H. pylori*. Immune cells infiltrating the stomach were analyzed by flow cytometry and qPCR respectively on days 7 and 6 post infection. (A) At sacrifice, the stomachs were recovered and *H. pylori* colonization was assessed using RUT (optical density [OD] at 550 nm). (B) Absolute number of cells (B.1) and frequency of $CD4^+$ T cells ($CD45^+CD3^+CD4^+$) (B.2) in the stomach of mice 7 days post infection. (C) Gastric mucosal expression of mRNA encoding csf2 and il17. (D) Relative and absolute number of gastric pathogenic Th17 cells ($CD45^+CD3^+CD4^+IL\text{-}17^+GM\text{-}CSF^+$) on day 7 post *H. pylori* infection. Each dot represents one mouse. $^*p < .05$, $^{***}p < .001$ (Mann–Whitney test). Bar graphs show the mean ± SEM. The results are representative of two independent experiments

Therefore, we checked whether GM-CSF and IL-22 HGD increase the gastric expression levels of mRNA encoding AMPs. It is known that IL-22 upregulates RegIIIβ expression by gastric epithelial cells.[23] As expected, we found that IL-22 HGD increases the gastric expression levels of mRNA encoding not only RegIIIβ but also of RegIIIγ in chronically infected mice (Figure 4A). Contrastingly, GM-CSF HGD did not increase the gastric expression levels of RegIIIβ nor RegIIIγ. However, GM-CSF HGD stimulated the production of mRNA encoding βdefensin3 in the gastric mucosa of *H. pylori* infected mice (Figure 4B). Lastly, by performing immuno-chemistry, we detected an increased expression of βdefensin3 in the glands of the stomach of chronically infected mice injected with GM-CSF HGD as compared to IL-22 HGD or mice injected with an empty plasmid (Figure 4C).

Taken together, our results show that the GM-CSF-induced reduction of *H. pylori* infection burden is associated with an increased βdefensin3 gastric expression.

3.5 | GM-CSF stimulates gastric epithelial cells to produce AMPs, and to kill *H. pylori*

To determine whether GM-CSF has a direct effect on gastric epithelial cells in promoting βdefensin3 expression and *H. pylori* killing, we performed a series of in vitro experiments. We have developed a new in vitro co-culture assay using AKP cells, a mouse gastric epithelial cell line, and *H. pylori* bacteria. Polarized AKP cell monolayers were cultivated on filters allowing for basolateral

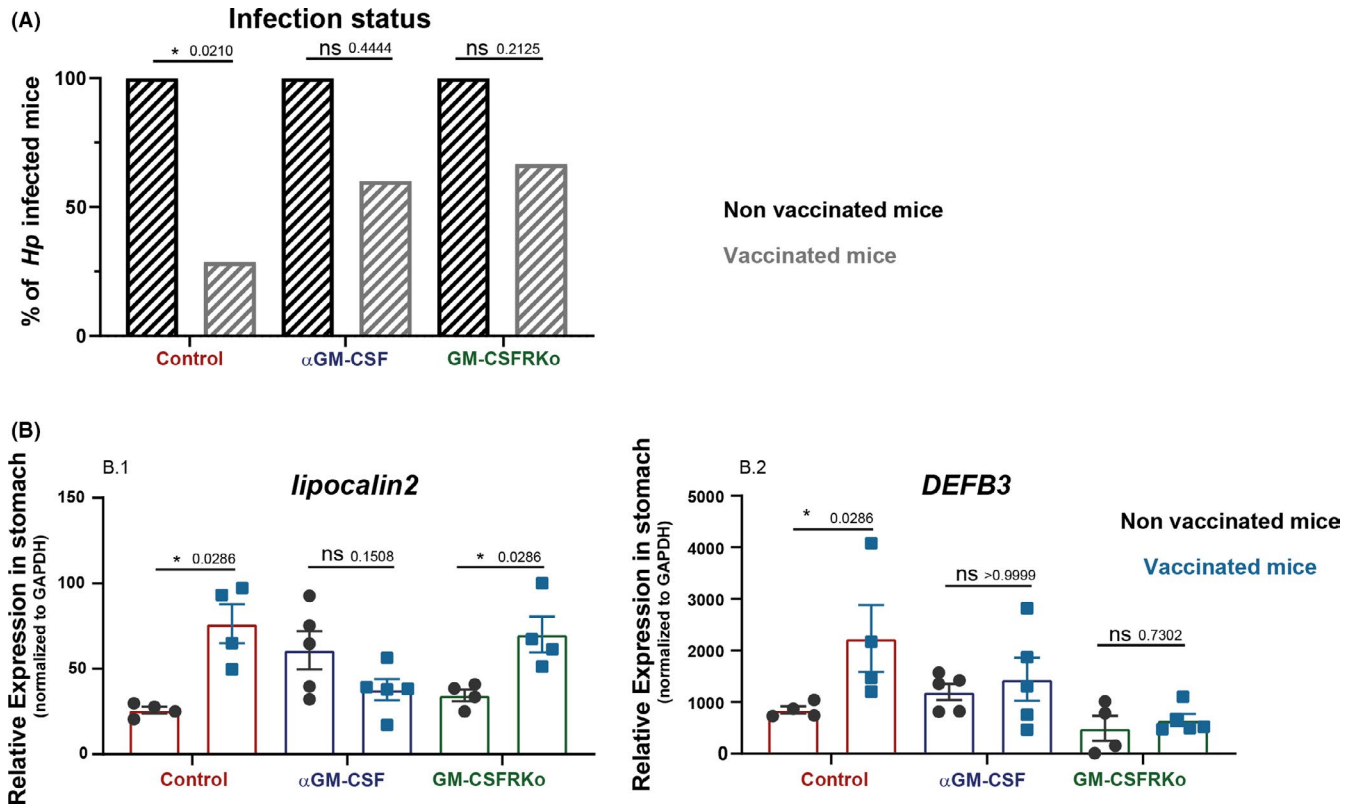

FIGURE 2 GM-CSF deficiency jeopardizes the efficacy of the vaccine-induced reduction of *Helicobacter pylori* infection. Eight-week-old WT or GM-CSFRko Balb/c mice were either vaccinated with urease and cholera toxin or treated with cholera toxin alone as a control, and two weeks later infected with *H. pylori*. WT Balb/c mice received either αGM-CSF mAb or its isotype control on days −1 and 2 post *H. pylori* infection. Mice were sacrificed 7 days post *H. pylori* infection. (A) At sacrifice, the stomachs were recovered. *H. pylori* colonization was assessed by RUT and results are expressed in percentage of *H. pylori* infected mice. Each group contains 5 to 6 mice. (B) Gastric mucosal expression of the mRNA encoding lipocalin2 (B.1) and DEFB3 (βdefensin3) (B.2). Each dot represents one mouse. ns, not significant; $^{*}p < .05$ (Mann–Whitney test). Bar graphs show the mean ± SEM. The results are representative of two independent experiments

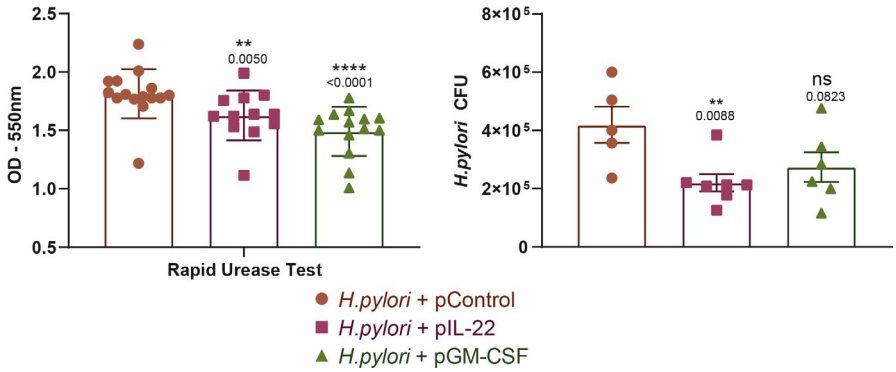

FIGURE 3 Therapeutic administration of GM-CSF reduces *Helicobacter pylori* colonization. Eight-week-old Balb/c mice were infected with *H. pylori*. Twenty-eight days later, mice received hydrodynamic gene delivery (HGD) injection of 20 μg plasmid encoding IL-22 (pIL-22), GM-CSF (pGM-CSF) or an empty plasmid (pControl). Mice were sacrificed 12 days post injection. *H. pylori* colonization was assessed by RUT and CFU. Graphs are from two different experiments. Each symbol represents one mouse; statistics are related to the control condition. ns, not significant; $^{**}p < .01$; $^{****}p < .0001$ (Mann–Whitney test). Bar graphs show the mean ± SEM

activation with GM-CSF or IL-22 and apical infection with *H. pylori*. Epithelial cell induced *H. pylori* killing was then evaluated by CFU counting.

The AKP cells expressed different molecular markers characteristic of antral gastric epithelial cells, as they expressed mRNA encoding gastrin, somatostatin, Olfactomedin 4 (Olfm4), leucine rich repeat containing g protein-coupled receptor 5 (lrg5), but not the mRNA encoding villin, H+/K+ATPase, and gastric intrinsic factor (Gif) (data not shown). AKP cells also express mRNA encoding IL-22R1α chain and the GM-CSFRα and βc chains (data not shown).

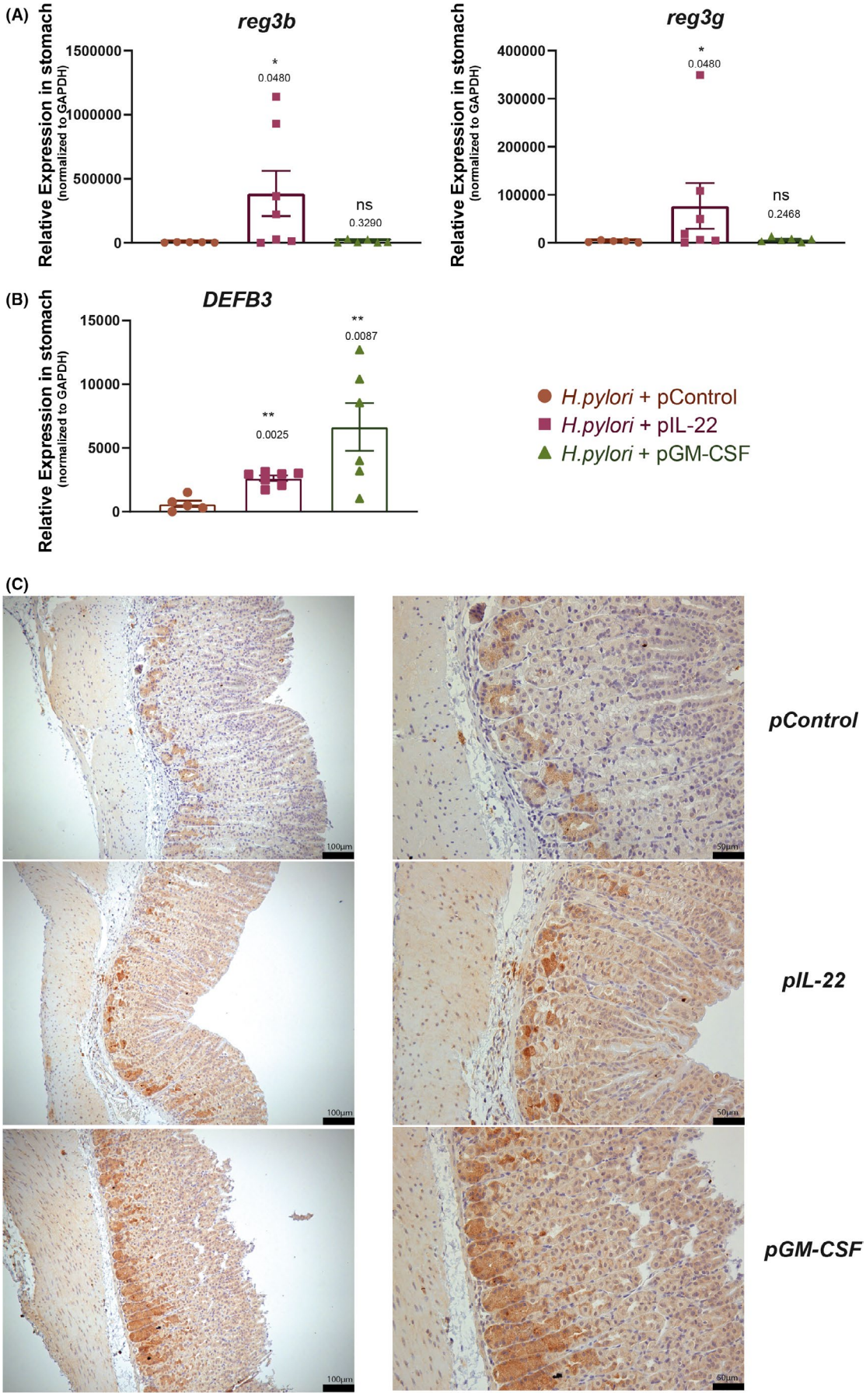
(A)
reg3b
Relative Expression in stomach (normalized to GAPDH)
1500000
1000000
500000
0
*
0.0480
ns
0.3290
reg3g
400000
300000
200000
100000
0
*
0.0480
ns
0.2468
(B)
DEFB3
15000
10000
5000
0
**
0.0025
**
0.0087
H.pylori + pControl
H.pylori + pIL-22
H.pylori + pGM-CSF
(C)
100µm
50µm
pControl
pIL-22
pGM-CSF

FIGURE 4 Therapeutic administration of GM-CSF upregulates gastric βdefensin3 expression. Eight-week-old Balb/c mice were infected with *H. pylori*. Twenty-eight days later, mice received hydrodynamic gene delivery (HGD) injection of 20μg plasmid encoding IL-22 (pIL-22), GM-CSF (pGM-CSF) or an empty plasmid (pControl). (A & B) Gastric mucosal expression of mRNA encoding reg3b (RegIIIβ), reg3g (RegIIIγ) and DEFB3 (βdefensin3), 12 days post HGD injection. (C) Detection of βdefensin3 by immunohistochemical staining on Day 7 post *H. pylori* infection. Stomach tissue sections of mice were decorated with anti-βdefensing3 Rabbit antibodies followed by biotinylated goat anti-rabbit and system-HRP for detection. Pictures are representative of data obtained from three individual mice per group. Bars: 50 μm (left panel) or 100 μm (right panel). Each dot represents one mouse. ns, not significant; $^{*}p < .05$ (Mann–Whitney test). Bar graphs show the mean ± SEM

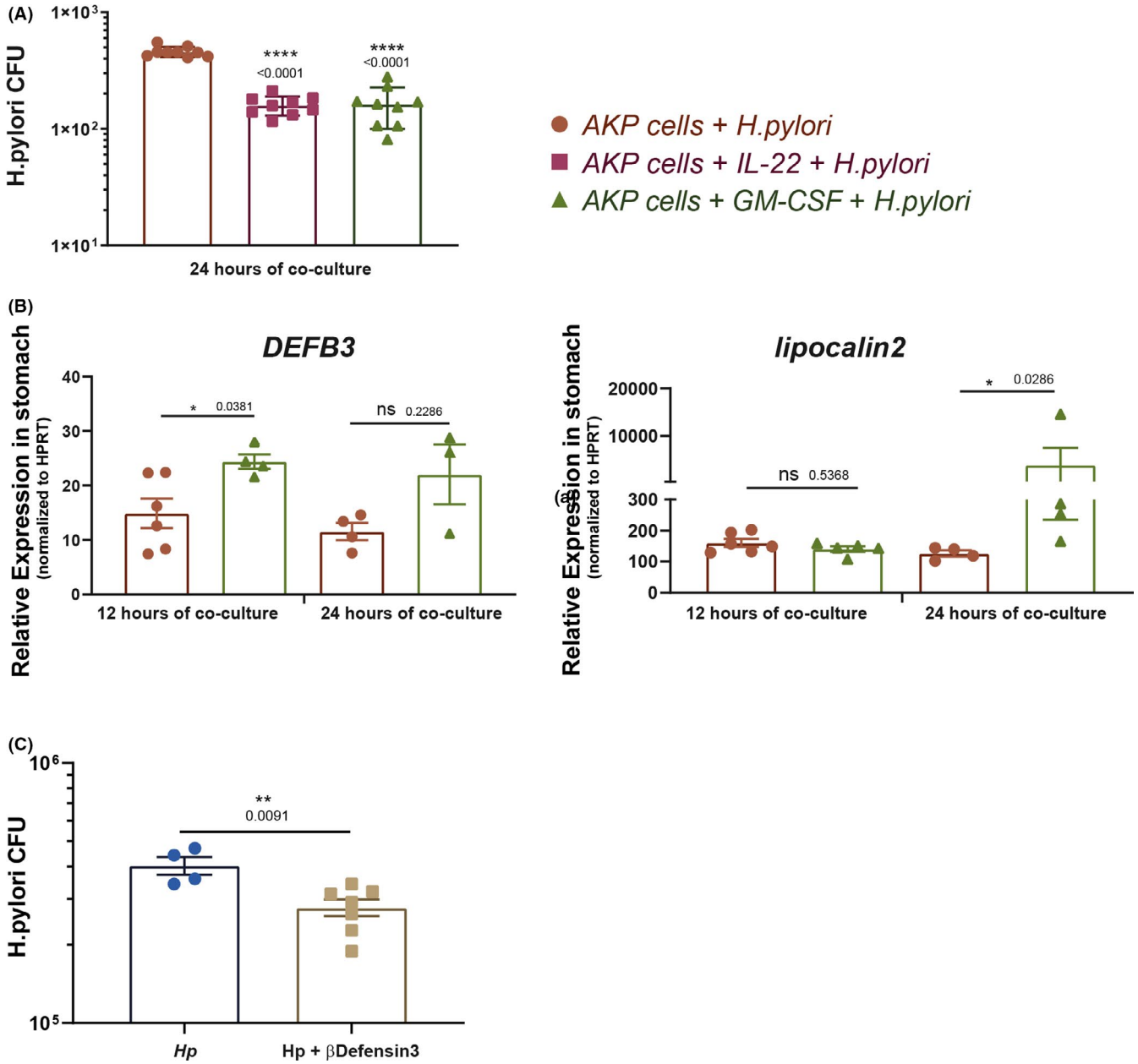

FIGURE 5 βdefensin3 displays antimicrobial properties against *Helicobacter pylori* in vitro. AKP cells were activated for 24 h with IL-22 or GM-CSF before co-culture with *H. pylori*. (A) *H. pylori* killing was assessed by CFU 24 h after beginning the co-culture. Results from 3 different experiments (B) GM-CSF stimulated AKP cell expression of mRNA encoding lipocalin2 and DEFB3 (βdefensin3) 12 or 24 h post co-culture with *H. pylori*. (C) 1×10^5 *H. pylori* bacteria were cultured in presence of 50 μg/μl of mouse βdefensin3 for one hour. *H. pylori* killing by βdefensin3 was assessed by CFU. Each dot represents one well. $^{*}p < .05$, $^{**}p < .01$, $^{****}p < .0001$ (Mann–Whitney test). Bar graphs show the mean ± SEM. The results are representative of three independent experiments

Remarkably, in line with our in vivo observations, we confirmed that AKP cells stimulated by IL-22 induce *H. pylori* killing. Similarly, AKP cells stimulated by GM-CSF also promote *H. pylori* killing (Figure 5A). Moreover, we documented the increased expression of mRNA encoding βdefensin3 in AKP cells upon GM-CSF stimulation for 12 h and 24 h (Figure 5B). These results are reminiscent of our in vivo results showing that GM-CSF induces the production of βdefensin3 by gastric epithelial cells (Figures 2B and 4). To firmly establish a link between βdefensin3 expression and *H. pylori* killing, *H. pylori* was co-incubated with 50 μg/μl of recombinant mouse βdefensin3 for one hour and plated to enumerate CFU. As expected, we observed that βdefensin3 displays direct antimicrobial activities against *H. pylori*, leading to the conclusion that GM-CSF-induced βdefensin3 may play a key role in the decrease of *H. pylori* infection burden (Figure 5C). Taken together, our results highlight that GM-CSF can directly stimulate gastric epithelial cells to trigger βdefensin3 production. In addition, we demonstrated that βdefensin3 displays AMP properties against *H. pylori* and is therefore involved in the vaccine-induced reduction of *H. pylori* infection.

4 | DISCUSSION

Improving *H. pylori* vaccine and/or treatment efficacy relies on the elucidation of immune mechanisms involved in the vaccine-induced reduction of *H. pylori* infection. The identification of key molecules modulating both innate and adaptive immune responses toward the promotion of bactericidal *H. pylori* activities will undoubtedly improve the development of new strategies to treat *H. pylori* infection.

In this study, we interrogated whether GM-CSF plays a role in the vaccine-induced reduction of *H. pylori* infection. Firstly, we clearly established that GM-CSF+IL-17+ pathogenic Th17 cells accumulate in the stomach mucosa during the vaccine-induced reduction of *H. pylori* infection. Secondly, we provided evidence that vaccinated GM-CSF deficient mice only modestly reduce *H. pylori* infection. Conversely, we observed that increased availability of GM-CSF reduces *H. pylori* burden in chronically infected mice. Thirdly, we showed that GM-CSF, by acting on gastric epithelial cells, promotes the production of βdefensin3, which exhibits *H. pylori* bactericidal activities.

It is well described that vaccine-primed *H. pylori*-specific CD4+ T cells are major contributors to vaccine efficacy.[19,20,48] Indeed, the CD4+ T cells but not CD8+ T cells or B cells are crucial for vaccine-induced protection against *H. pylori*.[48] As patients demonstrate a strong Th1 response upon *H. pylori* infection,[49] the role of Th1 response in vaccine-induced protection was first investigated. Several studies showed that the Th1 response was associated with protection but paradoxically independently of interferon-γ (IFNγ).[18,50] Several studies showed that Th2 response is dispensable for the vaccine-induced protection.[17,50] More recently, IL-17, IL-22 and the Th17, Th22 $CD4^+$ T cells subsets were associated with the protective response.[19,20] In addition to IL-17, Th17 cells secrete GM-CSF. These GM-CSF producing Th17 cells have already been identified in the literature as pathogenic Th17 cells. The differentiation of Th17 cells into pathogenic Th17 cells has been recently shown to occur in inflamed tissue where Th17 cells are recruited and the differentiation into pathogenic Th17 cells can be mediated by the local production of serum amyloid A (SAA) proteins.[51] Interestingly, SSA proteins are known to be upregulated in the stomach mucosa of Helicobacter infected mice.[52] Therefore, it can be hypothesized that the vaccine-induced *H. pylori*-specific Th17 cells, during their homing into the gastric mucosa of *H. pylori* infected hosts, will differentiate into pathogenic Th17 cells and will efficiently reduce *H. pylori* infection burden.

Interestingly enough, although these cells have been mainly characterized in the context of autoimmunity and inflammatory diseases,[28,53] our study is the first to show that pathogenic GM-CSF-producing Th17 are recruited into the stomach mucosa of vaccinated and infected mice during the vaccine-induced reduction of *H. pylori* infection. Until now, the anti-bacterial effect of GM-CSF has been mainly described in the lung and was mostly attributed to a direct effect of GM-CSF on macrophages, dendritic cells and/or neutrophils.[54] In addition, several studies have probed for roles of a lung-protective effect of GM-CSF by a direct impact on alveolar epithelial cells leading to improvement of epithelial repair processes.[55] Similarly, we show that the anti-bacterial effect of GM-CSF is also dependent on the direct activation of epithelial cells. Altogether, our study reveals for the first time that GM-CSF mobilizes the bactericidal activities of gastric epithelial cells by increasing the production of βdefensin3. Strongly rooted in our observations from this study, GM-CSF could be a potential alternative to treat *H. pylori* infection alone or in combination with other drugs to avoid antibiotic resistance and/or increase therapeutic vaccine efficacy. Indeed, recombinant human GM-CSF (sargramostim) is FDA-approved for multiple cancer immunotherapies[56] and has been envisioned as a therapeutic strategy against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)[57] and as vaccine adjuvant.[58] The adjuvant activity of GM-CSF relies on the local recruitment and maturation of DCs leading to an increased antigen presentation in the secondary lymphoid organs draining the vaccine injection site.[59] It is well known that DCs play important functions during *H. pylori* infection, meaning that the use of GM-CSF as adjuvant in vaccination against *H. pylori* could be relevant.

In addition, as GM-CSF triggers βdefensin3 secretion, this AMP in combination with RegIIIβ[23] could also be an alternative treatment for *H. pylori* clearance. In humans, the murine βdefensin3 ortholog is β-defensin2. Interestingly, several studies have revealed that human β-defensin2 also displays antimicrobial *H. pylori* properties.[60,61] Indeed, several synthetic AMP analogs have been synthetized and have been shown to efficiently kill different type of bacteria.[24] For instance, many studies highlight the promising potential of cathelicidin AMPs analogs for *H. pylori* clearance in combination or not with antibiotics.[62–64] However, due to the physiological conditions in the stomach, designing and administrating by oral route an AMP analog remain very challenging. Concerning the synthesis of defensin analogs, challenges remain in their size and complexity of disulfide pairing.[24] Nevertheless, even some efforts left to generate stable and efficient analogs of defensins, Pero et al[65] recently demonstrate

that this challenge is achievable, supporting the notion that our preclinical data may be translatable to humans and could be envisioned to alleviate or ameliorate antibiotic therapies for *H. pylori* eradication.

Our results show that pathogenic Th17 cells are involved in the vaccine-induced reduction of *H. pylori* infection and are reminiscent of the results of Annemann M et al[66] showing that pathogenic Th17 cells are protective against *citrobacter rodentium* infection. In the context of vaccine development, it is important to consider this information to select an adjuvant that promotes Th17 responses.[67] Bacterial components, including muramyl dipeptide (MDP), lipopolysaccharide (LPS), and CpG, are known to augment Th17 responses[68–71] and are very good candidates to be considered in vaccine formulation. CTB (e.g., Dukoral® vaccine), the *Escherichia coli* LT subunit B (LTB), or double mutant LT (dmLT) are also promising mucosal adjuvants to trigger specific Th17 immune responses. CTB as adjuvant has been almost evaluated fused to the vaccine antigen.[72–75] In addition to CTB, LTB and dmLT, recent adjuvant alternatives have been also evaluated such as mucosal administration of cyclic dinucleotides,[75] suggesting that the stimulation of mucosal *H. pylori*-specific Th17 responses could be envisioned in the future clinical trials.

In this study, we demonstrate for the first time the role of GM-CSF, most probably originating from pathogenic Th17 cells, in the vaccine-induced reduction of *H. pylori* infection. Mechanistically, we show that GM-CSF directly acts on gastric epithelial cells to induce the production of βdefensin3 and to kill *H. pylori*. Altogether, these findings highlight several potential alternatives and/or combination therapies to eradicate *H. pylori* infection in humans.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

D.V. conceived the project and designed the study. L.V., B.M., and D.V. analyzed the data and prepared the manuscript. L.V., P. O., and E.O.F. performed experiments.

INSTITUTIONAL REVIEW BOARD STATEMENT

Animal studies were approved by the State of Vaud Veterinary Office (authorization no. 836.11/2).

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SUPPORTING INFORMATION

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