Helicobacter *pylori*-induced Rev-erbα fosters gastric bacterial colonization by impairing host innate and adaptive defense

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Abstract

Background: Helicobacter pylori (H. pylori) is a human pathogen that infects nearly half of the world's population, however, the persistent colonization of H. pylori in gastric mucosa remains poorly understood. Nowadays it is believed that impairment of host defense of gastric epithelium induced by H. pylori plays key roles in H. pylori-associated pathology. The nuclear receptor Rev-erbα represents a powerful transcriptional repressor involved in host immunity. However, the regulation, function, and clinical relevance of Rev-erbα in H. pylori infection are presently unknown. Here we demonstrated a pro-colonization role of Rev-erbα in H. pylori infection.

Results: Rev-erbα was increased in gastric mucosa of H. pylori-infected patients and mice. H. pylori induced gastric epithelial cells (GECs) to express Rev-erbα via the phosphorylated cagA that activated extracellular signal-regulated kinase (ERK) signaling pathway to mediate transcription factor nuclear factor kappa-B (NF-κB) directly binding to Rev-erbα promoter. Human gastric Rev-erbα expression correlated with H. pylori colonization, and mouse Rev-erbα from non-bone marrow-derived cells promoted gastric H. pylori burden. Importantly, H. pylori colonization was attenuated in Rev-erbα-/- mice and the mice with in vivo pharmacological inhibition of Rev-erbα. Mechanistically, Rev-erbα in GECs not only directly suppressed Reg3b and β-defensin-1 expression via binding to Reg3b and β-defensin-1 promoter respectively, which resulted in impaired bactericidal effects against H. pylori of these antibacterial proteins in vitro and in vivo; but also directly inhibited chemokine CCL21 expression via binding to CCL21 promoter, which led to decreased gastric influx of CD45+CD11c-Ly6G-CD11b+CD68- myeloid cells by CCL21-CCR7-dependent migration and, as a direct consequence, reduced bacterial clearing capacity of H. pylori-specific T helper type 1 (Th1) cell response.

Conclusions: Overall, this study identifies a model involving Rev-erbα, which collectively ensures gastric bacterial persistence by suppressing host gene expression required for local innate and adaptive defense against H. pylori, and also highlight a pathological role and an immunosuppressive mechanism of Rev-erbα in persistent H. pylori infection.

Introduction

Helicobacter pylori (*H. pylori*) is a human pathogen that infects nearly half of the world's population and produces a persistent infection that can lead to gastric ulcers and gastric cancer [1]. Although the persistent colonization of *H. pylori* in gastric mucosa remains poorly understood, it is believed that the impaired host defense induced by *H. pylori* is a key contributing factor. Gastric epithelial cells (GECs) are not only the first-contacted cell type to exert host defense in gastric mucosa during *H. pylori* infection but also the targets modulated by *H. pylori* to re-create gastric microenvironment that may favor gastric *H. pylori* persistence. Among the many altered molecules in GECs in response to *H. pylori* infection are the nuclear receptors [2].

Rev-erbα (also called Nuclear Receptor Subfamily 1 Group D Member 1, NR1D1), an orphan nuclear receptor, encoded by *Rev-erbα* and belonged to the thyroid hormone receptor-like superfamily nuclear receptors [3], is part of the clock-keeping machinery [4] and plays an important role in regulating metabolism [5] and immunity [6]. It can physiologically modulate genes involved in lipid [7], bile acid [8] and glucose metabolism [9] in liver and adipose tissues. Under non-infectious conditions, Rev-erbα controls excessive inflammatory responses in liver [4] or pulmonary [10] inflammation. In infectious diseases, Rev-erbα plays roles in antimycobacterial function by repressing IL-10 expression [11, 12] and has protective effects in *vesicular stomatitis virus* (*VSV*)-induced encephalitis model through the inhibition of CCL2 expression [13], respectively. To date, virtually nothing is known about the regulation, function, and clinical relevance of Rev-erbα in GECs during *H. pylori* infection in either humans or mice.

In the current study, we have, for the first time, demonstrated a pro-colonization role of Rev-erbα in *H. pylori* infection. Increased Rev-erbα is detected in gastric mucosa of *H. pylori*-infected patients and mice, and Rev-erbα expression is induced in GECs by *H. pylori* in a *cagA*-dependent manner via extracellular signal-regulated kinase (ERK)- nuclear factor kappa-B (NF-κB) pathway activation. On the one hand, Rev-erbα impairs host innate defense to promote gastric *H. pylori* infection by directly suppressing the expression of antibacterial proteins Reg3b and β-defensin-1. On the other hand, Rev-erbα directly inhibits CCL21 production, which in turn reduces $CD45^{+}CD11c^{-}Ly6G^{-}CD11b^{+}CD68^{-}$ myeloid cell chemotaxis, as a direct consequence, impairs *H. pylori*-specific Th1 cell response leading to increasing *H. pylori* colonization. Collectively, these data highlight a pathological role for Rev-erbα in persistent *H. pylori* infection.

Results

Rev-erbα is increased in gastric mucosa of H. pylori-infected patients and mice

Although Rev-erbα was reported to play antimycobacterial role [11], it is currently not known whether it does also play a role during *H. pylori* infection. To evaluate the potential role of Rev-erbα, we first compared the mRNA expression profiles of thyroid hormone receptor-like superfamily nuclear receptors in human primary gastric mucosa of *H. pylori*-infected and uninfected donors. Among them, Rev-erbα was the most increased one in gastric mucosa infected with *H. pylori* compared to paired uninfected counterparts (Fig. 1a). We then confirmed that, compared to uninfected donors, the overall Rev-erbα mRNA level was higher in gastric mucosa of *H. pylori*-infected patients (Fig. 1b), suggesting an increased Rev-erbα in gastric mucosa of *H. pylori*-infected patients.

The presence of *cagA* is strongly associated with the pathogenicity of *H. pylori* [14]. Notably, we found that Rev-erbα expression in *cagA*-positive patients was significantly higher than that in *cagA*-negative individuals (Fig. 1c). Furthermore, a positive correlation between Rev-erbα expression and *H. pylori* colonization in gastric mucosa of *H. pylori*-infected patients (Fig. 1d) suggested Rev-erbα induction by *H. pylori*. Consistent with our findings in humans, Rev-erbα expression was also much higher in WT *H.*

pylori-, but not in *ΔcagA*-infected mice, reaching a peak from 8 weeks p.i. (Fig. 1e), indicating a key role for *cagA* to induce Rev-erbα during *H. pylori* infection *in vivo*. Furthermore, immunofluorescence staining (Fig. 1f; Supplementary Fig. 1a), immunohistochemical staining (Fig. 1g) and western blot analysis (Fig. 1h) also showed that the level of Rev-erbα protein was higher in gastric mucosa of *cagA*-positive *H. pylori*-infected patients and WT *H. pylori*-infected mice, compared to either uninfected or *cagA*-negative patients and *ΔcagA*-infected counterparts. Furthermore, infection with WT *H. pylori ex vivo*, the levels of Rev-erbα mRNA and protein in human primary gastric mucosa were also significantly increased compared to those in the samples either not infected or infected with *ΔcagA* (Fig. 1i). Similar observations were made when infecting with *H. pylori* 26695 (Supplementary Fig. 1b). Taken together, these findings suggest that Rev-erbα is increased in *H. pylori*-infected gastric mucosa of patients and mice.

Gastric epithelial cells infected by H. pylori express Rev-erbα

GECs, the first-contacted cells in gastric mucosa during *H. pylori* infection [14], might be responsible for Rev-erbα expression after *H. pylori* infection. Notably, within gastric mucosa of *H. pylori*-infected patients, Rev-erbα was expressed in the cytoplasm and nucleus of $CD326^+$ GECs (Fig. 4a; Supplementary Fig. 2a), suggesting that GECs express Rev-erbα in gastric mucosa during *H. pylori* infection.

Next, to explore the Rev-erbα induction in GECs, we confirmed increased Rev-erbα expression in the nucleus of AGS cells infected with *H. pylori* (Fig. 2b; Supplementary Fig. 2b, c). We also confirmed by microarray that, Rev-erbα, one member of the thyroid hormone receptor-like superfamily nuclear receptors, was the most increased nuclear receptor induced by *H. pylori* in AGS cells (Fig. 2c). Notably, compared to uninfected or *ΔcagA*-infected, WT *H. pylori*-infected AGS cells and human primary GECs potently increased Rev-erbα expression (Fig. 2d). Similar observations were made when other human GEC lines were infected with *H. pylori* (Supplementary Fig. 2e-h). We further demonstrated that *H. pylori*-infected AGS cells (Fig. 2e) and human primary GECs (Fig. 2f) increased Rev-erbα expression in a time-dependent and infection dose-dependent manner. Collectively, these results demonstrate that *H. pylori* infection induces Rev-erbα expression in GECs.

H. pylori induce gastric epithelial cells to express Rev-erbα via ERK-NF-κB pathway

To further explore which signaling pathways might operate in the induction of Rev-erbα in GECs by *H. pylori*, we first pre-treated AGS cells with corresponding pathway inhibitors and then infected them with *H. pylori*. The results showed that only blocking the signal transduction of ERK pathway with inhibitor

U0126 effectively decreased Rev-erbα expression (Fig. 3a; Supplementary Fig. 3a, b). Furthermore, ERK1/2, a direct ERK pathway downstream substrate, was phosphorylated in the infected AGS cells in an infection dose-dependent (Supplementary Fig. 3c) and time-dependent manner (Supplementary Fig. 3d), which was abolished when pretreated with inhibitor U0126 (Fig. 3a; Supplementary Fig. 3h). Next, to confirm whether *cagA* could induce Rev-erbα via ERK pathway, we infected AGS cells with WT *H. pylori* or *ΔcagA*, or transfected AGS cells with *cagA*-pcDNA3.1. We found increased Rev-erbα mRNA and protein and increased ERK1/2 phosphorylation in AGS cells either infected with WT *H. pylori* or transfected with *cagA*-pcDNA3.1 compared to those infected with *ΔcagA* or transfected with the vector (pcDNA3.1) (Fig. 3a-c). Importantly, these were abrogated by pretreatment with the ERK pathway inhibitor U0126 or with *cagA* EPIYA motif phosphorylation inhibitor PP2 [15] (Fig. 3a-c; Supplementary Fig. 3i). To further investigate the effect of *H. pylori* on Rev-erbα gene transcription, we synthesized a series of Rev-erbα-luc promoter constructs and cloned them into pGL3-basic vectors. Compared to *ΔcagA* or pcDNA3.1, WT *H. pylori* infection or *cagA*-pcDNA3.1 transfection enhanced luciferase activity in AGS cells transfected with full length (-2000/0) Rev-erbα-luc promoter construct plasmid, which was abolished when pretreatment with the ERK pathway inhibitor U0126 (Fig. 3d). Interestingly, WT *H. pylori* infection only enhanced luciferase activity in cells transfected with the Rev-erbα-luc promoter construct flanking regions -2000 to -500 (Fig. 3e). Further detailed luciferase reporter assay revealed that only the Rev-erbα promoter construct flanking regions -1249 to -1000 produced an increase in cellular luciferase activity upon WT *H. pylori* infection (Fig. 3f). The PROMO tool V.8.3 of TRANSFAC (Beverly, MA, USA) showed that Rev-erbα promoter (-1249/-1000) contains a NF-κB binding site (comprising the sequence: GGGAAATGAC). Subsequently, chromatin immunoprecipitation (ChIP) assay showed that, compared to no infection or *ΔcagA* infection, WT *H. pylori* infection significantly increased binding to the Rev-erbα promoter in AGS cells, which was abolished when pretreated with the ERK pathway inhibitor U0126 (Fig. 3g). Taken together, these findings clearly demonstrate that *cagA*-mediated ERK signaling pathway activation modulates NF-κB's transcriptional regulation on Rev-erbα expression in GECs during *H. pylori* infection.

Rev-erbα suppresses antibacterial proteins Reg3b and β-defensin-1 leading to increased bacterial burden in gastric mucosa during H. pylori infection

To evaluate the possible biological effects of Rev-erbα in *H. pylori*-associated pathogenesis *in vivo*, we compared the levels of bacterial colonization in gastric mucosa 8 weeks p.i. in WT and *Rev-erbα*$^{-/-}$ mice, and found that lacking endogenous Rev-erbα in *Rev-erbα*$^{-/-}$ mice effectively reduced *H. pylori* colonization when compared to that in WT mice (Fig. 4a left panel). Next, we again compared bacterial colonization in mice with *in vivo* pharmacological activation or inhibition of Rev-erbα by injecting exogenous Rev-erbα agonist or Rev-erbα antagonist, and found that activation of Rev-erbα significantly increased *H. pylori* colonization, conversely, inhibition of Rev-erbα significantly reduced gastric colonization when compared

to that in control mice (Fig. 4a middle panel). Finally, consistent with our previous findings that GECs are the source cells that express Rev-erbα in gastric mucosa during *H. pylori* infection (Fig. 2), by generating BM chimera mice, we found that non-BM-derived Rev-erbα-expressing cells (including GECs) largely contribute to increasing bacterial colonization in this model (Fig. 4a right panel). Taken together, our data demonstrate that Rev-erbα plays an essential role in promoting bacterial colonization *in vivo*.

Given powerful transcriptional repressing effects of Rev-erbα was observed in other infectious diseases [11, 13], we hypothesized that Rev-erbα might exert inhibiting effects on genes encoding proteins contributing to host defense. The β-defensins [16] and Reg3 proteins [17] play roles in mucosal defense during *H. pylori* infection. We therefore screened these proteins in gastric mucosa 8 weeks p.i. in WT and *Rev-erbα*$^{-/-}$ mice, and found that lacking Rev-erbα in *Rev-erbα*$^{-/-}$ mice led to increased Reg3b and β-defensin-1 expression when compared to that in WT mice (Fig. 4b and Fig. 4c, d left panel). Similar observations were made when using *in vivo* pharmacological activation or inhibition of Rev-erbα experiments (Fig. 4c, d middle panel). Again, BM chimera experiments confirmed that non-BM-derived Rev-erbα-expressing cells were largely responsible for inhibiting Reg3b and β-defensin-1 in gastric mucosa during *H. pylori* infection (Fig. 4c, d right panel). Furthermore, similar observations were made when analyzing Reg3b and β-defensin-1 protein by immunohistochemical staining (Fig. 4e; Supplementary Fig. 5a, b).

To further investigate the roles of Reg3b and β-defensin-1 in *H. pylori* infection, we performed *in vitro* and *in vivo* bactericidal assay. First, *in vitro* bactericidal assay showed that Reg3b and β-defensin-1 exerted killing activity against *H. pylori* in a time-dependent and infection dose-dependent manner (Fig. 4f). Next, *in vivo* gain-of-function experiments showed that Reg3b and/or β-defensin-1 administration significantly reduced *H. pylori* colonization in gastric mucosa of WT mice (Fig. 4g). As for the negative correlations between Rev-erbα and Reg3b/β-defensin-1 (Supplementary Fig. 5e) in gastric mucosa of *H. pylori*-infected mice and host defense against *H. pylori* from GEC-derived antibacterial proteins [16], we next found that, upon *H. pylori* infection, GECs derived from *Rev-erbα*$^{-/-}$ mice produced more Reg3b and β-defensin-1 compared to those from WT mice (Supplementary Fig. 5d), and that supernatants from *H. pylori*-infected GECs of *Rev-erbα*$^{-/-}$ mice exerted more pronounced killing activity against *H. pylori* than those of WT mice (Fig. 4h), which could be abrogated by blocking Reg3b and/or β-defensin-1 (Fig. 4h). Finally, to explore the molecule mechanism of β-defensin-1 inhibition in GECs by Rev-erbα (human have no *Reg3b* gene), we performed luciferase reporter assays, and the results showed decreased activity of β-defensin-1-luc when transfected together with the *Rev-erbα*-pcDNA3.1, and retrieved activity when the Rev-erbα binding site on the β-defensin-1 promoter was mutated (Fig. 4i); further ChIP assays showed that WT *H. pylori* infection significantly increased binding activity to the β-defensin-1 promoter with the Rev-erbα Abs (Fig. 4j), together suggesting that in WT *H. pylori*-infected AGS cells, Rev-erbα directly inhibited β-defensin-1 expression. Taken together, our data demonstrate that, in *H. pylori*-infected gastric mucosa, Rev-erbα in GECs inhibits Reg3b and β-defensin-1 directly, which likely contributes to bacterial persistence.

Rev-erbα inhibits $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cell accumulation and CCL21 production in gastric mucosa during H. pylori infection

To investigate whether increased Rev-erbα regulated immune cell infiltration into the gastric mucosa during *H. pylori* infection, we compared the levels of $CD45^+CD11c^+Ly6G^-$ dendritic cells (DCs), $CD45^+CD11c^-Ly6G^+$ neutrophils, $CD45^+CD11c^-Ly6G^-CD3^+NK1.1^-$ T cells, $CD45^+CD11c^-Ly6G^-CD3^-NK1.1^+$ natural killer cells (NK cells), $CD45^+CD11c^-Ly6G^-CD3^-NK1.1^-CD19^+$ B cells, and $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cells in gastric mucosa 8 weeks p.i. in WT and *Rev-erbα*$^{-/-}$ mice, and found that lacking Rev-erbα in *Rev-erbα*$^{-/-}$ mice only led to increased $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cells (Fig. 5a; Supplementary Fig. 6a, b). These results were also confirmed by our *in vivo* pharmacological activation or inhibition of Rev-erbα experiments and BM chimera experiments in which non-BM-derived Rev-erbα-expressing cells were largely responsible for this myeloid cell reduction in gastric mucosa during *H. pylori* infection (Fig. 5a). Similar observations were made when analyzing the number of $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cells per million total cells in gastric mucosa (Fig. 5b; Supplementary Fig. 6c). Furthermore, blood, spleen, or bone marrow $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cell levels were not altered in these mouse models above (Supplementary Fig. 7). Taken together, our data demonstrate that Rev-erbα plays an essential role in the inhibition of $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cell accumulation in gastric mucosa during *H. pylori* infection.

Chemotaxis plays important roles in immune cell migration. We were therefore interested to know if Rev-erbα regulated chemokine production in gastric mucosa. We screened chemokines in gastric mucosa 8 weeks p.i. in WT and *Rev-erbα*$^{-/-}$ mice, and found that only CCL21 expression was increased in *Rev-erbα*$^{-/-}$ mice (Fig. 5c; Supplementary Fig. 8a). Again, these results were confirmed by our *in vivo* pharmacological activation or inhibition of Rev-erbα experiments and BM chimera experiments in which non-BM-derived Rev-erbα-expressing cells were largely responsible for CCL21 inhibition in gastric mucosa during *H. pylori* infection (Fig. 5d; Supplementary Fig. 8b). Taken together, our data demonstrate that Rev-erbα plays an essential role in inhibiting CCL21 production in gastric mucosa during *H. pylori* infection.

To understand the negative correlation between Rev-erbα and CCL21 (Supplementary Fig. 8f) and CCL21 expression in $CD326^+$ cells (Supplementary Fig. 8g) in gastric mucosa of *H. pylori*-infected patients and mice, we then explored the molecular mechanism by which Rev-erbα down-modulate CCL21 expression in GECs. Luciferase reporter assay showed decreased activity of CCL21-luc when transfected together with the *Rev-erbα*-pcDNA3.1, and retrieved activity when the Rev-erbα binding site on the CCL21 promoter was mutated (Fig. 5e); further ChIP assays showed that WT *H. pylori* infection significantly increased binding activity to the CCL21 promoter by the Rev-erbα Abs (Fig. 5f), together suggesting that in WT *H. pylori*-infected AGS cells, Rev-erbα directly inhibited CCL21 expression. Finally, CCL21 production from AGS cells as well as from mouse primary GECs was negatively regulated in a Rev-erbα-dependent manner (Fig. 5g; Supplementary Fig. 8d). Taken together, our data demonstrate that Rev-erbα inhibits $CD45^+CD11c^-$

Ly6G$^-$CD11b$^+$CD68$^-$ myeloid cell accumulation and CCL21 production in gastric mucosa during *H. pylori* infection.

Rev-erbα inhibits CD45$^+$CD11c$^-$Ly6G$^-$CD11b$^+$CD68$^-$ myeloid cell accumulation in vivo and migration in vitro during H. pylori infection via CCL21-CCR7 axis

Next, we tried to determine whether CD45$^+$CD11c$^-$Ly6G$^-$CD11b$^+$CD68$^-$ myeloid cell migration and accumulation during *H. pylori* infection was regulated by Rev-erbα-CCL21 axis. We first showed that CD45$^+$CD11c$^-$Ly6G$^-$CD11b$^+$CD68$^-$ myeloid cells expressed high levels of the monocyte-associated marker Ly6C and F4/80 (Fig. 6a) and exhibited a mono-lobar nucleus characteristic of monocytes by Wright staining (Supplementary Fig. 6a). Next, gastric (Fig. 6a) or blood (Fig. 6b) CD45$^+$CD11c$^-$Ly6G$^-$ CD11b$^+$CD68$^-$myeloid cells from *H. pylori*-infected humans and mice showed higher expression of CCR7, the chemokine receptor of CCL21. Finally, we conducted a series of loss- and gain-of-function experiments *in vivo* involving CCL21 and/or CCR7, and evaluated the CD45$^+$CD11c$^-$Ly6G$^-$CD11b$^+$CD68$^-$ myeloid cells in gastric mucosa 8 weeks p.i.. CCL21 administration significantly increased CD45$^+$CD11c$^-$ Ly6G$^-$CD11b$^+$CD68$^-$ myeloid cell accumulation; conversely, neutralization of CCL21 and/or CCR7 significantly reduced CD45$^+$CD11c$^-$Ly6G$^-$CD11b$^+$CD68$^-$ myeloid cell accumulation in gastric mucosa (Fig. 6c).

To further evaluate the contribution of a Rev-erbα-CCL21-CCR7 axis to the migration of these monocyte-like myeloid cells *in vitro*, human CD45$^+$CD11c$^-$CD66b$^-$CD11b$^+$CD68$^-$ myeloid cell chemotaxis assays were performed. It was demonstrated that culture supernatants from *ΔcagA*-infected AGS cells pre-treated with non-specific control siRNA (NC) or from WT *H. pylori*-infected AGS cells pre-treated with *Rev-erbα* siRNA induced significantly more CD45$^+$CD11c$^-$CD66b$^-$CD11b$^+$CD68$^-$ myeloid cell migration than those from WT *H. pylori*-infected AGS cells pre-treated with NC; and this effect was lost upon pre-treatment with neutralizing Abs against CCL21 and/or CCR7 (Fig. 6d).

Similarly, culture supernatant from *ΔcagA*-infected primary GECs of WT mice or from WT *H. pylori*-infected primary GECs of *Rev-erbα*$^{-/-}$ mice also induced significantly more mouse CD45$^+$CD11c$^-$Ly6G$^-$ CD11b$^+$CD68$^-$ myeloid cell migration than those from WT *H. pylori*-infected primary GECs of WT mice; and this effect was also lost upon pre-treatment with neutralizing Abs against CCL21 and/or CCR7 (Fig. 6e). Collectively, these results therefore suggest that a Rev-erbα-CCL21-CCR7 axis contributes to CD45$^+$CD11c$^-$Ly6G$^-$CD11b$^+$CD68$^-$ myeloid cell accumulation within gastric mucosa during *H. pylori* infection.

Rev-erbα impairs specific Th1 cell response to promote bacterial colonization in gastric mucosa during H. pylori infection

Th1 cell response and its effector IFN-γ play key roles in control *H. pylori* infection [18, 19]. First, there were negative correlations between Rev-erbα and IFN-γ in gastric mucosa of *H. pylori*-infected patients and mice (Supplementary Fig. 9a, b) and IFN-γ was increased in gastric mucosa 8 weeks p.i. in *Rev-erbα*$^{-/-}$ mice when compared to that in WT mice (Fig. 7a; Supplementary Fig. 9c). Again, these results were confirmed by our *in vivo* pharmacological activation or inhibition of Rev-erbα experiments and BM chimera experiments in which non-BM-derived Rev-erbα-expressing cells were largely responsible for IFN-γ inhibition in gastric mucosa during *H. pylori* infection (Fig. 7a; Supplementary Fig. 9c). Next, Th1 cell response level was also increased in *Rev-erbα*$^{-/-}$ mice (Fig. 7b). Notably, myeloid cell/T-cell co-cultures showed that gastric $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cells from *H. pylori*-infected mice induced more Th1 cells from blood or spleen $CD4^+$T cells of *H. pylori*-infected mice to proliferate and produce IFN-γ than those from uninfected counterparts (Fig. 7c), suggesting a promoting effect of $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cells on antigen-specific Th1 cell response during *H. pylori* infection.

To determine the potential contributions of Rev-erbα-mediated inhibition of specific Th1 cells to *H. pylori* colonization, we conducted a series of *in vivo* adoptive transfer experiments and evaluated bacterial colonization in gastric mucosa 8 weeks p.i.. First, $CD4^+$ T cells from *H. pylori*-infected WT donors into WT recipients effectively reduced *H. pylori* colonization when compared to WT mice received $CD4^+$ T cells from uninfected WT donors, suggesting specific $CD4^+$ T cells contribute to reduced bacterial colonization (Fig. 7d). Next, transferring $CD4^+$ T cells from *H. pylori*-infected WT donors into uninfected (or naïve) *Rev-erbα*$^{-/-}$ recipients effectively reduced *H. pylori* colonization when compared to those in the uninfected WT mice received the same $CD4^+$ T cells, suggesting Rev-erbα-mediated inhibition on *H. pylori*-specific $CD4^+$ T cells leading to increased bacterial colonization (Fig. 7d). Finally, transferring $CD4^+$ T cells from *H. pylori*-infected WT donors into uninfected *Rev-erbα*$^{-/-}$ recipients effectively reduced *H. pylori* colonization when compared to those in the uninfected *Rev-erbα*$^{-/-}$ recipients received $CD4^+$ T cells from *H. pylori*-infected *IFN-γ*$^{-/-}$ donors, suggesting Rev-erbα-mediated inhibition on IFN-γ production of *H. pylori*-specific $CD4^+$ T cells (Th1 cells) leading to increased bacterial colonization (Fig. 7d), which was also reflected by increased colonization in *IFN-γ*$^{-/-}$ mice (Fig. 7e). Overall, these results indicate that Rev-erbα promotes gastric *H. pylori* colonization, most likely through inhibiting *H. pylori*-specific Th1 cells' IFN-γ response.

Discussion

Our results from both *in vivo* and *in vitro* gain- and loss-of-function experiments identify Rev-erbα as a pathological regulator that benefits bacterial colonization and contributes to gastric *H. pylori* persistence.

This is supported by several observations. First, Rev-erbα is preferentially expressed in GECs during *H. pylori* infection, such as gastric mucin$^+$ cells, and *Rev-erbα*$^{-/-}$ mice have notably decreased *H. pylori* colonization associated with a marked increase in antibacterial protein content, chemokine production and Th1 cell response within gastric mucosa. We observed similar alterations upon knocking down Rev-erbα in human GEC line AGS cells (higher Reg3b and β-defensin-1 content and impaired CCL21-derived chemotaxis function). By contrast, pharmacological activation of Rev-erbα *in vivo* in mice infected with *H. pylori* and *in vitro* in *H. pylori*-infected AGS cells resulted in the opposite phenotype, highlighting direct Rev-erbα effect on GECs. Second, our data indicate that *H. pylori* induces Rev-erbα expression in GECs through the ERK-NF-κB signaling pathway, and that *H. pylori* virulence factor *cagA* plays an essential role in this process. Finally, we show that GEC Rev-erbα regulates several genes involved in different steps of the host defense process, including genes more specifically dedicated to innate (*Reg3b* and *β-defensin-1*) and adaptive defense (*CCL21* and *IFN-γ*). Together, these data support the concept that Rev-erbα acts through a two-pronged mechanism, involving both suppression of host innate defense and inhibition of host adaptive defense. Thus, during *H. pylori* infection, when Rev-erbα is induced, there is a progressive impaired host defense within gastric mucosa, allowing for gastric *H. pylori* persistence.

In this study, we not only identified a previously unrecognized role for Rev-erbα during *H. pylori* infection, but also found that Rev-erbα expression was readily induced upon *H. pylori* infection *ex vivo* in primary gastric tissues. This response is consistent with previous observations on Rev-erbα expression in inflamed tissues, such as liver [20] and skeletal muscle [21]. The regulation of Rev-erbα by *H. pylori* provides a molecular mechanism whereby *H. pylori* controls Rev-erbα expression through its virulence factor *cagA*. The *cagA* is crucial in the pathogenicity of *H. pylori* that is injected into GECs via the type IV secretion system (T4SS) to modulate the phenotype and function of GECs [14]. Lack of *cagA* activity in *H. pylori* is associated with impaired expression of Rev-erbα in GECs, attenuated inhibition of Rev-erbα-mediated CCL21 expression and decreased CD45$^+$CD11c$^-$Ly6G$^-$CD11b$^+$CD68$^-$ myeloid cell chemotaxis. Thus, our findings that *H. pylori*-associated virulence factor *cagA* was necessary to induce maximal Rev-erbα expression may suggest that intrinsic factors encoded by the infection itself are likely to be important in influencing the role of Rev-erbα. In addition, we revealed that ERK pathway activation-mediated *Rev-erbα* promoter transcription initiation is essential for the induction of Rev-erbα in GECs in response to *H. pylori*. Of note, it is reported that several inflammatory factors such as TGF-β [22] and transcription factors such as NF-κB [23] are involved in Rev-erbα induction in hepatic stellate cells or under oxidative stress, which resembles our data on Rev-erbα regulation by NF-κB in *H. pylori*-infected GECs.

Host defense is a dynamic host-pathogen interaction process that ensures the selective clearance of invading pathogens, which is particularly important in infected cells such as GECs. It has been reported that Rev-erbα ameliorates *Mycobacterium tuberculosis* clearance through regulating autophagy-related genes [11], whereas our data demonstrate that Rev-erbα impedes *H. pylori* clearance by regulating several genes involved in innate and adaptive defense. Several antibacterial proteins including LL-37 [24] and β-defensin-2 [16] exerts antimicrobial activity against *H. pylori*, here we add Reg3b and β-defensin-1 onto

that list which are selectively suppressed by *H. pylori*-induced Rev-erbα [25] that represses *β-defensin-1* gene directly triggering decreased bactericidal effect against *H. pylori* to maintain gastric bacterial persistence. Several data have shown that Rev-erbα represses many cytokine and chemokine gene expression such as IL-6 [26], IL-17 [27] and CCL2 [28] in inflammatory diseases. In our model, Rev-erbα downregulates CCL21 expression, leading to impaired chemotaxis of $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cells and therefore decreased stimulation on *H. pylori*-specific Th1 cells. IFN-γ, one of the key effector molecules of the Th1 cells, is reported to play key roles in controlling *H. pylori* infection in mice [18] and humans [29]. Moreover, IFN-γ deficiency indeed results in uncontrolled colonization of *H. pylori* in gastric mucosa [19]. Notably, our findings mechanistically demonstrate for the first time that *H. pylori*-induced Rev-erbα in GECs impairs host *H. pylori*-specific Th1 response resulting in increased bacterial colonization. Together, these data support a prominent role for Rev-erbα in *H. pylori* infection on connecting innate and adaptive host defense. Given the apparent relationship between Rev-erbα levels and the extent of bacterial colonization in *H. pylori*-infected patients observed in this study, Rev-erbα should be considered as a novel diagnostic biomarker for *H. pylori* infection-associated diseases. Specifically, our *in vitro* and *in vivo* data together provide a multi-step model of *H. pylori* persistent infection involving direct and indirect interactions among *H. pylori*, GECs, Rev-erbα, Reg3b, β-defensin-1, CCL21, $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cells and Th1 cells within the gastric mucosa (Fig. 7f).

Although eradication therapy for *H. pylori* by oral antibiotics progressed in recent years [30, 31], it is noteworthy that *H. pylori* colonization commonly persists because of increased antimicrobial resistance and impaired host defense. In this regard, our findings suggest a possible therapeutic target, Rev-erbα, for decreasing gastric *H. pylori* persistence. In addition to the direct action of Rev-erbα on genes associated with host defense, Rev-erbα deletion or over-expression causes disturbed circadian rhythmicity [32] may contribute to the observed phenotype. In conclusion, Rev-erbα is a novel pathological regulator of the function of host defense during *H. pylori* infection. Thus, pharmacological inhibition of Rev-erbα may be a promising approach for the treatment of gastric diseases caused by *H. pylori* infection.

Conclusions

We demonstrated for the first time that Rev-erbα acted through a two-pronged mechanism, involving both suppression of host innate defense and inhibition of host adaptive defense to exert a pro-colonization role in *H. pylori* infection. Also, we revealed a pathological immunosuppressive mechanism, that during *H. pylori* infection, when Rev-erbα was induced, there would be a progressive impaired host defense within gastric mucosa, allowing for gastric *H. pylori* persistence.

Methods

Patients and specimens

The gastric biopsy specimens and blood were collected from 92 *H. pylori*-infected and 32 uninfected patients who underwent upper esophagogastroduodenoscopy for dyspeptic symptoms at XinQiao

Hospital (Supplementary Table 1). *H. pylori* infection was determined by [^{14}C] urea breath test and rapid urease test of biopsy specimens taken from the antrum, and subsequently conformed by real-time PCR for 16s rDNA and serology test for specific anti-*H. pylori* antibodies (Abs) [33]. For isolation of human primary GECs, fresh non-tumor gastric tissues (at least 5-cm distant from the tumor site) were obtained from gastric cancer patients who underwent surgical resection and were determined as *H. pylori*-negative individuals as above at the Southwest Hospital. None of these patients had received chemotherapy or radiotherapy before sampling. Individuals with atrophic gastritis, hypochlorhydria, antibiotics treatment, autoimmune disease, infectious diseases and multi-primary cancer were excluded. The study was approved by the Ethics Committee of XinQiao Hospital and Southwest Hospital of Third Military Medical University. A written informed consent was obtained from each subject.

Antibodies and other reagents

(Supplementary Table 2)

Mice

All breeding and experiments were undertaken with review and approval from the Animal Ethical and Experimental Committee of Third Military Medical University. C57BL/6 *Rev-erbα*$^{+/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, USA). C57BL/6 *Rev-erbα*$^{-/-}$ mice and their littermate control (wild-type, WT) mice were generated by breeding between C57BL/6 *Rev-erbα*$^{+/-}$ mice. C57BL/6 *interferon-γ*$^{-/-}$ (*IFN-γ*$^{-/-}$) mice were kindly provided by Dr. Richard A. Flavell (Yale University). All mice used in experiments were viral Ab negative for pathogenic murine viruses, negative for pathogenic bacteria including *Helicobacter* spp. and parasites, and were maintained under specific pathogen free (SPF) conditions in a barrier sustained facility and provided with sterile food and water.

Bacteria culture and infection of mice with bacteria

H. pylori NCTC 11637 (*cagA* positive) (WT *H. pylori*) and *cagA*-knockout mutant *H. pylori* NCTC 11637 (*ΔcagA*) or *H. pylori* 26695 were grown in brain-heart infusion plates containing 10% rabbit blood at 37°C under microaerophilic conditions. For infecting mouse, bacteria were propagated in Brucella broth with 5 % fetal bovine serum (FBS) with gentle shaking at 37°C under microaerobic conditions. After culture for 1 d, live bacteria were collected and adjusted to 10^9 colony-forming units (CFU)/ml. The mice were fasted overnight and orogastrically inoculated twice at a 1 d interval with 3×10^8 CFU bacteria. *H. pylori* infection status and *H. pylori*-induced gastritis in murine experiments were confirmed using real-time PCR of *H.*

pylori 16s rDNA, urease biopsy assays, Warthin-Starry staining and immunohistochemical staining for *H. pylori*, and evaluation of inflammation by haematoxylin and eosin (H&E) staining [33, 34].

Generation of bone marrow (BM) chimera mice

The following BM chimeric mice were created: male WT BM→female WT mice, male WT BM→female *Rev-erbα*$^{-/-}$ mice, male *Rev-erbα*$^{-/-}$ BM→female WT mice, and male *Rev-erbα*$^{-/-}$ BM→female *Rev-erbα*$^{-/-}$ mice. BM cells were collected from the femurs and tibia of donor mice by aspiration and flushing, and were suspended in PBS at the concentration of 5×10^7/ml. The BM in recipient mice was ablated with lethal irradiation (8 Gy). Then, the animals received intravenously 1.5×10^7 BM cells from donor mice in a volume of 300 μl sterile PBS under the anaesthesia. Thereafter, the transplanted BM was allowed to reconstitute for 8 weeks before subsequent experimental procedures. To verify successful engraftment and reconstitution of the BM in the host mice, genomic DNA was isolated from tail tissues of each chimera mouse 8 weeks after BM transplantation. Quantitative PCR was performed to detect the *Sry* gene present in the Y chromosome (primers seen in Supplementary Table 3) and mouse β2-microglobulin gene as an internal control. The chimeric rates were calculated on the assumption that the ratio of the Sry to β2-microglobulin gene was 100% in male mice. We confirmed that the chimeric rates were consistently higher than 90%. After BM reconstitution was confirmed, mice were infected with bacteria as described above.

Antibodies/CCL21/Reg3b/β-defensin-1/Rev-erbα agonist/Rev-erbα antagonist administration

One day after infection with WT *H. pylori* as described above, WT mice were injected intraperitoneally with recombinant mouse CCL21 (25 μg) or Reg3b and/or β-defensin-1 (50 μg), or anti-mouse CCL21 and/or anti-mouse CCR7 or isotype control Abs (100 μg), or Rev-erbα agonist SR9009 (100 mg/kg, dissolved in cremophor) or cremophor control, or Rev-erbα antagonist SR8278 (25 mg/kg, dissolved in DMSO) or DMSO control and repeated every week until the mice were sacrificed.

T-cell adoptive transfer

One day before infection with WT *H. pylori*, WT or *Rev-erbα*$^{-/-}$ mice were injected intravenously (1×10^6 cells/mouse) with purified spleen $CD4^+$ T cells (StemCell Technologies) from uninfected WT mice, or WT

H. pylori-infected WT mice or WT *H. pylori*-infected *IFN-γ*$^{-/-}$ mice (8 week post infection (p.i.)). Then the recipient mice were infected with bacteria as described above and sacrificed for bacteria colonization evaluation at week 8 p.i..

Evaluation of bacteria colonization

The mice were sacrificed at the indicated time points. The stomach was cut open from the greater curvature and half of the tissue was cut into four parts for RNA extraction, DNA extraction, protein extraction, and tissue fixation for immunohistochemistry or immunofluorescence staining, respectively. DNA of the biopsy specimens were extracted with QIAamp DNA Mini Kit. As previously described [35], *H. pylori* colonization was quantified by measuring *H. pylori*-specific 16s rDNA using specific primer and probe (Supplementary Table 3) by the TaqMan method. The amount of mouse β2-microglobulin DNA in the same specimen was used to normalize the data. According to a previous study [36], the density of *H. pylori* was shown as the number of bacterial genomes per nanogram of host genomic DNA. Another half of stomach was used for isolation of single cells as described below. The isolated single cells were collected and analyzed by flow cytometry staining.

Isolation of single cells from tissues

Fresh tissues were washed three times with Hank's solution containing 1% FBS, cut into small pieces, collected in RPMI 1640 containing 1 mg/ml collagenase IV and 10 mg/ml DNase I, and then mechanically dissociated by using the gentle MACS Dissociator (Miltenyi Biotec). Dissociated cells were further incubated for 0.5-1 h at 37°C under continuous rotation. The cell suspensions were then filtered through a 70-μm cell strainer (BD Labware).

Cell/tissue culture, transfection and stimulation

Primary GECs were purified from gastric tissue single-cell suspensions from uninfected donors or mice with a MACS column purification system using anti-human or mouse CD326 magnetic beads. The sorted primary GECs were used only when their viability was determined >90% and their purity was determined >95%. For human GEC lines (AGS cells, GES-1 cells, HGC-27 cells, SGC-7901 cells, BGC-823 cells), 3×10^5 cells per well in 12-well cell culture plate (for real-time PCR) or 1×10^6 cells per well in 6-well cell culture plate (for western blot and ELISA) were starved in DMEM (Dulbecco's Modified Eagle Medium)/F-12 medium supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) for 6 h in a humidified

environment containing 5% CO_2 at 37 °C. Then the cells were incubated in antibiotic-free DMEM/F-12 medium supplemented with 10% FBS instead. The cell lines were used when their viability was determined >90%. Human GEC lines, primary GECs, or primary gastric mucosa tissues from uninfected donors or mice were infected with WT *H. pylori*, *ΔcagA* or *H. pylori* 26695 at a multiplicity of infection (MOI) of 100 for 24 h. AGS cells were also infected with WT *H. pylori* at different MOI (24 h) or at the indicated time points (MOI=100). For signal pathway inhibition experiments, AGS cells were pre-treated with 5 μl (20 μM) U0126 (an MEK-1 and MEK-2 inhibitor), SB203580 (a p38 MAPK inhibitor), SP600125 (a JNK inhibitor), FLLL32 (an STAT3 inhibitor), AG490 (a JAK inhibitor), Wortmannin (a PI3K-AKT inhibitor), PP2 (a *cagA* EPIYA motif phosphorylation inhibitor) or DMSO control for 2 h. For Rev-erbα activation or inhibition experiments, AGS cells were pre-treated with 5 μl (10, 20 μM) Rev-erbα agonist SR9009 or cremophor control, or Rev-erbα antagonist SR8278 or DMSO control for 2 h, or were pre-treated with *Rev-erbα* siRNA, non-specific control siRNA (NC) (40 nM), or lipofectamine 2000 only (Mock) for 24 h. In some cases, AGS cells were transfected with plasmids pcDNA3.1 or *cagA*-pcDNA3.1 by using lipofectamine 2000 according to the manufacturer's protocols. At 24 h after transfection, cells were treated with or without U0126 (5 μl, 20 μM) or DMSO control for 2 h and cultured for an additional 24 h. After co-culture, cells were collected for microarray, immunofluorescence, real-time PCR, and western blot, and the culture supernatants were harvested for ELISA.

Luciferase reporter assay

Promoter constructs containing the region from -2000 to 0, -1000 to 0, -500 to 0, -250 to 0, -100 to 0, and other 250 bp fragments of *Rev-erbα* gene were amplified from human genomic DNA using specifically designed primers (Supplementary Table 4) by PCR. The amplified full-length or fragmented sequences were cloned into the NheI and HindIII sites of the pGL3-basic vector respectively. Promoter constructs containing the region from -2000 to 0 of *CCL21* and *β-defensin-1* gene, and mutants of predicted Rev-erbα binding site of *CCL21* and *β-defensin-1* gene sequences were also synthesized with primers (Supplementary Table 5) and cloned into the NheI and HindIII sites of the pGL3-basic vector respectively. The above constructs were sequence verified. Plasmids over-expressing Rev-erbα (*Rev-erbα*-pcDNA3.1) were constructed by inserting target gene into plasmid pcDNA3.1, these plasmids were constructed and produced by Sangon Biotech (Shanghai) Co., Ltd. Cells were transfected with various combinations of the reporter plasmid, which contains the internal control pRL-TK (Promega) or the expression plasmid. Cells were harvested at 24 h (*H. pylori* infection assay) or 48 h (*cagA*-pcDNA3.1 or *Rev-erbα*-pcDNA3.1 plasmid transfection assay) after transfection. Luciferase activities of the lysates were measured using the Dual-Luciferase Reporter assay Kit following the manufacturer's protocol. Luciferase activity was normalized to Renilla luciferase activity.

Chromatin Immunoprecipitation (ChIP)

AGS cells infected with *H. pylori* (MOI=100, 24 h) were treated at room temperature for 10 min with 1% formaldehyde in cell culture media. Glycine (11 % in media) solution was then gently mixed in at room temperature for 5 min to terminate cross-linking. Cells were washed twice with ice-cold PBS and palleted at 3000g for 5 min. Membrane Extraction Buffer containing protease/phosphatase inhibitors was then added to each palleted sample. Cell lysates were pulse-sonicated on ice; supernatants containing the digested chromatin were collected into two tubes for input and immunoprecipitation. Anti-NF-κB p65 or anti-Rev-erbα Ab was added and IP reactions conducted overnight at 4°C with agitation. ChIP grade protein A/G magnetic beads were then added to each IP reaction. Two hours later beads were collected, washed, bounded IP materials eluted with 5 M NaCl containing 20 μg/ml Proteinase K. The cross-linking was reversed by heating up to 65°C for 1.5 h and DNA was purified. Purified DNA samples were analyzed by PCR with designed primers (Supplementary Table 6).

In vitro bactericidal assay

Two hundred microliters of 5×10^6 CFU/ml WT *H. pylori* suspension was incubated with 20 μg/ml mouse Reg3b and/or β-defensin-1 for 3, 6, or 12 h, or incubated with 5, 10, or 20 μg/ml mouse Reg3b and/or β-defensin-1 for 24 h. PBS was used as control. In another set of experiments, primary GECs from WT or *Rev-erbα*$^{-/-}$ mice were infected with WT *H. pylori* (MOI=100) for 24 h. The culture supernatants were filtered through 0.4-μm filters and collected. Then two hundred microliters of 5×10^6 CFU/ml WT *H. pylori* suspension were incubated with the above collected culture supernatants with or without anti-Reg3b and/or anti-β-defensin-1 Abs (20 μg/ml) for 24 h. Bacteria were serially diluted and plated on brain-heart infusion plates containing 10% rabbit blood and incubated for 3-5 days at 37°C under microaerophilic conditions and CFU was enumerated. The results was determined by counting CFU of alive bacteria with agar plating and expressed as the survival rate of WT *H. pylori* after incubation with Reg3b/β-defensin-1 or PBS, or was determined by counting CFU of alive bacteria with agar plating and expressed as the survival rate of WT *H. pylori* after incubation with supernatants from *H. pylori*-infected GECs of *Rev-erbα*$^{-/-}$ mice or WT mice with or without anti-Reg3b and/or anti-β-defensin-1 Abs.

Chemotaxis assay

Human $CD45^+CD11c^-CD66b^-CD11b^+CD68^-$ myeloid cells or mouse $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cells from blood of *H. pylori*-infected donors or WT *H. pylori*-infected mice (8 week p.i.) were sorted by fluorescence activating cell sorter (FACS) (FACSAria II; BD Biosciences). AGS cells were pre-treated with *Rev-erbα* siRNA or non-specific control siRNA (NC) (both at 40 nM) for 24 h, and then infected

with WT *H. pylori* or *ΔcagA* (MOI=100) for 24 h. The culture supernatants were filtered through 0.4-μm filters, collected and used as source of chemoattractants in a human myeloid cell chemotaxis assay. In another set of experiments, mouse primary GECs were purified from gastric tissue single-cell suspensions of uninfected WT or *Rev-erbα*$^{-/-}$ mice with anti-mouse CD326-conjugated MACS magnetic beads, and then infected with WT *H. pylori* or *ΔcagA* (MOI=100) for 24 h. The culture supernatants were then collected mentioned above. These culture supernatants were then used as source of chemoattractants in a mouse myeloid cell chemotaxis assay.

In a chemotaxis assay, FACS-sorted myeloid cells (1×10^5) were transferred into the upper chambers of the transwells (5-μm pore). CCL21 (100 ng/ml) and culture supernatants from various cultures were placed in the lower chambers. After 6 h culture, migration was quantified by counting cells in the lower chamber and cells adhering to the bottom of the membrane. In some cases, blocking Ab for human/mouse CCL21 (20 μg/ml) or corresponding control IgG/IgG2a (20 μg/ml) were added into the culture supernatants, and blocking Ab for human/mouse CCR7 (20 μg/ml) or corresponding control IgG2a (20 μg/ml) were added into myeloid cell suspensions and incubated for 2 h before chemotaxis assays.

In vitro T cell culture system

Purified mouse peripheral or spleen $CD4^+$ T cells from uninfected or WT *H. pylori*-infected WT mice (8 week p.i.) were labeled with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured (1×10^5 cells/well) with FACS-sorted gastric $CD45^+CD11c^-Ly6G^-CD11b^+CD68^-$ myeloid cells from WT *H. pylori*-infected WT mice (8 week p.i.) at 2:1 ratio in 200 μl RPMI 1640 medium containing mouse recombinant IL-2 (20 IU/ml), anti-CD3 (2 μg/ml), and anti-CD28 (1 μg/ml) Abs. After a 5-d incubation, cells were collected and analyzed by intracellular cytokine staining, and the culture supernatants were harvested for ELISA.

Immunohistochemistry

Paraformaldehyde-fixed and paraffin-embedded samples were cut into 5 μm sections. For immunohistochemical staining, the sections were incubated with rabbit anti-human/mouse Rev-erbα, rabbit anti-human/mouse p-ERK, sheep anti-mouse Reg3b or rabbit anti-mouse β-defensin-1 followed by HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-sheep IgG and later its substrate diaminobenzidine. All the sections were finally counterstained with haematoxylin and examined using a microscope (Nikon Eclipse 80i; Nikon).

Immunofluorescence

Paraformaldehyde-fixed cryostat tissue sections or AGS cells were washed in PBS, blocked for 30 min with 20% goat serum in PBS, stained for Rev-erbα, Rev-erbα and CD326, or CCL21 and CD326. Slides were examined with a confocal fluorescence microscope (LSM 510 META, Zeiss).

Real-time PCR

DNA of the biopsy specimens were extracted with QIAamp DNA Mini Kit and RNA of biopsy specimens and cultured cells were extracted with TRIzol reagent. The RNA samples were reversed transcribed into cDNA with PrimeScript^{TM} RT reagent Kit. Real-time PCR was performed on an IQ5 (Bio-Rad) with Real-time PCR Master Mix according to the manufacturer's specifications. The mRNA expression of 16s rDNA, *cagA*, Rev-erbα, chemokine, IFN-γ, β-defensin and Reg3 genes was measured using the TaqMan and/or SYBR green method with the relevant primers (Supplementary Table 3). For mouse samples, mouse β2-microglobulin mRNA level served as a normalizer, and its level in the stomach of uninfected or WT mice served as a calibrator. For human samples, human β-actin mRNA level served as a normalizer, and its level in the uninfected cells/tissues or stomach of uninfected donors served as a calibrator. The relative gene expression was expressed as fold change of relevant mRNA calculated by the ΔΔCt method.

Flow cytometry

Cell surface markers were stained with specific or isotype control Abs. For intracellular molecules measurements, the cells were stimulated for 5 h with PMA (50 ng/ml) plus ionomycin (1 μg/ml) in the presence of Golgistop. Intracellular cytokine staining was performed after fixation and permeabilization, using Perm/Wash solution. Then, the cells were analyzed by multicolor flow cytometry on a FACSCanto II (BD Biosciences). Data were analyzed with Flowjo software (TreeStar) or FACSDiva software (BD Biosciences).

ELISA

Isolated human and mouse gastric tissues were homogenized in 1 ml sterile Protein Extraction Reagent, and centrifuged. Tissue supernatants were collected for ELISA. Concentrations of CCL21, Reg3b, β-defensin-1 or IFN-γ in the tissue supernatants; concentrations of CCL21, Reg3b or β-defensin-1 in the

gastric epithelial cell culture supernatants; concentrations of IFN-γ in the T cell culture supernatants were determined using ELISA kits according to the manufacturer's instructions.

Western blot analysis

Western blots were performed on 10%-15% SDS-PAGE gel transferred PVDF membranes with equivalent amounts of cell or tissue lysate protein for each sample. Five percent skim milk was used for blocking the PVDF membranes. Mouse Rev-erbα, ERK1/2, and p-ERK1/2 were detected with rabbit anti-Rev-erbα Ab, rabbit anti-ERK1/2 Ab, and rabbit anti-p-ERK1/2 Ab; human Rev-erbα, ERK1/2, and p-ERK1/2 were detected with rabbit anti-Rev-erbα Ab, rabbit anti-ERK1/2 Ab, and rabbit anti-p-ERK1/2 Ab; *cagA* was detected with rabbit anti-*cagA* Ab respectively. This was followed by incubation with HRP-conjugated secondary Abs. Bound proteins were visualized by using SuperSignal® West Dura Extended Duration Substrate kit.

Microarray experiments

Gene expression profiles of WT *H. pylori*-infected and uninfected AGS cells were analyzed with the human Exon 1.0 ST GeneChip (Affymetrix), strictly following the manufacturer's protocol. Microarray experiments were performed at the Genminix Informatics (China) with the microarray service certified by Affymetrix.

Statistical analysis

Results are expressed as mean ± SEM. Student *t* test was generally used to analyze the differences between two groups, but when the variances differed, the Mann-Whitney U test was used. For multiple comparisons, the 1-way ANOVA was used. Correlations between parameters were assessed using Pearson correlation analysis and linear regression analysis, as appropriate. SPSS statistical software (version 13.0) was used for all statistical analysis. All data were analyzed using two-tailed tests, and $P<0.05$ was considered statistically significant. Microarray data analysis was performed with the assistance of Genminix Informatics. Raw data from each array were analyzed using TwoClassDif.

Abbreviations

H. pylori: *Helicobacter pylori*; GECs: gastric epithelial cells; Th cell: helper T cell; IFN: interferon; extracellular signal-regulated kinase: ERK; nuclear factor kappa-B: NF-κB; WT: wild-type; BM: bone

marrow; p.i.: post infection; antibody: Ab; chromatin immunoprecipitation: ChIP; colony-forming units: CFU; haematoxylin and eosin: H&E; fetal bovine serum: FBS.

Declarations

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Not applicable.

Author contributions

YZ conceived and designed the project. YZ, FYM, YPL, CJH, YST, YGL, PC and TL performed experiments. SMY provided human samples. YZ and WC wrote the manuscript, and QMZ supervised the project. All authors discussed the results and commented on the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The biopsy specimens were obtained under protocols approved by the ethics committees of XinQiao Hospital and Southwest Hospital and informed consent was obtained from all patients. All animal experiments were undertaken with approval from the Animal Ethical and Experimental Committee of Third Military Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

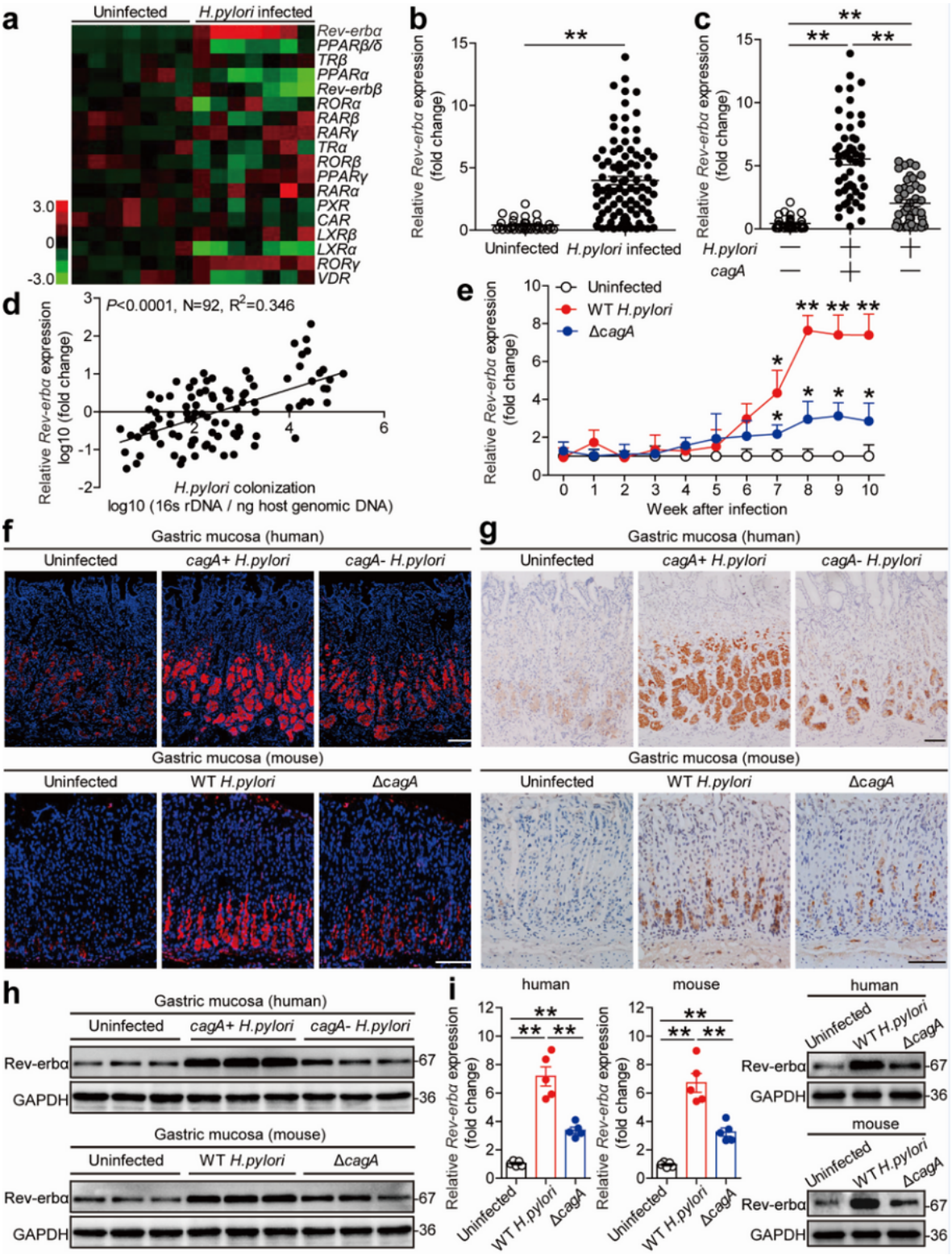

Figure 1

Rev-erbα is increased in gastric mucosa of H. pylori-infected patients and mice. a The mRNA expression profiles of thyroid hormone receptor-like superfamily nuclear receptors in human primary gastric mucosa of H. pylori-infected patients (n=7) and uninfected donors (n=7) was analyzed by real-time PCR. b Rev-erbα mRNA expression in gastric mucosa of H. pylori-infected (n=92) and uninfected donors (n=32) was compared. c Rev-erbα mRNA expression in gastric mucosa of cagA+ H. pylori-infected (n=51), cagA- H.

pylori-infected (n=41), and uninfected donors (n=32) was compared. d The correlation between Rev-erbα mRNA expression and H. pylori colonization in gastric mucosa of H. pylori-infected patients was analyzed. e Dynamic changes of Rev-erbα mRNA expression in gastric mucosa of WT H. pylori-infected, ΔcagA-infected, and uninfected mice. n=5 per group per time point in e. f-h Rev-erbα protein in gastric mucosa of cagA+ H. pylori-infected, cagA- H. pylori-infected, and uninfected donors or in gastric mucosa of WT H. pylori-infected, ΔcagA-infected, and uninfected mice 8 week p.i. was analyzed by immunofluorescence staining (f), immunohistochemical staining (g) and western blot (h). Scale bars: 100 microns. i Rev-erbα mRNA expression and Rev-erbα protein in human or mouse primary gastric mucosa from uninfected donors infected with WT H. pylori or ΔcagA ex vivo analyzed by real-time PCR and western blot (n=5). *P<0.05, **P<0.01 for groups connected by horizontal lines, or compared with uninfected mice.

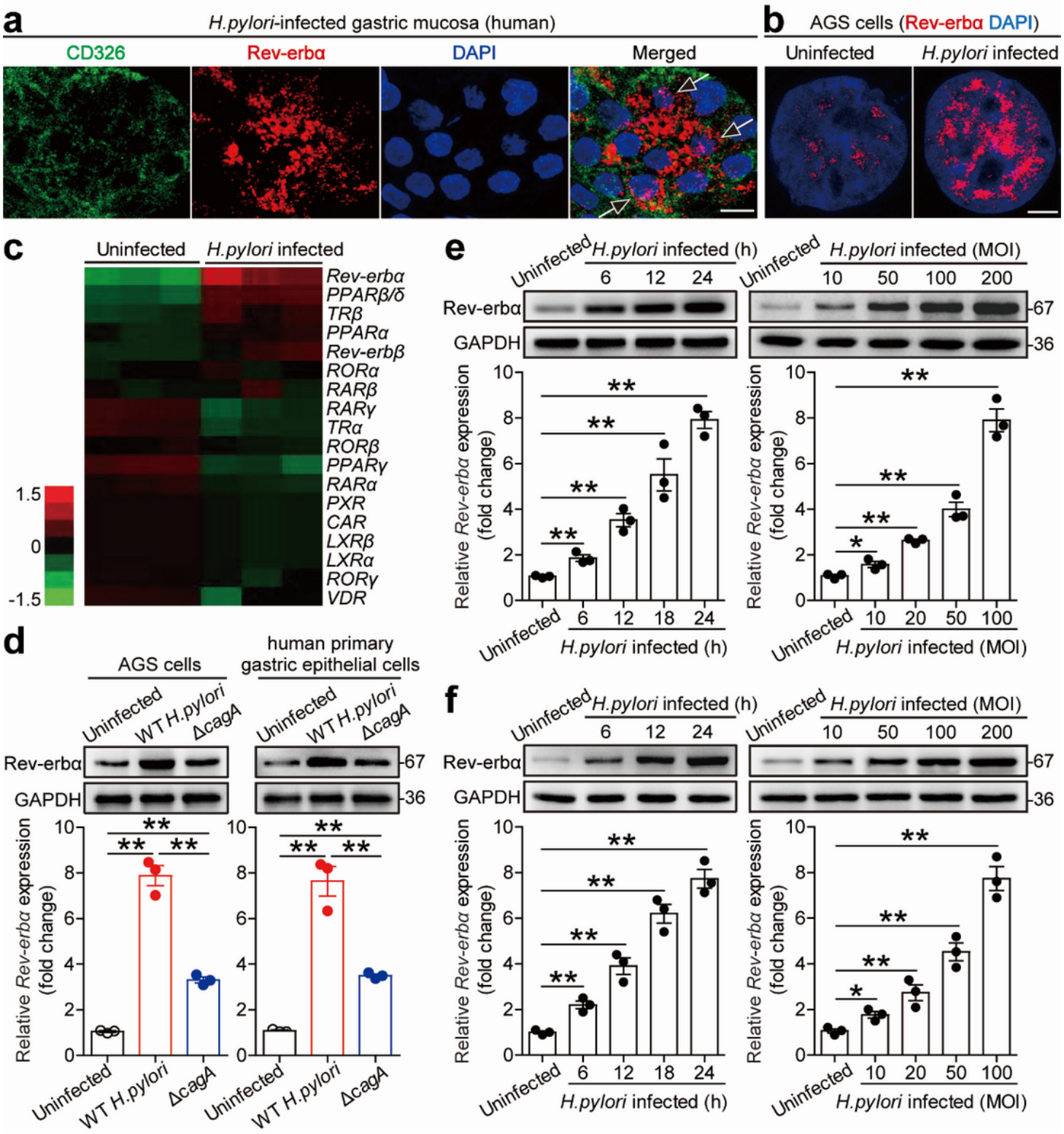

Figure 2

Gastric epithelial cells (GECs) express Rev-erbα during H. pylori infection. a Representative immunofluorescence staining images showing Rev-erbα expression (red) in cytoplasm and nucleus (arrow) of CD326+ cells (green) in gastric mucosa of H. pylori-infected patients. Scale bars: 10 microns. b Representative immunofluorescence staining images showing Rev-erbα expression (red) in WT H. pylori-infected, and uninfected AGS cells (MOI=100, 6 h). Scale bars: 1 micron. c The mRNA expression profiles

of thyroid hormone receptor-like superfamily nuclear receptors in WT H. pylori-infected and uninfected AGS cells (MOI=100, 6 h) were compared by microarray (n=3). d Rev-erbα mRNA expression and Rev-erbα protein in WT H. pylori-infected, ΔcagA-infected, and uninfected AGS cells or human primary GECs (MOI=100, 24 h) were analyzed by real-time PCR and western blot (n=3). e-f Rev-erbα mRNA expression and Rev-erbα protein in WT H. pylori-infected and uninfected AGS cells (e) or human primary GECs (f) at different time point (MOI=100) or with different MOI (24 h) were analyzed by real-time PCR or western blot (n=3). *P<0.05, **P<0.01 for groups connected by horizontal lines.

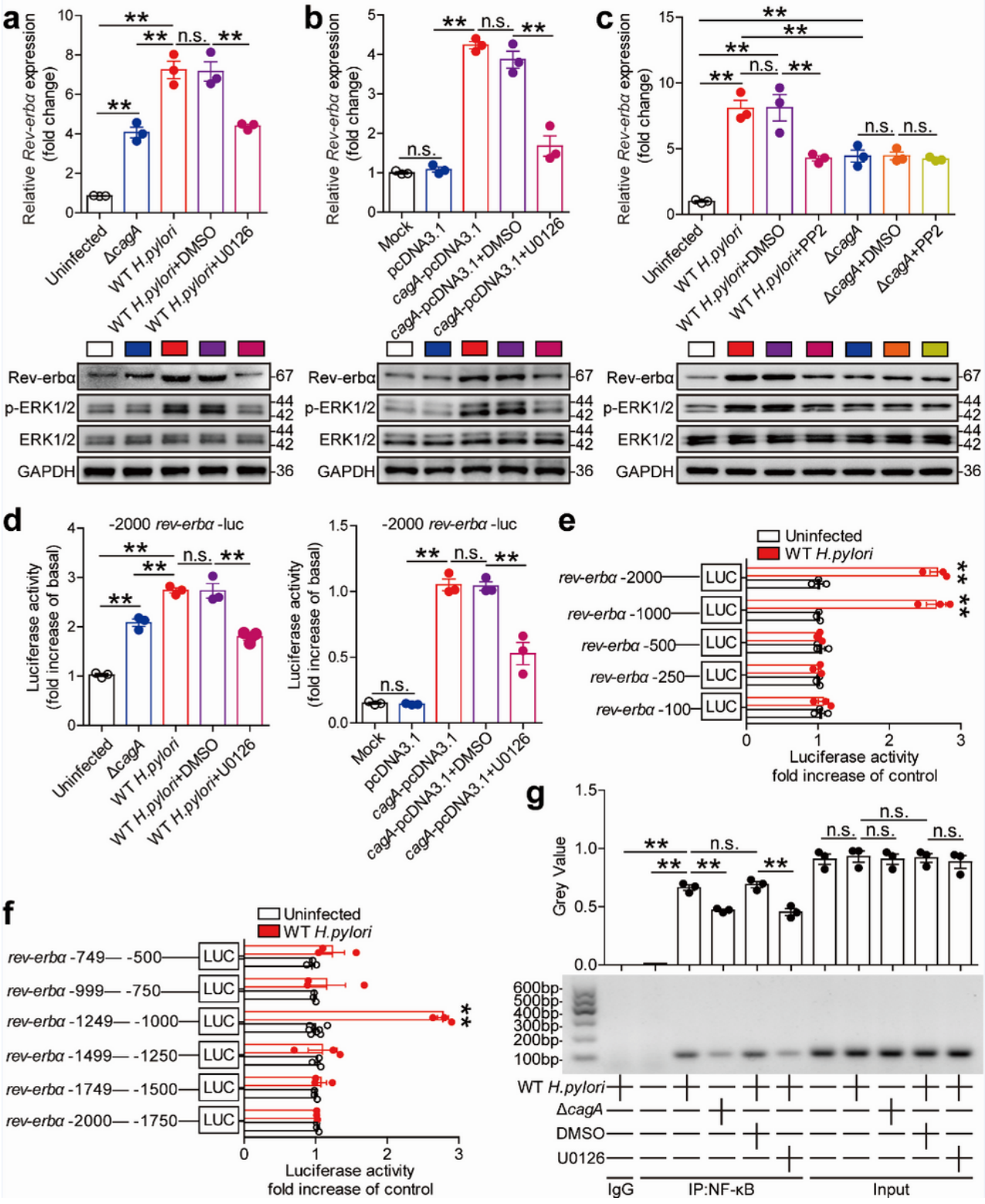

Figure 3

H. pylori induces Rev-erbα expression via ERK-NF-κB pathway. a AGS cells were pre-treated with or without U0126 and then infected with WT H. pylori or ΔcagA (MOI=100) for 24 h. Rev-erbα mRNA expression in these cells was compared (n=3). Rev-erbα, ERK1/2 and p-ERK1/2 proteins were analyzed by western blot. b AGS cells were transfected with plasmids pcDNA3.1 or cagA-pcDNA3.1 for 24 h, then treated with or without U0126 for 2 h and cultured for an additional 24 h. Rev-erbα mRNA expression in these cells was compared (n=3). Rev-erbα, ERK1/2 and p-ERK1/2 proteins were analyzed by western blot. c AGS cells were pre-treated with or without PP2 and then infected with WT H. pylori or ΔcagA (MOI=100) for 24 h. Rev-erbα mRNA expression in these cells was compared (n=3). Rev-erbα, ERK1/2 and p-ERK1/2 proteins were analyzed by western blot. d AGS cells were transfected with luciferase reporter constructs containing the Rev-erbα-luc promoter for 4 h. Luciferase activity was measured to assess promoter activity after WT H. pylori (pre-treated with or without U0126) or ΔcagA infection (MOI=100) for 24 h (n=3). AGS cells were co-transfected with Rev-erbα-luc construct and cagA-pcDNA3.1 (pre-treated with or without U0126) or pcDNA3.1 (control vector) for 48 h. Luciferase activity was measured to assess Rev-erbα promoter activity (n=3). e-f AGS cells were transfected with Rev-erbα-luc constructs for 4 h. Luciferase activity was measured to assess promoter activity after WT H. pylori infection (MOI=100) for 24 h (n=3). g Representative data and statistical analysis of chromatin immunoprecipitation assay in AGS cells infected with WT H. pylori (pre-treated with or without U0126) or ΔcagA, followed by regular PCR with primers designed for NF-κB binding site of Rev-erbα promoter region (n=3). *P<0.05, **P<0.01, n.s. P>0.05 for groups connected by horizontal lines, or compared with uninfected cells.

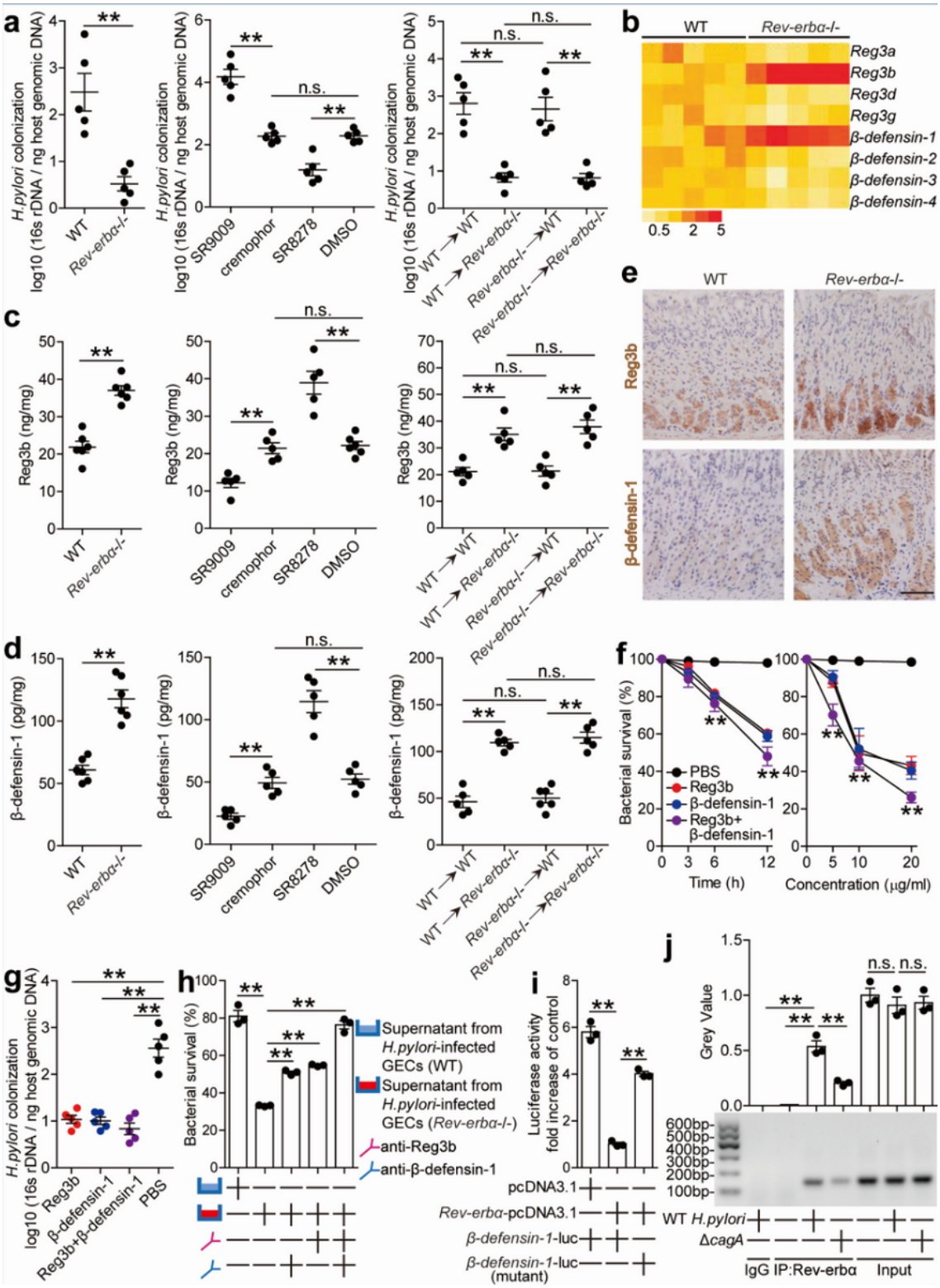

Figure 4

Rev-erbα suppresses antibacterial pr oteins Reg3b and β-defensin-1 and increases bacterial burden in gastric mucosa during H. pylori infection. a-d The bacteria colonization (a), concentrations of Reg3b (c) and β-defensin-1 (d) in gastric mucosa of WT H. pylori-infected WT and Rev-erbα-/- mice, in gastric mucosa of WT H. pylori-infected mice with in vivo by injecting Rev-erbα agonist SR9009 or cremophor control, or Rev-erbα antagonist SR8278 or DMSO control, or in gastric mucosa of WT H. pylori-infected

BM chimera mice was compared 8 weeks p.i. (n=5). The mRNA expression profiles of β-defensins and Reg3 proteins (b) in gastric mucosa of WT H. pylori-infected WT and Rev-erbα-/- mice 8 weeks p.i. was analyzed by real-time PCR (n=5). e Representative immunohistochemical staining images showing Reg3b or β-defensin-1 expression (brown) in gastric mucosa of WT H. pylori-infected WT and Rev-erbα-/- mice 8 weeks p.i.. Scale bars: 100 microns. f-h In vitro (f, h) and in vivo (g) bactericidal assay was performed as described in Methods and statistically analyzed (n=3). i AGS cells were co-transfected with β-defensin-1-luc construct or a mutant construct and Rev-erbα-pcDNA3.1 or pcDNA3.1 (control vector) for 48 h. Luciferase activity was measured to assess β-defensin-1 promoter activity (n=3). j Representative data and statistical analysis of chromatin immunoprecipitation assay in AGS cells infected with WT H. pylori or ΔcagA, followed by regular PCR with primers designed for Rev-erbα binding site of β-defensin-1 promoter region (n=3). *P<0.05, **P<0.01, n.s. P>0.05 for groups connected by horizontal lines, or compared with samples treated with PBS.

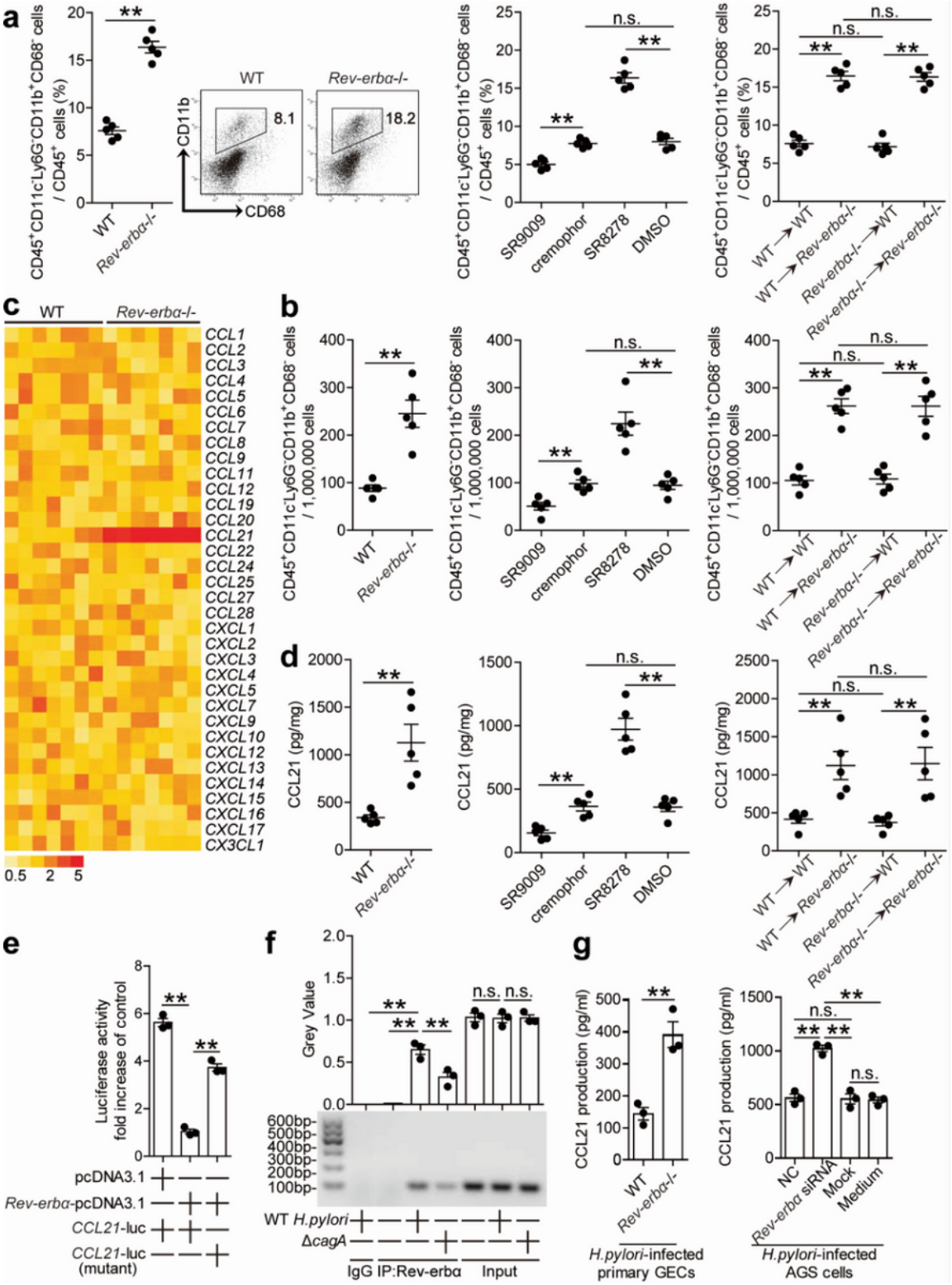

Figure 5

Rev-erbα inhibits CD45+CD11c-Ly6G-CD11b+CD68- myeloid cell accumulation and CCL21 production in gastric mucosa during H. pylori infection. a-b CD45+CD11c-Ly6G-CD11b+CD68- myeloid cell levels (a) or CD45+CD11c-Ly6G-CD11b+CD68- myeloid cell numbers (b) in gastric mucosa of WT H. pylori-infected WT and Rev-erbα-/- mice, in gastric mucosa of WT H. pylori-infected mice injected with Rev-erbα agonist SR9009 or cremophor control, or Rev-erbα antagonist SR8278 or DMSO control, or in gastric mucosa of

WT H. pylori-infected BM chimera mice were compared 8 weeks p.i. (n=5). c The mRNA expression profiles of chemokines in gastric mucosa of WT H. pylori-infected WT and Rev-erbα-/- mice 8 weeks p.i. was analyzed by real-time PCR (n=5). d Concentrations of CCL21 in gastric mucosa of WT H. pylori-infected WT and Rev-erbα-/- mice, in gastric mucosa of WT H. pylori-infected mice injected with Rev-erbα agonist SR9009 or cremophor control, or Rev-erbα antagonist SR8278 or DMSO control, or in gastric mucosa of WT H. pylori-infected BM chimera mice were compared 8 weeks p.i. (n=5). e AGS cells were co-transfected with CCL21-luc construct or a mutant construct and Rev-erbα-pcDNA3.1 or pcDNA3.1 (control vector) for 48 h. Luciferase activity was measured to assess CCL21 promoter activity (n=3). f Representative data and statistical analysis of chromatin immunoprecipitation assay in AGS cells infected with WT H. pylori or ΔcagA, followed by regular PCR with primers designed for Rev-erbα binding site of CCL21 promoter region (n=3). g Primary gastric epithelial cells (GECs) from uninfected Rev-erbα-/- and WT mice, and Rev-erbα siRNA, non-specific control siRNA (NC), or lipo2000 only (Mock) pre-treated AGS cells or AGS cells without treatment (medium) were infected with WT H. pylori (MOI=100) for 24 h. CCL21 production was measured in cell culture supernatants by ELISA (n=3). *P<0.05, **P<0.01, n.s. P>0.05 for groups connected by horizontal lines.

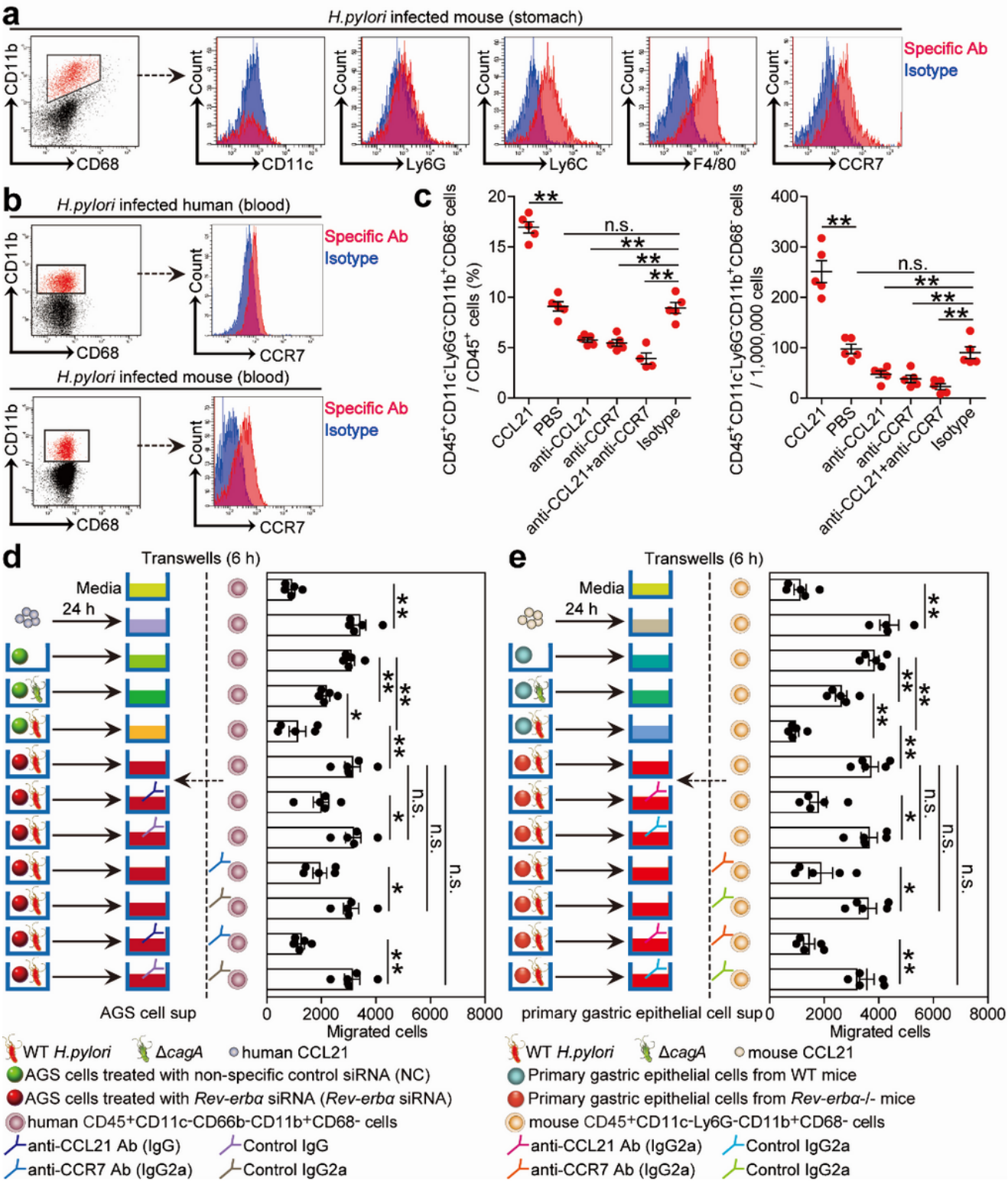

Figure 6

Rev-erbα inhibits CD45+CD11c-Ly6G-CD11b+CD68- myeloid cell accumulation in vivo and migration in vitro during H. pylori infection via CCL21-CCR7 axis. a Expression of CD11c, Ly6G, Ly6C, F4/80 and CCR7 on gastric CD45+CD11c-Ly6G-CD11b+CD68- myeloid cells from WT H. pylori-infected WT mice 8 week p.i.. Red histograms represent staining of the molecules of interest; blue, isotype control. b Representative dot plots of CCR7 expression on blood CD45+CD11c-CD66b-CD11b+CD68- myeloid cells in blood of H.

pylori-infected patients or CCR7 expression on CD45+CD11c-Ly6G-CD11b+CD68- myeloid cells of WT H. pylori-infected mice 8 weeks p.i.. c CD45+CD11c-Ly6G-CD11b+CD68- myeloid cell levels or numbers in gastric mucosa of WT H. pylori-infected mice injected with CCL21 or PBS control, or Abs against CCL21 and/or CCR7 or corresponding isotype control Ab were compared 8 weeks p.i. (n=5). d-e Human CD45+CD11c-CD66b-CD11b+CD68- myeloid cell migration (d) and mouse CD45+CD11c-Ly6G-CD11b+CD68- myeloid cell migration (e) was assessed via a transwell assays as described in Methods and statistically analyzed (n=5). *P<0.05, **P<0.01, n.s. P>0.05 for groups connected by horizontal lines.

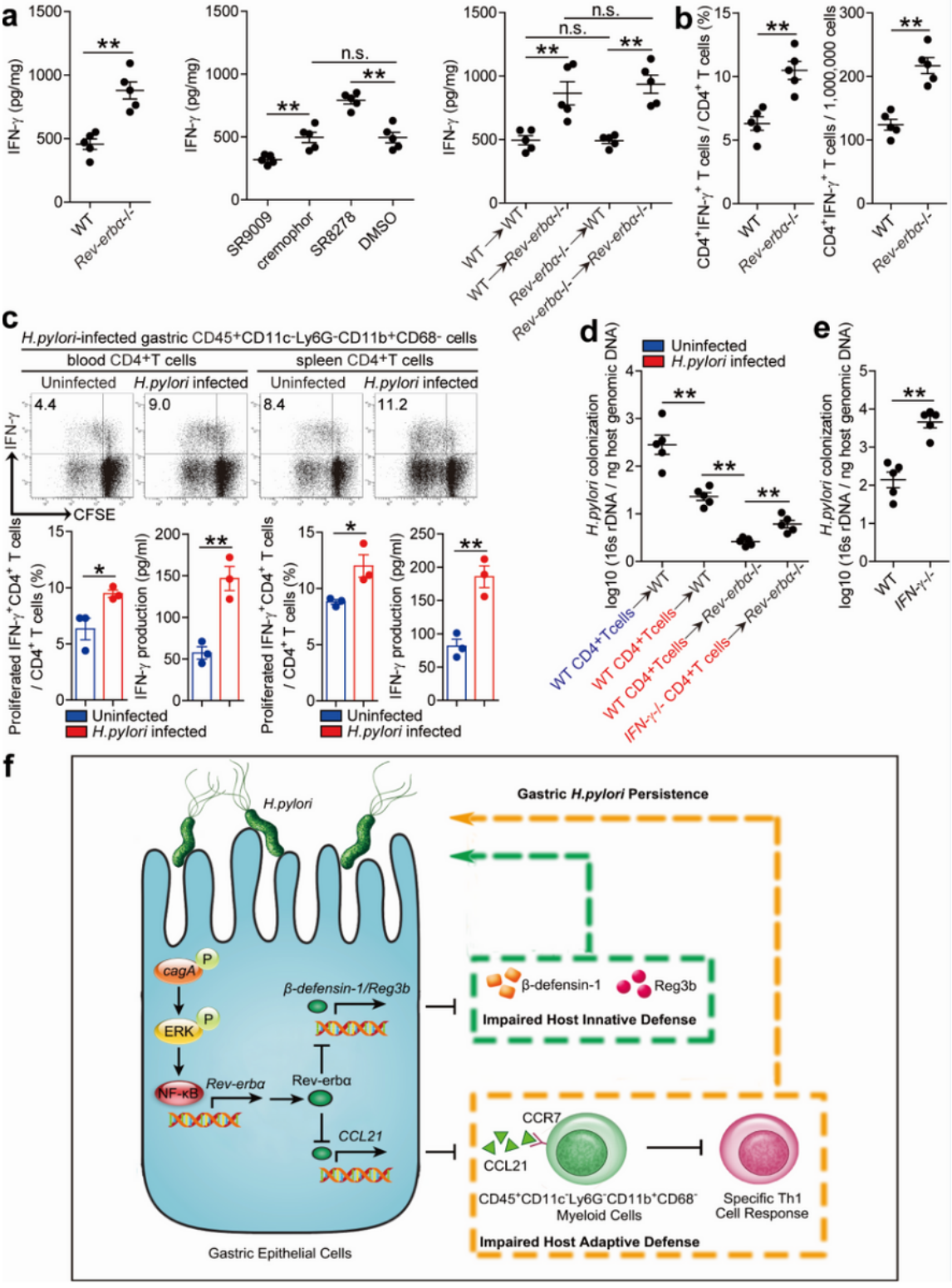

Figure 7

Rev-erbα impairs specific Th1 cell response to promote bacterial colonization in gastric mucosa during H. pylori infection. a Concentrations of IFN-γ in gastric mucosa of WT H. pylori-infected WT and Rev-erbα-/- mice, in gastric mucosa of WT H. pylori-infected mice injected with Rev-erbα agonist SR9009 or cremophor control, or Rev-erbα antagonist SR8278 or DMSO control, or in gastric mucosa of WT H. pylori-infected BM chimera mice were compared 8 weeks p.i. (n=5). b CD4+IFN-γ+ T cell levels or numbers in gastric mucosa of WT H. pylori-infected WT and Rev-erbα-/- mice were compared 8 weeks p.i. (n=5). c CFSE-labeled peripheral or spleen CD4+ T cells from uninfected or WT H. pylori-infected mice (8 weeks p.i.) were co-cultured for 5 days with FACS-sorted gastric CD45+CD11c-Ly6G-CD11b+CD68- myeloid cells from WT H. pylori-infected mice (8 weeks p.i.). Representative data and statistical analysis of CD4+IFN-γ+ T cell level and IFN-γ production were shown (n=3). d The bacteria colonization in gastric mucosa of WT H. pylori-infected WT and Rev-erbα-/- mice adoptively transferred with spleen CD4+ T cells from uninfected or WT H. pylori-infected WT or IFN-γ-/- mice (8 weeks p.i.) was compared 8 weeks p.i. (n=5). e The bacteria colonization in gastric mucosa of WT H. pylori-infected WT and IFN-γ-/- mice was compared 8 weeks p.i. (n=5). f A proposed model of crosstalk among H. pylori, GECs, Rev-erbα, Reg3b, β-defensin-1, CCL21, CD45+CD11c-Ly6G-CD11b+CD68- myeloid cells, and Th1 cell response leading to Rev-erbα-mediated pro-colonization in gastric mucosa during H. pylori infection. *P<0.05, **P<0.01, n.s. P>0.05 for groups connected by horizontal lines.

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