Helicobacter

Helicobacter ISSN 1523-5378
doi: 10.1111/hel.12102

Helicobacter pylori Induces Increased Expression of the Vitamin D Receptor in Immune Responses

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Keywords
H. pylori, vitamin D receptor, cathelicidin, 1α, $25(OH)_2D_3$.

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Abstract

Background: Vitamin D receptor (VDR) is a member of the nuclear receptor family of transcription factors that play a critical role in innate immunity. This study examined the role of VDR in gastric innate immune defence against the gastric pathogen *Helicobacter pylori*.

Materials and Methods: Seventeen *H. pylori*-infected patients and sixteen controls participated in the study. The GES-1 cells were transfected with siRNA or incubated with or without $1\alpha,25(OH)_2D_3$ (100 nmol/L) then infected with *H. pylori*. VDR, cathelicidin antimicrobial protein (CAMP), and cytokine mRNA expression levels in normal and *H. pylori*-infected gastric mucosa and GES-1 cells was determined by qRT-PCR and correlated with the histopathologic degree of gastritis. Bactericidal activity was measured by using a colony-forming unit assay.

Results: Vitamin D receptor mRNA expression levels were significantly up-regulated in *H. pylori*-infected patients and positively correlated with chronic inflammation scores. There was a significant positive correlation between VDR and CAMP mRNA expression in *H. pylori*-positive gastric mucosa. VDR siRNA reduced *H. pylori*-induced CAMP production and conversely increased IL-6 and IL8/CXCL8 expression levels. The vitamin D agonist $1\alpha,25(OH)_2D_3$ increased CAMP expression and reduced cytokine activation in GES-1 cells infected with *H. pylori*. $1\alpha,25(OH)_2D_3$ could enhance the intracellular killing of the replicating bacteria, but the presence of siVDR and siCAMP led to a decline in its bactericidal ability.

Conclusions: The expression of VDR and CAMP in the gastric epithelium is up-regulated in the case of *H. pylori* infection; thus, VDR plays an important role in gastric mucosa homeostasis and host protection from *H. pylori* infection.

Helicobacter pylori is a spiral-shaped gram-negative microaerophilic bacterium which colonizes the human stomach and causes a number of gastrointestinal disorders, including gastritis, peptic ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma [1]. Host defence mainly involves the action of the innate immune system via neutrophils and lymphocytes. The role of the vitamin D receptor (VDR) in the antimicrobial activity against some bacteria has been reported. $1,25(OH)_2D_3$ signals through the vitamin D receptor, a ligand-stimulated transcription factor that recognizes specific DNA sequences called vitamin D response elements. $1,25(OH)_2D_3$ is a direct regulator of antimicrobial innate immune responses, upregulation and activation of VDR [2,3]. VDR is a member of the nuclear receptor family [4]; it is tightly associated with its heterodimeric partner, RXR, and only this liganded VDR-RXR heterodimer can penetrate the deep groove of DNA molecules and recognize vitamin D responsive elements (VDREs) in the DNA sequence of vitamin D-regulated genes [5]. The VDR/RXR complex controls more than 900 genes involved in

a wide array of physiologic functions including calcium homeostasis, growth control, differentiation and apoptosis of many cell types, regulation of immune responses and cytokine production [6,7]. Moreover, vitamin D deficiency is adversely associated with autoimmune diseases and inflammation [8].

The target genes of the VDR signal pathway include those of the enzyme Cyp24 and antimicrobial peptides (AMPs) β-defensin and cathelicidin (CAMP, also known as LL37, CAP18 or FALL39). Diverse combinations of cationic AMPs, including α- and β-defensins and cathelicidins, form a major component of the innate immune system in mammals [9,10]. Because bacteria have difficulty developing resistance against AMPs and are quickly killed in the presence of AMPs, this class of antimicrobial agents is being commercially developed as a source of peptide antibiotics [11–13].

The CAMP gene is directly regulated by binding of the VDR to a VDRE located in its promoter region, and its expression has been shown to be upregulated by VDR signaling in multiple cell types, including epithelial cells [14]. CAMP plays a role in several important activities including bactericidal action, antiseptic action, chemoattraction, and promotion of angiogenesis and wound healing [14]. *H. pylori* infection leads to upregulation of the production of CAMP via the gastric epithelium; this could mean that CAMP contributes to regulating the balance between host mucosal defence and *H. pylori* survival mechanisms that govern chronic infection with this gastric pathogen [9,10,15].

Previous studies have shown that the vitamin D agonist $1\alpha,25(OH)_2D_3$ induces AMP gene expression in isolated human keratinocytes, monocytes and neutrophils, and human cell lines and that $1\alpha,25(OH)_2D_3$ along with LPS synergistically induces CAMP expression in neutrophils [2,16]. Functional analyses indicate that $1\alpha,25(OH)_2D_3$ is a potent regulator of cytokine production by uterine neutral killer (uNK) cells; this indicates the potential role of vitamin D in immunoregulation [17].

It has been shown in vitro that the VDR-mediated antimicrobial response against *M. tuberculosis* infection involves the production of CAMP as part of the antimicrobial peptide response against the infection [18]. However, to our knowledge, the role of VDR-mediated CAMP expression in the antimicrobial activity against *H. pyroli* infection has not been reported so far.

The aim of this study was to determine the role of VDR and its target genes in gastric epithelial cell lines and gastric mucosa tissues infected with *H. pylori*. To this end, we studied the expression of VDR, CAMP, the cytokines IL-6 and IL8/CXCL8, DEFB4, and CYP24A1 in the study samples. The findings indicate that VDR plays an important role in immune defence against *H. pylori* infection and that the CAMP gene is a direct target of the transcription factor VDR.

Methods

Subjects and Tissue Samples

This study prospectively enrolled patients with *H. pylori* infection from among patients who underwent gastroscopy. Exclusion criteria were as follows: age <18 or >80 years, pregnancy, body mass index >30 kg/m^2, diabetes mellitus, cachectic state (including cancer), systemic infection, liver disease, renal impairment, use of medications effective against *H. pylori* during the preceding 3 months, alcohol abuse, drug addiction, and use of chronic corticosteroid or nonsteroidal anti-inflammatory medication, proton-pump inhibitors, bismuth salts or antibiotics in the 2 weeks prior to the gastroscopy. None of the subjects had undergone gastrointestinal surgery before. Before gastroscopy was performed, all the patients underwent a C13/C14 urea breath test to assess *H. pylori* status. During gastroscopy, two biopsy specimens were obtained from the gastric antrum along the lesser curvature. One sample was immediately frozen in liquid nitrogen until RNA isolation. The other was fixed in 10% formalin and embedded in paraffin for histopathologic analysis. Patients were considered positive for *H. pylori* infection if all of these examinations yielded positive results. On the other hand, patients were considered to be *H. pylori*-negative if all the test results were negative. This study was approved by the Ethical Committee of First Affiliated Hospital of Zhejiang University, Hangzhou, China. All samples were obtained with the written informed consent of the patients prior to their inclusion, in accordance with the Helsinki Declaration. The degree of inflammation in all the samples was verified by pathologic analysis. Patients who were found to have gastric cancer on enrollment or during follow-up were excluded. The chronic inflammation score on a scale of 0–3 (absence: 0; presence: score 1–3) was determined using the updated Sydney System [19].

Cell Culture Conditions

The human gastric epithelial cell line-GES-1 was obtained from Tumor Center of Cancer Institute & Hospital, Chinese Academy of Medical Sciences. The GES-1 cells were grown in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10% (vol/vol) fetal calf serum (Invitrogen, Carlsbad, CA, USA), penicillin (100 U/mL), streptomycin (100 μg/

mL), and 2 mmol/L L-glutamine at 37 °C in an atmosphere containing 5% CO_2. For the experiments, GES-1 cells were seeded at a density of 5×10^5 cells/mL of medium in six-well plates and grown to 80% confluence prior to the experiments.

Bacterial Strains and Growth Conditions

Helicobacter pylori strain SS1 (both $VacA^+$ and $CagA^+$) was obtained from the National Institute for Communicable Disease Control and Prevention (NICDC), Beijing, China. The strains were grown in a microaerobic humidified atmosphere (5% O_2, 10% CO_2, 85% N_2) on 10% lysed sheep blood Columbia agar at 37 °C. After 48–72 h, bacteria were harvested in phosphate-buffered saline (PBS) (pH 7.4) or in RPMI-1640 medium without antibiotics, resuspended to a concentration of 6×10^8 CFU/mL and used immediately.

Coculturing of GES-1 Cells with Bacteria

Subconfluent GES-1 cells were cultured alone or with various doses of freshly harvested *H. pylori* (1×10^4–6×10^8 CFU/mL) for various periods of time. At the end of the treatment, GES-1 cells were harvested and processed for the preparation of whole-cell extracts and western blotting.

Total RNA Extraction, cDNA Synthesis, and Real-Time Quantitative PCR

Total RNA was isolated from GES-1 cells or gastric mucosa tissues using the Trizol reagent (BBI) according to the manufacturer's instructions. The first-strand cDNAs were synthesized from total RNA using reverse transcriptase (Takara, Dalian, China) according to the manufacturer's instructions. All PCR primers were synthesized by Bio Basic Inc. (Shanghai, China) (Table 1). cDNA samples in each treatment group were pooled in subsequent experiments and reactions d set in a 15-μL reaction mixture in 96-well plates. Real-time RT-PCR quantitation for individual target mRNA was performed on an ABI Model 7500 Sequence Detector (Applied Biosystems, Foster City, CA) using a TaKaRa real-time PCR kit. RT-PCRs were performed using the following parameters: 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 34 s and 72 °C for 15 s. For each sample, a melting curve was generated at the end of the reaction to ensure specificity. Gene expression levels were normalized to those of GAPDH, and the data were analyzed using comparative cycle threshold calculations. Data were expressed as fold changes relative to the control group. Each real-time PCR experiment was run three times. The comparative $2^{-\Delta\Delta CT}$ method was used for quantification and statistical analysis (the results were expressed as fold changes relative to normal controls).

Table 1 Primers for real-time PCR

Primers	Sequences
VDR	Forward: 5′- AGCGGAAGGCACTATTCACC-3′
	Reverse: 5′-CATCATGCCGATGTCCACACA-3′
CAMP	Forward: 5′-TGG GCC TGG TGA TGC CT-3′
	Reverse: 5′-CGA AGG ACA GCT TCC TTG TAG C-3′
IL8/CXCL8	Forward: 5′-ACTGAGAGTGATTGAGAGTGGAC-3′
	Reverse: 5′-AACCCTCTGCACCCAGTTTTC-3′
IL-6	Forward: 5′-CCTGAACCTTCCAAAGATGGC -3′
	Reverse: 5′-TTCACCAGGCAAGTCTCCTCA -3′
DEFB4	Forward: 5′-CTCCTCTTCTCGTTCCTCTTCA -3′
	Reverse: 5′-GCAGGTAACAGGATCGCCTAT-3′
CYP24A1	Forward: 5′-GGTGGCGAGACTCAGAACG-3′
	Reverse: 5′-GTCGTGCTGTTTCTTGAGACC-3′
GAPDH	Forward: 5′-CTCACCGGATGCACCAATGTT-3′
	Reverse: 5′-CGCGTTGCTCACAATGTTCAT-3′

siRNA Silencing

GES-1 cells were transfected with either nonspecific siRNA oligomers or siRNAs targeting the VDR mRNA (Invitrogen, Shanghai, China) by using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The cells were seeded into 24-well plates and grown in phenol red-free RPMI1640 supplemented with 5% FBS. Liposomes containing control or VDR siRNA were synthesized by incubating 40 pmol of each siRNA duplex with 2 μL of Lipofectamin™2000 for 20 min at room temperature in a total volume of 500 μL of phenol red-free DMEM without antibiotics. The liposomes were added to the cells and siRNA treatment was continued for 24 h, and then, the cells were treated with *H. pylori* at a multiplicity of infection (MOI) of 100 for 24 h and finally exposed to either a solvent (ethanol, <0.1% final concentration) or 1 nmol/L $1\alpha,25(OH)_2D_3$ for the indicated time periods.

Western Blot Analysis

GES-1 cells were treated with siVDR or $1\alpha,25(OH)_2D_3$ the next day with or without *H. pylori* for 24 h for a time-course study. At the end of the incubation period, cells were washed with PBS. The cells were scraped into the lysis buffer (Sangon Biotech Inc., Shanghai) and centrifuged at ~14,000 ×*g* for 10 min to pellet the cell debris. Total protein was quantified using the Bradford assay, and equal amounts of protein were separated by

12% SDS–PAGE and then transferred to a polyvinylidene difluoride membrane (Milipore, Buckinghamshire, UK). The membranes were blocked at room temperature for 1 h with 5% nonfat milk in 1 × TBST (TBS + 0.05% Tween 20) and subsequently incubated with mouse anti-VDR at 1:500 (sc-13133, Santa Cruz Biotechnology Inc.), mouse anti-CAMP at 1:1000 (sc-130552, Santa Cruz Biotechnology Inc.) or mouse anti-GAPDH at 1:10,000 (sc-130301, Santa Cruz Biotechnology Inc.) at 4 °C overnight. After washing with TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibody at room temperature for 1 h, and the antibody binding was visualized using the ECL detection system (MultiSciences).

Bactericidal Assays

GES-1 cells were infected with *H. pylori* SS1 (1×10^8 bacteria/mL) in the presence or absence of siVDR, siCAMP or $1,25(OH)_2D_3$. After incubation for 2 h at 37 °C in an atmosphere containing 5% O_2, 10% CO_2, and 85% N_2, GES-1 cells were washed two times and treated with 150 μg/mL gentamicin for 2 h to kill extracellular bacteria. The infected cells were washed two times and then incubated with gentamicin-containing (25 μg/mL) medium before the samples were harvested. The cells were lysed with 1 mL of 0.01% saponin in Dulbecco's phosphate-buffered saline (DPBS) and diluted and then plated on Columbia agar plates; the number of visible colonies was counted after 3–5 days of incubation. The CFU data are derived from triplicate wells in three independent experiments using three separate donors.

Statistical Analysis

All data are expressed as the mean ± standard deviation (SD) value from at least three independent experiments. Statistical analysis was performed using two-tailed unpaired Student's *t*-test or one-way ANOVA with Bonferroni's multiple comparison test or two-tailed Pearson's correlation coefficient analysis to analyze statistically significant differences between groups by the SPSS software (version 16.0). Differences at $p < .05$ were considered significant.

Results

Patients and Gastric Mucosal Specimens

A total of 33 outpatients (mean age, 53.6 years; range, 24–78 years) consisting of 12 men and 21 women who underwent upper gastrointestinal endoscopy were enrolled in this study. Based on the results of the C^{13}/C^{14} urea breath test and histopathologic analysis, 17 subjects were designated as positive and 16 as negative for *H. pylori* infection. Table 2 shows the number of biopsy samples that were found to be representative of each histopathologic category (chronic inflammation and intestinal metaplasia) and each grade (normal, mild, moderate, and marked) of gastritis in accordance with the updated Sydney system (18). Baseline characteristics, including age, gender, alcohol intake, smoking habits, and body mass index, did not differ significantly between the *H. pylori*-positive and *H. pylori*-negative groups.

H. pylori Induces Upregulation of VDR, CAMP and Cytokine mRNA Levels in Gastric Mucosa Tissues

Vitamin D receptor, CAMP, IL-6, IL8/CXCL8, DEFB4, and CYP24A1 mRNA levels were significantly elevated in the gastric mucosa of *H. pylori*-positive patients, compared with *H. pylori*-negative patients (Fig. 1). Moreover, a significant positive correlation between VDR, DEFB4, and CYP24A1 mRNA levels and chronic inflammation scores (correlation coefficient $r = .536$, $p < .01$; $r = .390$, $p = .025$; $r = .398$, $p = .022$, respectively, Fig. 2A–C) was observed. The CAMP levels in turn were found to have a significant positive correlation with the VDR levels ($r = .814$, $p < .001$, Fig. 2D). Moreover, the IL-6 and IL8/CXCL8 mRNA expression levels also showed a significant positive correlation with chronic inflammation scores.

H. pylori Induces VDR and CAMP Expression In Vitro

To further characterize the effect of *H. pylori* on the expression of VDR and CAMP, GES-1 cells were exposed to *H. pylori* at an MOI ranging from 0 to 100

Table 2 Histopathologic characteristics of the biopsy samples

	H. pylori		
Category	Positive	Negative	Total
Chronic inflammation			
Normal	0	0	0
Mild	0	13	13
Moderate	13	3	16
Marked	4	0	4
Intestinal metaplasia			
Normal	11	16	27
Mild	3	0	3
Moderate	0	0	0
Marked	3	0	3

for 0–24 h. VDR and CAMP expression during infection was measured by quantitative real-time PCR and western blot analysis. GES-1 cells infected with *H. pylori* showed increased expression of VDR, in an MOI- and time-dependent manner (Fig. 3A,B). VDR was expressed at a significantly higher level in the *H. pylori*-infected group than in the normal group. The expression of CAMP showed no significant changes in cells infected with *H. pylori* at low MOI (MOI = 10) or for short durations (0–12 h).

Interestingly, expression patterns of IL-6 and IL8/CXCL8 mRNA showed an association with MOI and incubation time: IL-6 and IL8/CXCL8 expression increased to maximum levels at an MOI of 10 for 24 h, but higher concentrations of *H. pylori* did not result in a further increase in expression (Fig. 4A). In addition, IL-6 and IL8/CXCL8 expression reached maximum levels after 12 h of stimulation and subsequently declined (Fig. 4B).

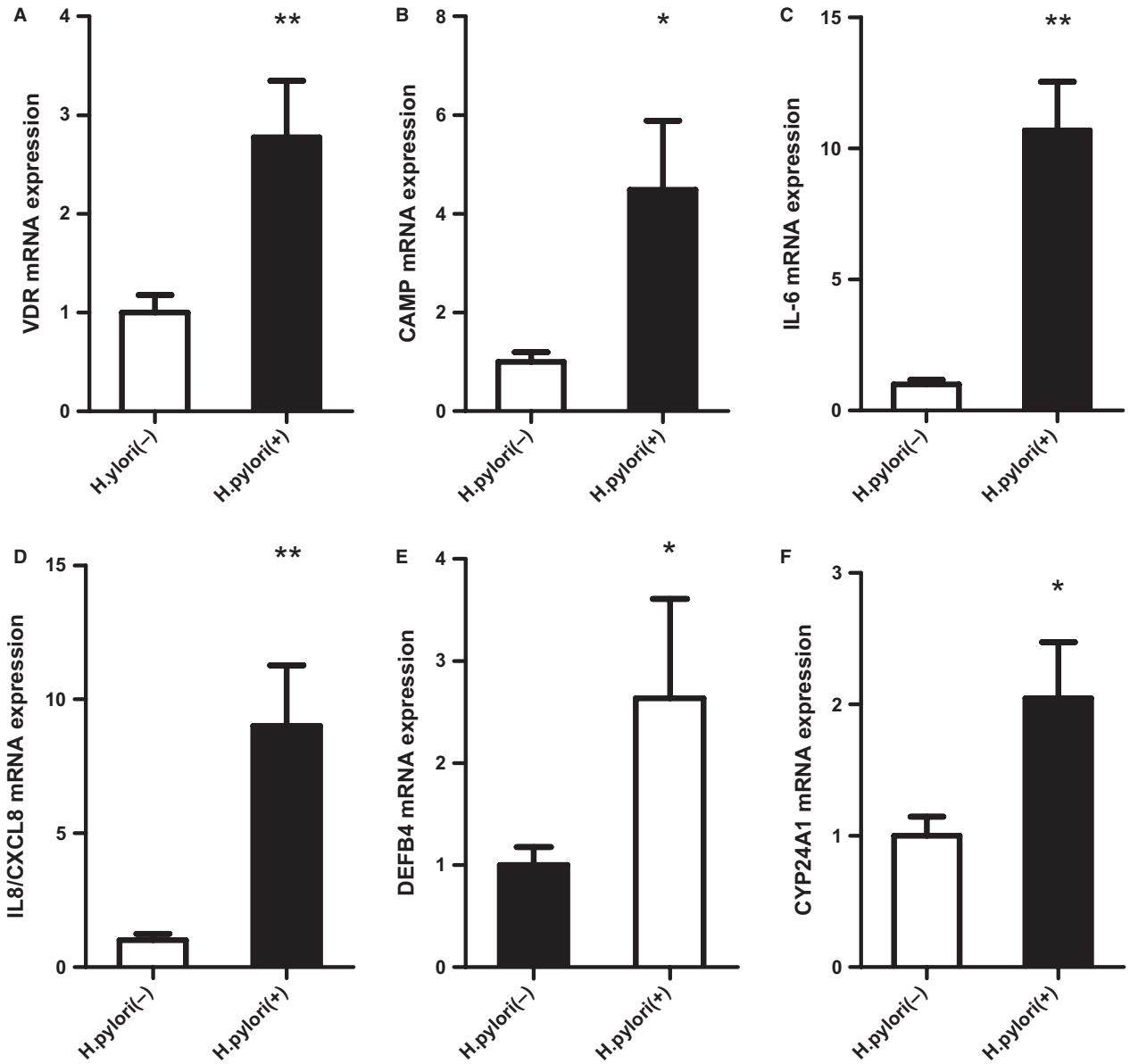

Figure 1 Comparison of the expression level of VDR (*A*), CAMP (*B*), IL-6 (*C*), IL8/CXCL8 (*D*), DEFB4 (*E*) and CYP24A1 (*F*) between gastric tissues infected with *H. pylori* and normal tissue samples. The expression levels were determined by qRT-PCR and were normalized to that of GAPDH. The expression of VDR, CAMP, IL-6, IL8/CXCL8, DEFB4 and CYP24A1 was found to be significantly higher in *H. pyroli*-infected tissues compared with normal tissues. $*p < .05$, $**p < .01$.

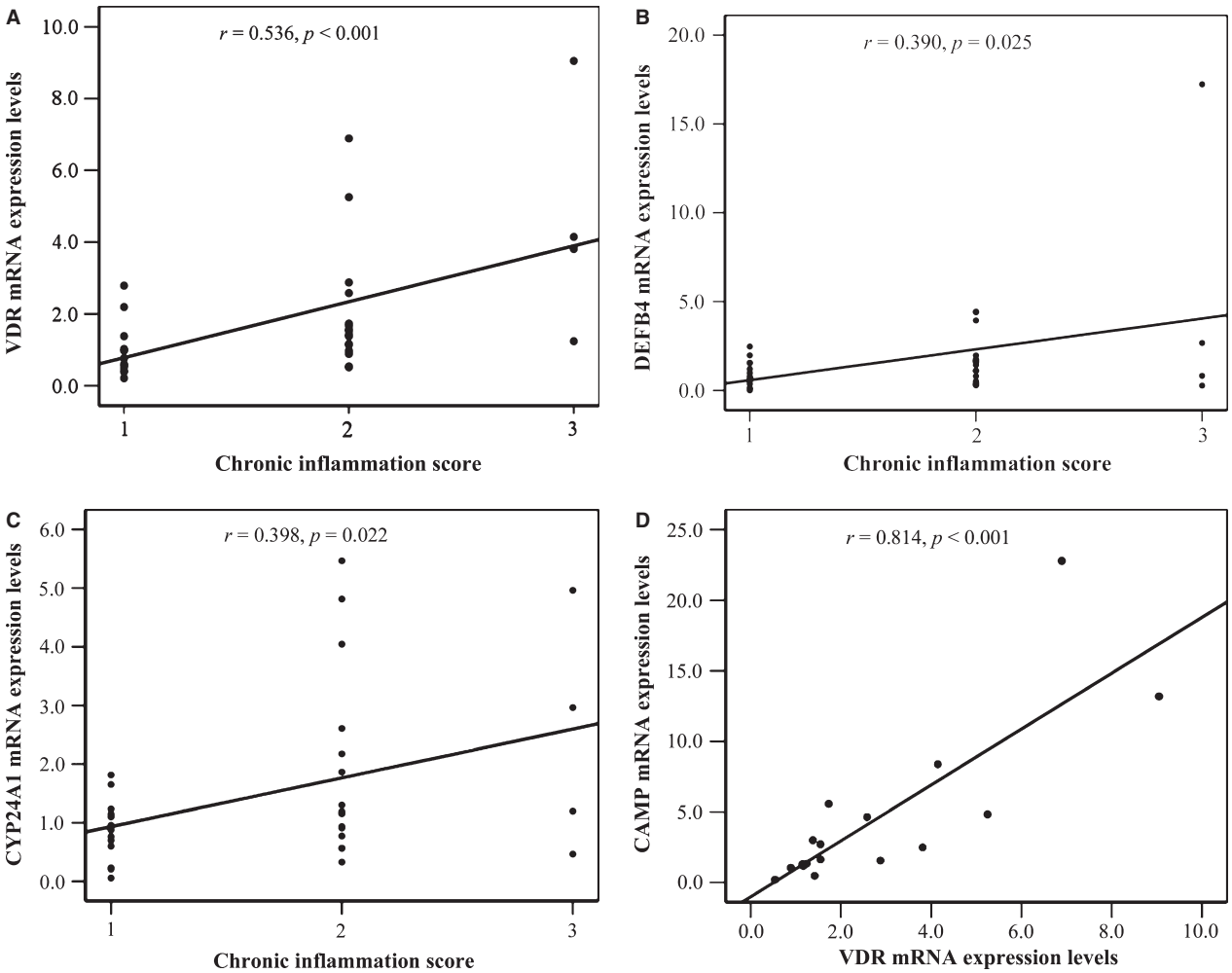

Figure 2 Relationship between VDR mRNA expression in the gastric mucosa and chronic inflammation scores and CAMP mRNA expression. (*A*, *B*, *C*) Analysis of correlation between VDR, DEFB4 and CYP24A1 mRNA expression and the chronic inflammation score in *H. pylori*-infected sample tissues. (*B*) Analysis of correlation between VDR and CAMP expression in *H. pylori*-infected chronic gastritis tissues.

Genetic Manipulation of VDR Expression Affects CAMP and Cytokine Expression Regulation After *H. pylori* Infection

We next used siRNAs to investigate the role of VDR in the downstream modulation of antimicrobial activity against *H. pylori*. VDR silencing effectively knocked down the expression of VDR by 80% (Fig. 5A,B). Inhibition of VDR expression resulted in appreciable downregulation of CAMP mRNAs and proteins compared with the negative control siRNA-treated group (Fig. 5A, B). To address the regulatory role of VDR in antimicrobial activity, siVDR and nonspecific control-transfected GES-1 cells were also infected with *H. pylori* at an MOI of 100 for 24 h. The results revealed that there was a significant decrease in CAMP expression with VDR siRNA compared with the negative control siRNA.

We next analyzed the gene expression profile of antimicrobial genes isolated from the negative control siRNA- and VDR siRNA-transfected cells. We found significant downregulation of DEFB4 and CYP24A1 with siVDR, in the presence and absence of *H. pylori* infection (Fig. 5C,D).

$1\alpha,25(OH)_2D_3$ Regulates IL-6, IL8/CXCL8, and CAMP Expression

We investigated the effects of $1\alpha,25(OH)_2D_3$ (100 nm) on immune response to *H. pylori* and assessed the cytokine levels by qRT-PCR. IL-6 and IL8/CXCL8 production was decreased by more than 50% ($p < .05$). These results implied that $1\alpha,25(OH)_2D_3$ is able to modify the cytokine response to *H. pylori* toward an anti-inflammatory profile (Fig. 6A).

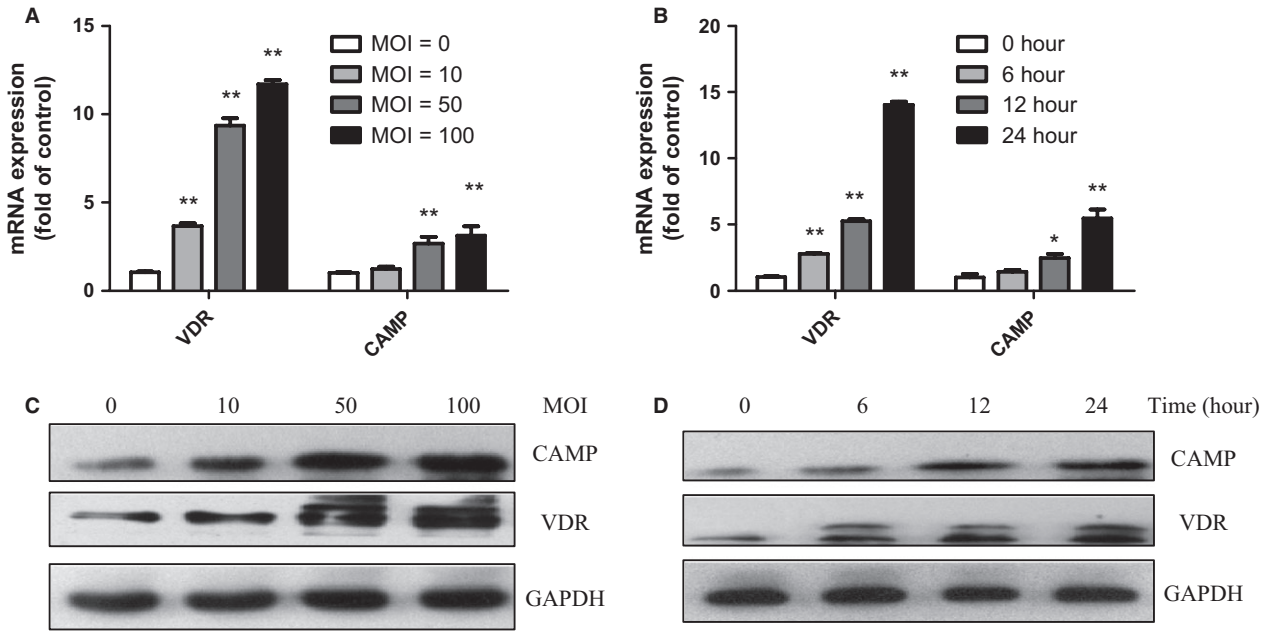

Figure 3 Effect of *H. pylori* infection at different MOIs and for different times on the expression of VDR and CAMP in gastric epithelial cell lines. (*A*) SYBR Green real-time PCR results showed a significant increase in VDR expression with increase in MOI; the increase in CAMP expression was significant only after a certain MOI value. (*B*) SYBR Green real-time PCR results showed a significant increase in VDR expression with increase in time; the increase in CAMP expression was significant only after a certain time point. All the data were normalized to that of GAPDH mRNA. (*C*) and (*D*) show the corresponding western blotting results for (*A*) and (*B*) respectively. $*p < .05$, $**p < .01$.

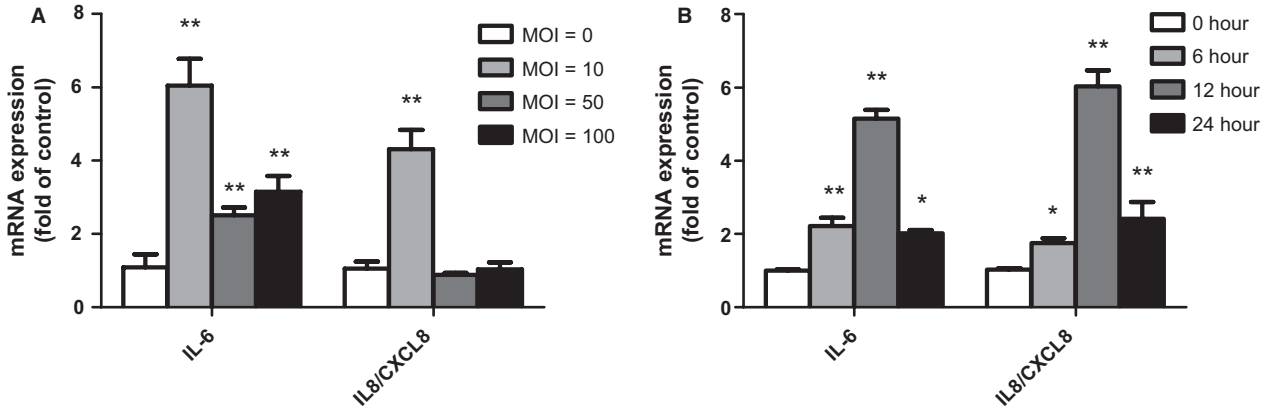

Figure 4 Effect of *H. pylori* infection at different MOIs and for different times on IL-6 and IL8/CXCL8 expression in vitro. (*A*) qRT-PCR results showed that the expression of IL-6 and IL8/CXCL8 vary with MOI. (*B*) The expression of both IL-6 and IL8/CXCL8 showed a similar pattern with incubation time. $*p < .05$, $**p < .01$.

In the absence or presence of $1\alpha,25(OH)_2D_3$ and *H. pylori* treatment of GES-1 cells, $1\alpha,25(OH)_2D_3$ significantly increased CAMP mRNA levels, as revealed by qRT-PCR (Fig. 6B) and western blotting (data not shown). More importantly, the medium from $1\alpha,25(OH)_2D_3$-treated cells acquired antibacterial activity indicative of enhanced secretion of functional AMPs.

Finally, we noted that the effects of $1\alpha,25(OH)_2D_3$ on AMP gene expression are not limited to *camp*, as we also found that $1\alpha,25(OH)_2D_3$ stimulated the expression of DEFB4 and CYP24A1; moreover, the expression further increased in *H. pylori*-infected cells (Fig. 6C).

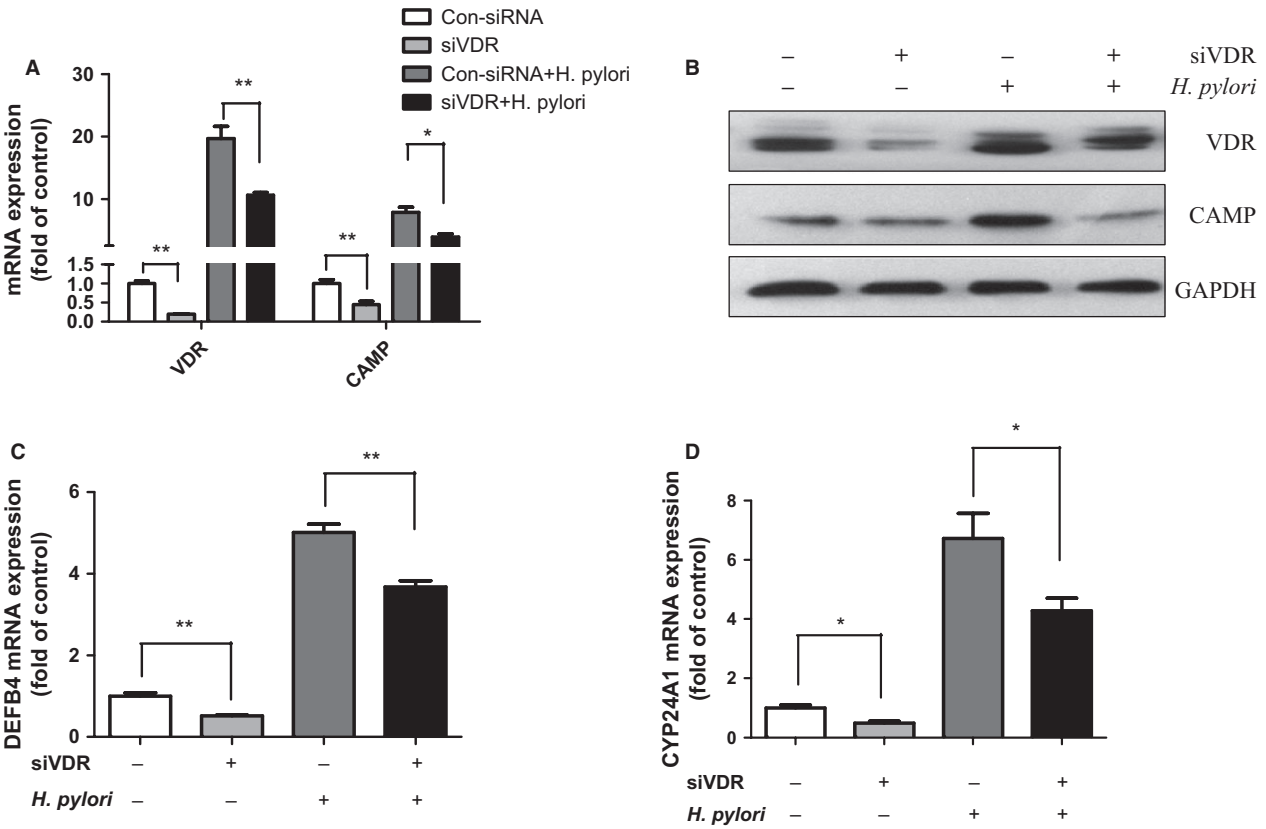

Figure 5 Effect of siRNA-mediated silencing of VDR expression in GES-1 cells on the expression of VDR, CAMP, IL-6, IL8/CXCL8, DEFB4 and CYP24A1 mRNA and protein. (*A*) qRT-PCR and (*B*) western blot results showed that VDR and CAMP expression was decreased in siVDR-transfected cells compared with the control siRNA-transfected cells in both *H. pylori*-infected and normal cells. qRT-PCR results showed that the expression of (*C*) DEFB4 and (*D*) CYP24A1 was decreased in siVDR-transfected cells compared with the control siRNA-transfected cells in both *H. pylori*-infected and normal cells. *$p < .05$, **$p < .01$.

Effect of siVDR on Antimicrobial Activity Against *H. pylori*

Because *H. pylori* infection was found to upregulate VDR production, we investigated the effect of VDR knockdown on antimicrobial activity against *H. pylori*. As shown in Fig. 7A, treatment with siVDR increased the viability of bacteria in the siRNA-transfected cells, as measured by CFU.

Because *H. pylori* infection up-regulated CAMP production, we tried to address the role of CAMP in antimicrobial activity by transfecting *H. pylori*-infected ES-1 cells (at an MOI of 100 for 2 h) with siCAMP and Con-siRNA. The cells were harvested and analyzed for viability by a CFU assay. As shown in Fig. 7B, knockdown of the CAMP gene resulted in reduction of the antimicrobial activity of GES-1 cells.

To determine whether $1\alpha,25(OH)_2D_3$ is active against *H. pylori*, we examined its antimicrobial activity by incubation of *H. pylori* SS1 with $1\alpha,25(OH)_2D_3$ (100 nmol/L). As shown in Fig. 7C, the bactericidal assay revealed that incubation of *H. pylori* SS1 with $1\alpha,25(OH)_2D_3$ (100 nmol/L) resulted in a decrease in bacterial viability within 2 h.

Discussion

In this study, we have provided evidence for the role of VDR-mediated CAMP and cytokine (IL-6 and IL8/CXCL8) expression in the antimicrobial activity of gastric cells against *H. pylori*.

We were able to confirm significantly increased VDR mRNA levels in *H. pylori*-infected gastric mucosa. Moreover, the mucosal VDR mRNA levels were positively correlated with the chronic inflammation scores. In the in vitro experiment, VDR expression was associated with MOI and incubation time. Similar roles of VDR have been reported in the antibacterial action against *M. tuberculosis* (7, 8) and the invasive enteric

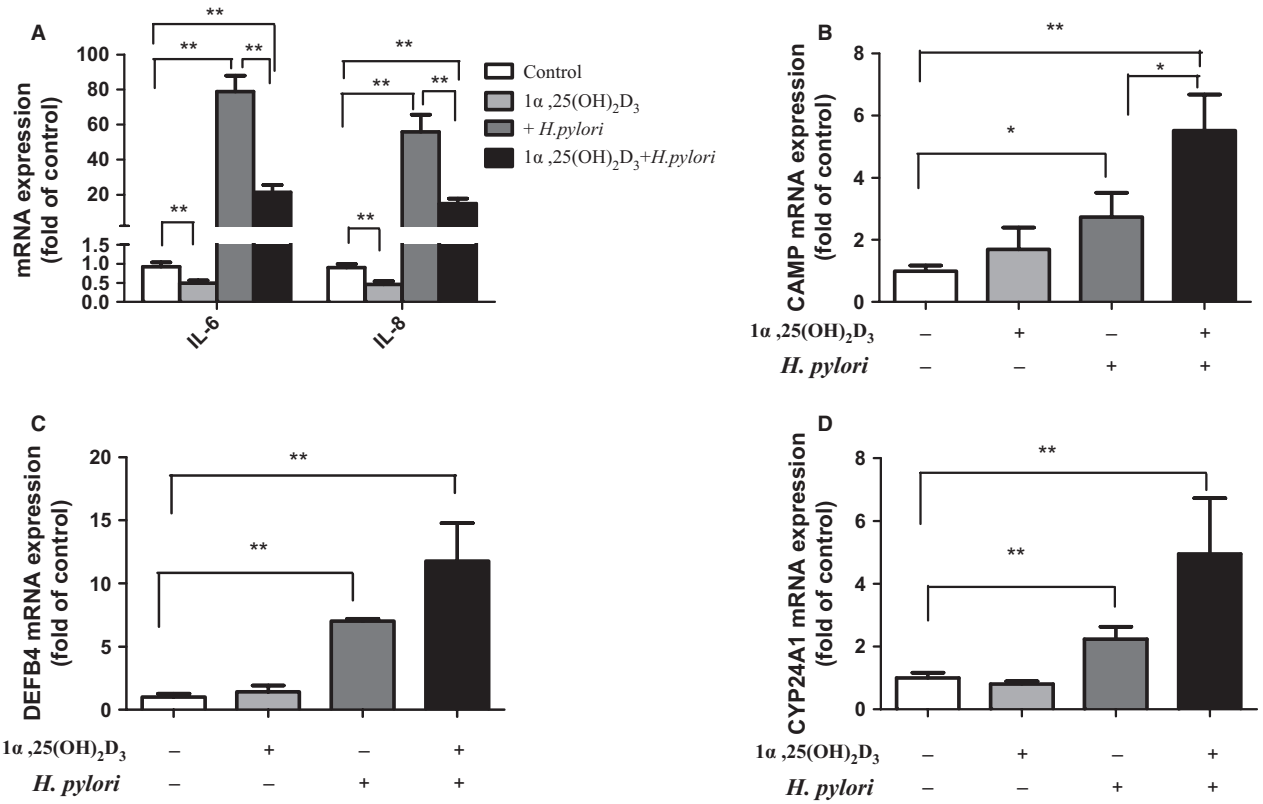

Figure 6 Effect of 1α, 25(OH)$_2$D$_3$ on the expression of cytokines, CAMP, DEFB4 and CYP24A1 in GES-1 cell lines. (*A*) RT-PCR results showed that incubation with 1α, 25(OH)$_2$D$_3$ (100 nmol/L) for 24 h decreased IL-6 and IL8/CXCL8 expression in *H. pylori*-infected and normal GES-1 cells. RT-PCR results showed that 1α, 25(OH)$_2$D$_3$ treatment increased the expression of CAMP (*B*), DEFB4 (*C*) and CYP24A1 (*D*) in *H. pylori*-infected and normal GES-1 cells. *$p < .05$, **$p < .01$.

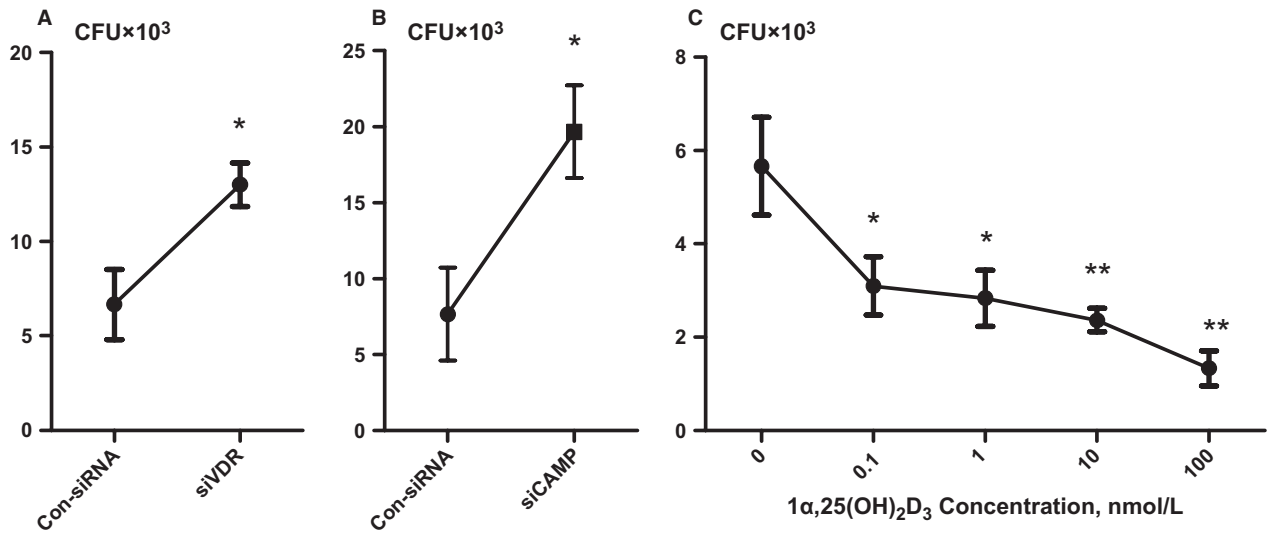

Figure 7 Effects of VDR knockdown, CAMP knockdown and 1α,25(OH)$_2$D$_3$ treatment on viability of *H. pylori* in infected cells, as determined by the CFU values. Treatment with both siVDR (*A*) and siCAMP (*B*) led to a significant increase in bacterial cell viability. (*C*) Treatment with increasing concentrations of 1α,25(OH)$_2$D$_3$ led to a corresponding progressive decrease in cell viability. *$p \leq .05$, ** $p \leq .01$.

pathogen *Salmonella typhimurium*, which can induce colonic VDR expression and localization in vivo, and stimulate VDR expression, transcription, and signaling in colonic epithelial cell lines and MEFs [20].

We found that *H. pylori* infection-induced upregulation of CAMP expression in the gastric mucosa, which was comparable with previously published results (3, 14). Moreover, our results showed that CAMP expression in gastric epithelial cells was also upregulated upon *H. pylori* infection with a sufficient bacterial load and duration of infection. Thus, CAMP can block *H. pylori*-induced inflammation [10]. CAMP was positively associated with VDR mRNA expression in *H. pylori*-positive mucosa and GES-1 cells. This is in agreement with a recent study which showed that activation of toll-like receptor-2 on human macrophages upregulated the expression of VDR and induced expression of human CAMP and killing of intracellular *M. tuberculosis* [7]. Activation of the CAMP gene occurred via a consensus VDRE in the promoter that is bound by VDR. Previous studies provide evidence that the CAMP gene is a direct target of the transcription factor VDR, which mediates strong upregulation of CAMP in response to treatment of cells with $1\alpha,25(OH)_2D_3$ and its analogs [14,21]. In our study too, we found that the VDR agonist $1\alpha,25(OH)_2D_3$ increased the production of CAMP. CAMP expression was further increased in *H. pylori*-infected cells, which is in agreement with data previously reported for the regulation of CAMP expression in vitamin D-mediated antimicrobial response [7]. Together, these findings suggest that increase in the production of the antimicrobial peptide CAMP may play a critical role in host defence against *H. pylori*. In addition, our results indicate that $1\alpha,25(OH)_2D_3$ has the ability to directly induce antimicrobial gene expression and activity of the antimicrobial peptide CAMP.

We also examined the effect of VDR on the production of IL-6 and IL8/CXCL8. We show here that knockdown of the VDR gene increased the levels of IL-6 and IL8/CXCL8. Therefore, $VDR^{-/-}$ cells are more susceptible to inflammatory stimuli in inflammatory responses. This observation is in agreement with previous reports that mouse fibroblasts lacking VDR exhibit increased NF-κB activity, leading to increased production of IL-6 [20]. NF-κB activation possesses an inherent self-amplifying potency via induction of IFN-γ and pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, IL8/CXCL8, and TNF-α [22,23]. Moreover, loss of VDR leads to more aggressive gross and histologic colonic injury, increases serum IL-6 levels, which are a marker of systemic inflammation, and enhances mortality after *Salmonella* infection [5]. We have also demonstrated that the proinflammatory cytokines IL-6 and IL8/CXCL8 are suppressed by $1\alpha,25(OH)_2D_3$. Collectively, these data suggest that cells that lack VDR appear to be in a preinflammatory or proinflammatory state. It will be very interesting to further investigate the inflammatory status of $VDR^{-/-}$ mice with *H. pylori* infection.

According to a case–control study, the average concentration of vitamin D in subjects with autoimmune gastritis was 9.8 ± 5.6 ng/mL; nonspecific gastritis patients, 22.2 ± 13.5 ng/mL; and *H. pylori* gastritis patients, 11.3 ± 8.4 ng/mL [24]. However, another Nutritional Deficiencies investigation showed that the 25-OH vitamin D_3 levels did not differ between *H. pylori*+ and *H. pylori*− patients ($p > .20$) [25]. Unfortunately, in our study, we were unable to obtain samples promptly to test the concentration of vitamin D. However, we were able to confirm that the vitamin D agonist $1\alpha,25(OH)_2D_3$ had in vitro antimicrobial activity against *H. pylori*.

In our study, we found that $1\alpha,25(OH)_2D_3$ leads to a decrease in IL-6 and IL8/CXCL8 levels. Similar to this, $1\alpha,25(OH)_2D_3$ was found to suppress the production of a spectrum of inflammatory cytokines in immune and other cells (such as keratinocytes), including IL-1, IL-2, IL-6, IL8/CXCL8 (29), INF-γ, and TNF-α [26]; this action forms the basis for its anti-inflammatory mechanism. Therefore, $1\alpha,25(OH)_2D_3$ is a marker of systemic inflammation in *H. pylori* infection. Moreover, $1\alpha,25(OH)_2D_3$ is involved in anti-inflammatory action through its agonistic effect on VDR, which targets the antimicrobial peptide CAMP gene in GES-1 cells. Taken together, our data show that $1\alpha,25(OH)_2D_3$ has multiple effects on the expression and release of antimicrobial peptides. We also found that the effects of $1\alpha,25(OH)_2D_3$ on the expression of VDR, CAMP, DEFB4 and CYP24A1. Similar to this, DEFB4 has been shown to be upregulated under *H. pylori* infection-associated inflammatory conditions in vivo and under cagA-positive *H. pylori* infection in AGS cells in vitro [27]; moreover, the DEFB4 promoter contains VDREs [28]. In agreement with all these findings, $1\alpha,25(OH)_2D_3$ is known to regulate anti-inflammatory activity and other facets of immunity, including the induction of innate immune responses [7,29].

In conclusion, this study has shown that VDR has an effect on antimicrobial activity against *H. pylori*. Our data are consistent with and explain at least in part, the critical role of the VDR/CAMP pathway in innate immunity. Moreover, these findings help improve our understanding of the anti-inflammatory mechanism of vitamin D. Given the importance of this subject, more studies are warranted to further understand the functional significance as well as the molecular mechanisms underlying this role of VDR.

Acknowledgements and Disclosures

This study was supported by National Natural Science Foundation of China (No. 30600281) and National 973 Program (2013CB911303).

Competing interests: The authors have no financial conflicts of interest.

Author Contributions

All the coauthors of this paper have contributed to the intellectual content of the paper.

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