Modulation of the $CD4^+$ T-Cell Response by *Helicobacter pylori* Depends on Known Virulence Factors and Bacterial Cholesterol and Cholesterol α-Glucoside Content

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Helicobacter pylori **blocks the proliferation of human $CD4^+$ T cells, facilitated by vacuolating exotoxin (VacA) and γ-glutamyl transpeptidase (GGT).** ***H. pylori*****–triggered T-cell reactions in mice correlate with bacterial cholesterol and cholesterol α-glucoside content but their role in human cells is unclear. We characterized the effect of VacA, GGT, and cholesterol on T-helper 1, T-helper 2, T-regulatory and T-helper 17 associated cytokines and T-cell proliferation. VacA, GGT, and bacterial cholesterol content exhibited differential and synergistic inhibitory effects on the expression of activation markers CD25 and CD69 and on interleukin 2, interleukin 4, interleukin 10, and interferon γ production. These factors did not affect the** ***H. pylori–mediated*** **abrogation of transforming growth factor β secretion or increased interleukin 6 production. Cholesterol α-glucosyltransferase–deficient bacteria exerted strongly reduced antiproliferative effects on primary human $CD4^+$ T cells. In conclusion,** ***H. pylori*** **shapes rather than suppresses human $CD4^+$ T-cell responses, and glucosylated cholesterol is a relevant bacterial component involved in this modulation.**

Helicobacter pylori resides in one-half of the world's population and can cause gastritis, ulcer, and gastric cancer [1]. T-cell responses against *H. pylori* are associated with gastric inflammation [2, 3] and, in animal models, confer protection against infection [4, 5]. *H. pylori* virulence factors have evolved to suppress protective T-cell responses. The constitutively expressed enzyme γ-glutamyl transpeptidase (GGT) is responsible for suppression of T cells [6]; however, vacuolating exotoxin (VacA) has also been implicated in the inhibition of T-cell proliferation [7]. *H. pylori* has been shown to inhibit interleukin 2 (IL-2) secretion from human T-cell lines in a VacA-dependent manner, with modest effects in primary human T cells [8]. A third mechanism used by *H. pylori* has also been proposed: *H. pylori* is a cholesterol auxotroph and therefore extracts this essential nutrient from epithelial and antigen-presenting cells [9]. In a murine model of *H. pylori* infection, this process affected the immune response against the bacteria by promoting phagocytosis by antigen-presenting cells and thereby enhancing antigen-specific T-cell responses [9].

T cells differentiate into diverse subsets upon activation by antigen-presenting cells [10], including T-helper 1 (Th1), T-helper 2 (Th2), T-helper 17 (Th17), and T-regulatory (Treg) cells [11]. Th1 cells characteristically produce interferon γ (IFN-γ) and are important for immunity against intracellular microorganisms, whereas Th2 cells produce interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 10 (IL-10), and interleukin 13 (IL-13) and are linked to immune responses against extracellular pathogens. Cellular immune responses against *H.*

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pylori are known to be predominantly mediated by Th1 cells [2–5]. Th17 cells depend on transforming growth factor β (TGF-β) and interleukin 6 (IL-6) for differentiation, produce interleukin 17A (IL-17A), and are important in the response to extracellular bacteria at mucosal surfaces, including *H. pylori* infection [12, 13]. Treg cells, which also require TGF-β for differentiation, are characterized by the expression of the transcription factor FoxP3 and their capacity to suppress immune responses [14] and are implicated in the persistence of *H. pylori* [15].

Previous investigations into the effects of *H. pylori* on $CD4^+$ T cells have mainly used cell lines, even though these results may not be fully applicable to primary human T cells [8]. In addition, whereas the effect of purified molecules (eg, VacA and GGT) has been tested on T cells, little is understood about their contribution to the modulation of immunity by whole bacteria [7, 8]. Moreover, because most studies have focused on IL-2 secretion [6–8], the effect of *H. pylori* on other cytokines remains poorly characterized. Finally, potential interactions between *H. pylori* virulence factors affecting T-cell immunity have not yet been addressed.

In this study, we evaluate the interdependence of the effects of VacA, GGT, and cholesterol/cholesterol α-glucosides on primary T cells using live *H. pylori* and relevant mutants. Analyzing Th1-, Th2-, and Th17-associated cytokines and Treg subsets of human $CD4^+$ T cells and proliferation, we demonstrate that *H. pylori* shapes rather than simply suppresses immune responses and bacterial cholesterol content, and the ability to form cholesterol α-glucosides contribute to this process.

MATERIALS AND METHODS

Bacteria

CagPAI$^+$ *H. pylori* wild-type strain P12 is a clinical isolate, which has been described elsewhere [16]. *H. pylori* P12 wild type was cultured on horse serum agar plates supplemented with vancomycin (10 μg/mL). The isogenic P12 mutant strains *H. pylori* Δ*vacA*, *H. pylori* Δ*ggt*, and *H. pylori* Δ*421* (lacking cholesterol α-glucosyl transferase) strains were cultivated on selective agar plates. Bacteria were incubated for 2 days before infection at 37°C in an incubator with 5% CO_2 and 5% O_2.

Isolation of Primary Human $CD4^+$ T Cells

Peripheral blood mononuclear cells were obtained following approval of the ethical committee of the Charité and informed consent from healthy human donors as described elsewhere [17]. $CD4^+$ T cells were isolated with a $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotech) according to the manufacturer's instructions. $CD4^+$ T cells were cultured in Roswell Park Memorial Institute 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS) or in Optimizer T-cell Expander serum-free medium (Gibco). The purified cells were 99% $CD4^+$ cells, as assessed by flow cytometry.

Incubation of $CD4^+$ T Cells With *H. pylori*

$CD4^+$ T cells (1×10^6 cells/mL) were incubated with *H. pylori* at different multiplicities of infection (MOIs) for 2 hours. *H. pylori* or *H. pylori* Δ*421* were coated with water-soluble cholesterol (C4951; Sigma) at a concentration of 100 μmol/L for 30 minutes at 37°C and washed prior to infection for 2 hours. Penicillin (100 U/mL), streptomycin (100 μg/mL), and gentamycin (100 μg/mL) were then added to kill extracellular bacteria. After 1 hour, the $CD4^+$ T cells were activated with immobilized anti-CD3 (1 μg/mL) and soluble anti-CD28 (3 μg/mL; BD Pharmingen) and incubated for 24 hours or 7 days.

$CD4^+$ T-Cell Proliferation Assay

$CD4^+$ T cells were suspended in phosphate-buffered saline (PBS) containing carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Invitrogen) at a concentration of 0.5 mmol/L, incubated for 3 minutes at room temperature, and washed twice with the appropriate culture medium. CFSE-stained $CD4^+$ T cells (1×10^6 cells/mL) were then suspended in Optimizer T-cell Expander serum-free medium (Gibco) and incubated with *H. pylori* or mutants, as indicated above. Cell proliferation was evaluated after 7 days by CFSE fluorescence. Dead cells were excluded from the analysis.

Measurement of Cytokine Levels

Secretion levels of IL-2, IL-10, and tumor necrosis factor α (TNF-α) were evaluated by the DuoSet enzyme-linked immunosorbent assay (ELISA) development system (R&D Systems). For measurement of IFN-γ, IL-6, IL-4, and IL-17A levels, sandwich ELISAs were performed as suggested by the supplier (U-Cytech, the Netherlands). The TGF-β1 ELISA was purchased from Bender MedSystems.

Flow Cytometry and Apoptosis Assay

$CD4^+$ T cells were washed with PBS and 2% FCS and incubated with anti-CD25 (Bekton Dickinson) or anti-CD69 (Dako) for 30 minutes at 4°C. After washing the cells with PBS and 2% FCS, fixation was performed with 4% paraformaldehyde for 20 minutes at room temperature. For each experimental condition, data from 10 000 cells were collected on a FACSCalibur flow cytometer (BD Biosciences). Live cells were gated based on forward and side-scatter properties. Apoptosis was evaluated with the Annexin V-FITC Apoptosis Detection Kit I from BD Pharmingen.

Statistical Analysis

Data shown are median values unless otherwise stated. Nonparametric tests (Mann-Whitney test or Wilcoxon paired test) or analysis of variance were used to determine statistical significance. Differences were considered significant if $P < .05$.

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RESULTS

Interdependent and Differential Inhibition of Early T-Cell Activation Markers IL-2, CD25, and CD69 by *H. pylori* Virulence Factors

We mimicked the ability of *H. pylori* to obtain cholesterol from the membranes of host cells [9] by incubating the bacteria with cholesterol. The effect of cholesterol saturation in combination with VacA or GGT on IL-2 production and expression of its receptor CD25 in human $CD4^+$ T cells was assessed by ELISA and fluorescence-activated cell sorting (FACS), respectively. Cells obtained from healthy volunteers were incubated with *H. pylori* and then activated. Levels of IL-2 secretion after 24 hours by $CD4^+$ T cells from different donors not exposed to *H. pylori* ranged from 450 to 1300 pg/mL (Figure 1). In comparison, levels of IL-2 production were significantly reduced upon incubation with *H. pylori* (Figure 1*A*); however, coating *H. pylori* with cholesterol significantly attenuated this inhibition (Figure 1*A*). Isogenic mutants lacking VacA elicited a similar, albeit weaker, phenotype as the cholesterol-saturated *H. pylori*. In agreement with previous studies [6], mutants lacking GGT activity inhibited IL-2 production (Figure 1*A*).

We also measured the expression of CD25, a component of the IL-2 receptor, upon exposure to cholesterol-saturated *H. pylori* or VacA or GGT mutants. FACS analysis revealed that, similar to IL-2 production, expression of CD25 after 24 hours was significantly reduced when T cells were incubated with wild-type *H. pylori,* but not cholesterol-coated *H. pylori* or the VacA or GGT mutant bacteria (Figure 1*B*, *upper panels*). After 7 days of incubation, CD25 remained down-regulated in viable *H. pylori*–exposed T cells. This long-term effect was partially reverted by precoating the bacteria with cholesterol (Figure 1*B*, *lower panel*). Consistent with the data from 24-hour incubation, expression of CD25 was also not reduced in cells incubated with VacA or GGT mutant bacteria for 7 days (Figure 1*B*, *lower panel*). *H. pylori* also hampered the up-regulation of CD69 (Figure 1*C*), another early activation marker transiently expressed upon stimulation of T cells [18]. Expression of CD69 was exclusively dependent on VacA, as its inhibition was reverted by neither cholesterol coating nor the absence of GGT (Figure 1*C*).

These effects were not due to differences in the ability of cholesterol-treated bacteria or mutants to interact with $CD4^+$ T cells or induce their apoptosis (Figure 2; Table 1). Therefore, these results reveal that even though *H. pylori* inhibits IL-2 and/or IL-2 receptor expression via the action of VacA and GGT, their effect is dramatically modulated by bacterial cholesterol content. In contrast, the inhibition of CD69 is exclusively mediated by VacA.

Interdependent Effects of *H. pylori* Virulence Factors on Th1- and Th2-Type Cytokine Release in Activated Human T Cells

$CD4^+$ T cells play a key role in the immune response against *H. pylori* [19], exerting their protective effect mainly through the secretion of cytokines. Hence, $CD4^+$ T cells were incubated with wild-type *H. pylori* or VacA and GGT mutants for 2 hours and activated. Levels of IL-10, IL-4, IFN-γ, and TNF-α were measured 24 hours later.

Cells from healthy donors released 2–350 pg/mL of IL-10. Cells from 2 donors did not produce detectable levels of the cytokine. Production of IL-10 was suppressed by *H. pylori* (Figure 3); however, this was restored by coating the bacteria with cholesterol or in the absence of VacA and GGT (Figure 3*A*). Coating of *H. pylori* Δ*vacA* or *H. pylori* Δ*ggt* with cholesterol did not further alter the release of IL-10 (data not shown).

Gastric epithelial cells from healthy individuals have been shown to produce more IL-4 than cells from *H. pylori*–infected patients [20]. In accordance, levels of IL-4 released by stimulated $CD4^+$ T cells (2–33 pg/mL) were significantly suppressed in the presence of *H. pylori*. This suppression was reverted when $CD4^+$ T cells were incubated with cholesterol-coated *H. pylori* (Figure 3*B*). VacA seemed to have the most significant impact on the inhibition of IL-4 (Figure 3*B*), and accordingly, coating of *H. pylori* Δ*vacA* with cholesterol had no additional effect (data not shown). By contrast, the absence of GGT did not increase IL-4 levels (Figure 3*B*). These data show that *H. pylori* suppresses IL-4 production by $CD4^+$ T cells mainly in a VacA-dependent manner. Furthermore, the amplitude of this suppression is modulated by bacterial cholesterol content.

Mucosal inflammation in *H. pylori* infection correlates with Th1 T-cell signatures [21], and IFN-γ–producing T cells are involved in gastric epithelial cell cytotoxicity [22]. Levels of IFN-γ secretion by $CD4^+$ T cells varied between 200 and 4500 pg/mL (Figure 3*C*). Cells from 1 donor did not release IFN-γ upon stimulation. $CD4^+$ T cells incubated with *H. pylori* released less IFN-γ than cells not exposed to the bacteria (Figure 3*C*). Suppression of IFN-γ and IL-4, however, was less pronounced than that observed for IL-10 (Figure 3*A*, *B*, and *C*). Interestingly, although the presence of VacA and GGT was required for the suppression of IFN-γ, bacterial cholesterol content did not play any role.

Production of TNF-α has been shown to correlate with gastritis and bacterial load in *H. pylori*–infected patients [23, 24]. In contrast to the effect of *H. pylori* on the other cytokines studied here, exposure of $CD4^+$ T cells to the bacterium did not modulate the secretion of this pro-inflammatory cytokine. Under all conditions, cells released TNF-α levels of 395–2, 820 pg/mL (Figure 3).

Our results show that *H. pylori* can suppress anti-inflammatory and pro-inflammatory cytokine production. However, modulation of the latter type is often less pronounced or may not occur. Bacterial cholesterol content affects the amplitude of the inhibition of anti-inflammatory cytokines only, whereas VacA and GGT are involved in the inhibition of both types of cytokines.

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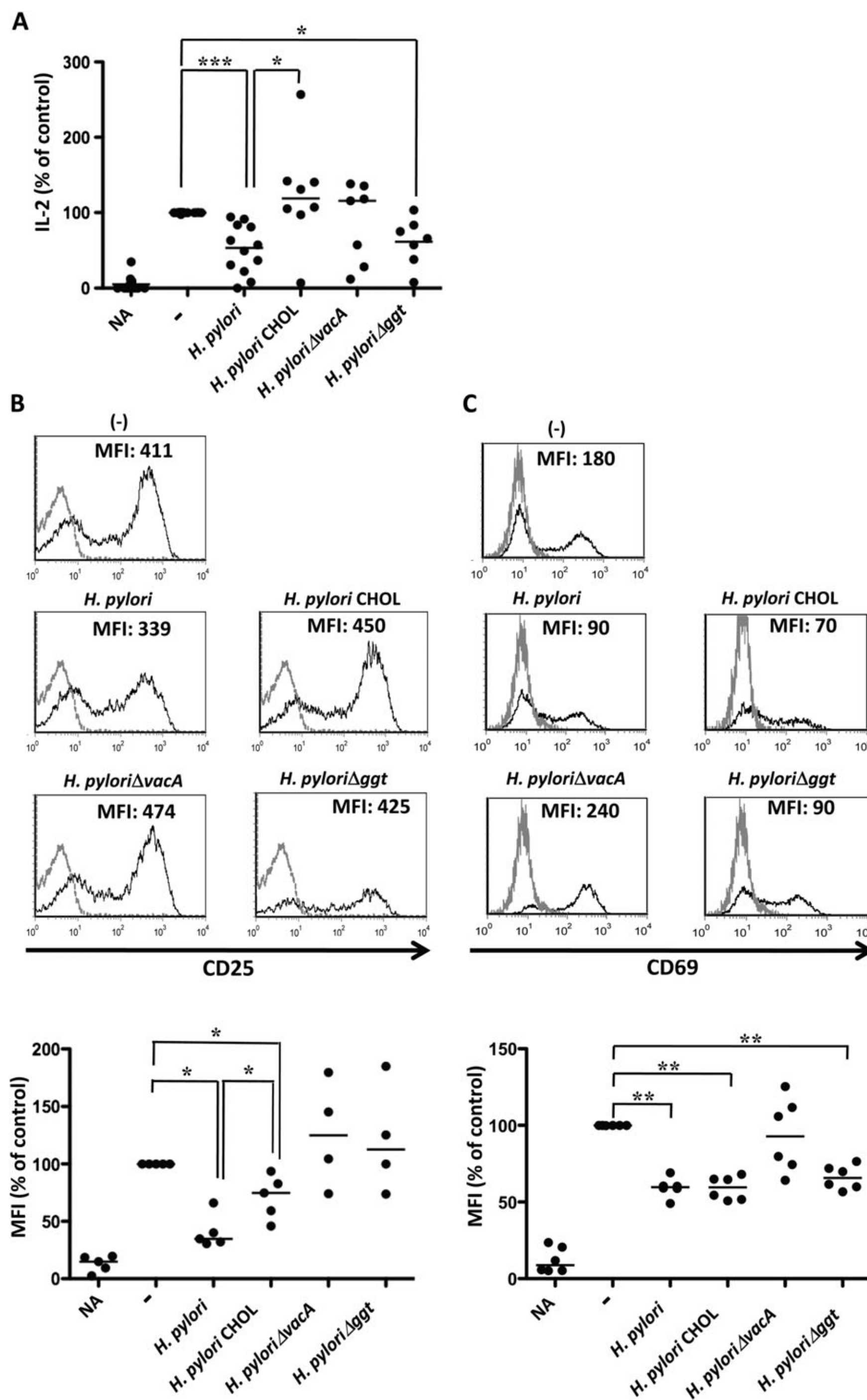

Figure 1. Interdependent modulation of early T-cell activation markers interleukin 2 (IL-2), CD25, and CD69 by *Helicobacter pylori* virulence factors. Human CD4$^+$ T-cells were incubated with *H. pylori*, cholesterol-coated *H. pylori* (*H. pylori* CHOL), mutant *H. pylori* lacking γ-glutamyl transpeptidase (*H. pylori* Δ*ggt*), or mutant *H. pylori* lacking vacuolating exotoxin (*H. pylori* Δ*vacA*) at a multiplicity of infection (MOI) of 50 for 2 hours and then activated with anti-CD3 and anti-CD28. *A,* IL-2 levels. Supernatants were harvested after 24 hours and the concentration of IL-2 was evaluated by enzyme-linked immunosorbent assay. Cells from 12 donors were assessed. Data are the median percentage relative to uninfected cells. NA, not activated. *$P < .05$; ***$P < .001$ (Mann–Whitney *U* test). *B,* Histograms showing the expression of CD25 after 24 hours of activation (*gray, isotype control*). A representative experiment of $n = 5$ is shown. The lower part shows the median fluorescence intensity (MFI) of CD25 surface expression obtained from cells incubated with *H. pylori, H. pylori* CHOL, *H. pylori* Δ*ggt,* or *H. pylori* Δ*vacA* for 7 days. Cells from 5 donors were assessed. The graph depicts the percentage of MFI values relative to uninfected cells. NA, not activated. *$P < .05$ (Mann–Whitney *U* test). (*C*) Expression of CD69 after 24 hours of activation (*gray, isotype control*); shown is a representative experiment of $n = 6$ (*upper panel*). Median MFI values of cells incubated with *H. pylori, H. pylori* CHOL, *H. pylori* Δ*ggt,* or *H. pylori* Δ*vacA* for 24 hours are shown. The graph depicts the percentage MFI relative to uninfected cells. Abbreviation: NA, not activated. *$P < .05$; **$P < .006$ (Mann–Whitney *U* test).

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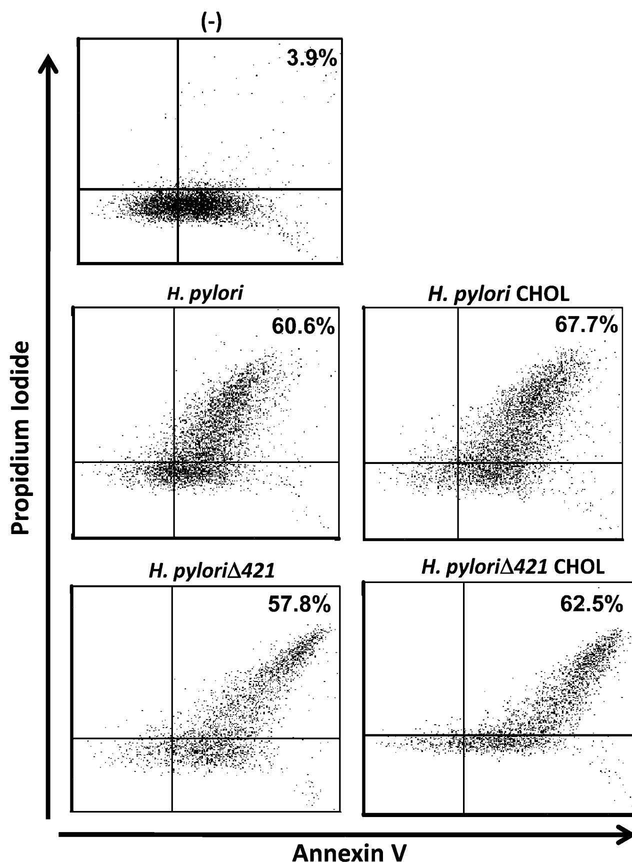

Figure 2. Apoptosis of human CD4$^+$ T cells incubated with *Helicobacter pylori*, cholesterol-coated *H. pylori* (*H. pylori* CHOL), a mutant strain lacking cholesterol α-glucosyl transferase (*H. pylori Δ421*) and cholesterol-coated *H. pylori Δ421 (H. pylori Δ421* CHOL). The cells were incubated with the bacteria at a multiplicity of infection of 50 for 2 hours and then activated with anti-CD3 and anti-CD28. Apoptosis was evaluated by propidium iodide and Annexin V staining after 24 hours. Histograms show the percentage of double positive cells. A representative experiment of $n = 5$ is shown. A mutant strain lacking γ-glutamyl transpeptidase (*H. pylori Δggt*), and a mutant strain lacking vacuolating exotoxin (*H. pylori ΔvacA*) were analyzed similarly (see Table 1).

VacA-, GGT-, and Cholesterol-Independent Modulation of Th17-Related Cytokine Release in Human CD4$^+$ T Cells

Previous work has shown that IL-17 is expressed at a higher level in the gastric mucosal and lamina propria of *H. pylori*–infected patients [13]. By contrast, here we found that *H. pylori* did not significantly affect the level of IL-17A produced by activated human CD4$^+$ T cells (20–285 pg/mL) (Figure 4). Coating the bacteria with cholesterol or exposure to *H. pylori* VacA or GGT mutants did not alter the levels of IL-17A produced (Figure 4*A*).

Because Th17 differentiation depends on TGF-β1 and IL-6 [11], we also measured the production of these cytokines. TGF-β1 secretion ranged from 5 to 52 ng/mL; however, cells from 8 of 14 donors did not release detectable levels of TGF-β1 (Figure 4*B*). In the presence of *H. pylori,* production of TGF-β1 was completely abolished (Figure 4*B*), an effect neither modulated by cholesterol coating of *H. pylori* nor dependent on VacA or GGT virulence factors (Figure 4*B*). Cells from 10 of 12 donors produced detectable levels of IL-6 upon activation ranging from 14 to 790 pg/mL. *H. pylori* enhanced the release of IL-6 under all conditions tested. Cholesterol coating or the absence of VacA or GGT had no effect on this *H. pylori*–enhanced IL-6 secretion (Figure 4*C*). Moreover, despite proliferation of CD4$^+$ T cells being suppressed by the bacteria (see below), IL-6 levels were significantly increased after 7 days of incubation with *H. pylori* (data not shown).

These data show that although IL-17A production, presumably by Th17 cells, is not modulated by *H. pylori,* T-cell–dependent TGF-β1 production was inhibited and IL-6 secretion was stimulated. In contrast to type 1 and 2 related cytokines, neither cholesterol coating nor VacA or GGT played a role in the modulation of TGF-β1 and IL-6, suggesting the existence of yet another set of factors targeting this arm of CD4$^+$ T-cell responses.

Cholesterol α-Glucosyltransferase–Deficient *H. pylori* at Low MOI Lack Antiproliferative Activity on Human T Cells

Previous work has implicated VacA and GGT in the *H. pylori*–induced blockage of T-cell proliferation, the latter factor exerting a more potent effect [6, 7]. Given the modulation of GGT- and VacA-mediated effects on cytokines by bacterial cholesterol content, we determined whether cholesterol coating of *H. pylori* would alter the proliferation of human CD4$^+$ T cells. CFSE-stained CD4$^+$ T cells were incubated with wild-type *H. pylori* or cholesterol-coated *H. pylori* prior to activation. In accordance with the results of previous work [25], *H. pylori* inhibited proliferation of primary T cells; however, this effect was dependent on GGT but independent on VacA, contradicting results of previous studies that used purified VacA [7] (Figure 5). Inhibition of T-cell proliferation was MOI-dependent, and consistent with our findings here, the effect of GGT was significantly modulated by bacterial cholesterol levels (Figure 5*C*), in particular at lower MOIs, which are probably more physiologically relevant.

Most of the cholesterol *H. pylori* requires for optimal growth becomes glucosylated through the action of a cholesterol α-glucosyl transferase encoded by open reading frame HP0421 [26]. In a mouse model of *H. pylori* infection, HP0421-deficient mutants are readily phagocytosed by antigen-presenting cells enhancing proliferation of specific T cells [9]. Therefore, we measured the proliferation of T cells upon exposure to an isogenic *H. pylori* strain lacking cholesterol α-glucosyl transferase (*H. pylori Δ421*) [9]. At high MOIs, T-cell proliferation was strongly inhibited by the mutant; however, this inhibitory effect was rapidly attenuated as MOI decreased (Figure 5*B*). The mutation had an effect similar to coating wild-type bacteria with cholesterol (Figure 5*C*). However, coating *H. pylori Δ421* with cholesterol did not modulate the inhibition of proliferation (Figure 5*C*). Similar results were obtained with a mutant of the gene HP0499, which

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Table 1. Apoptosis of CD4+ T Cells Induced by *H. pylori* Is Not Modulated by VacA, GGT, Alpha-Glucosyl Transferase or Cholesterol Loading

	No. of Annexin V– and propidium iodide–positive cells, %					
Treatment	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Mean (SD)
Not activated	6.3	7.3	4.9	3.1	5.3	5.4 (1.6)
Activated without bacteria	9.3	8.1	11.7	3.9	5.5	7.7 (3.1)
H. pylori	62.2	45.7	54.2	60.6	62.8	57.1 (7.2)
H. pylori CHOL	64.5	50.6	61.4	67.7	75.4	63.9 (9.1)
H. pylori Δ*421*	54.7	51.2	53.5	57.8	60.8	55.6 (3.7)
H. pylori Δ*421* CHOL	56.8	59.8	66.0	62.5	72.1	63.4 (5.9)
H. pylori Δ*vacA*	54.6	66.5	74.1	77.7	85.7	71.7 (11.8)
H. pylori Δ*ggt*	60.0	64.3	64.8	64.5	86.5	68.0 (10.5)

Human $CD4^+$ T cells were incubated with *Helicobacter pylori,* cholesterol-coated *H. pylori* (*H. pylori* CHOL), a mutant strain lacking cholesterol α-glucosyl transferase (*H. pylori* Δ*421*), cholesterol-coated *H. pylori* Δ*421* (*H. pylori* Δ*421* CHOL), a mutant strain lacking γ-glutamyl transpeptidase (*H. pylori* Δ*ggt*), or a mutant strain lacking vacuolating exotoxin (*H. pylori* Δ*vacA*) at a multiplicity of infection of 50 for 2 hours and then activated with anti-CD3 and anti-CD28. Apoptosis was evaluated by fluorescence-activated cell sorting after 24 hours. No significant differences were found after treatment between *H. pylori, H. pylori* CHOL, and the mutants (analysis of variance test).

is also involved in the synthesis of cholesterol α-glucosides [27] (data not shown). These data provide the first evidence that cholesterol α-glucosyl transferase activity is required for *H. pylori*–mediated direct inhibition of human $CD4^+$ T-cell proliferation.

DISCUSSION

To date, 2 *H. pylori* virulence factors, VacA and GGT, have been implicated in directly suppressing human T cells. Purified VacA

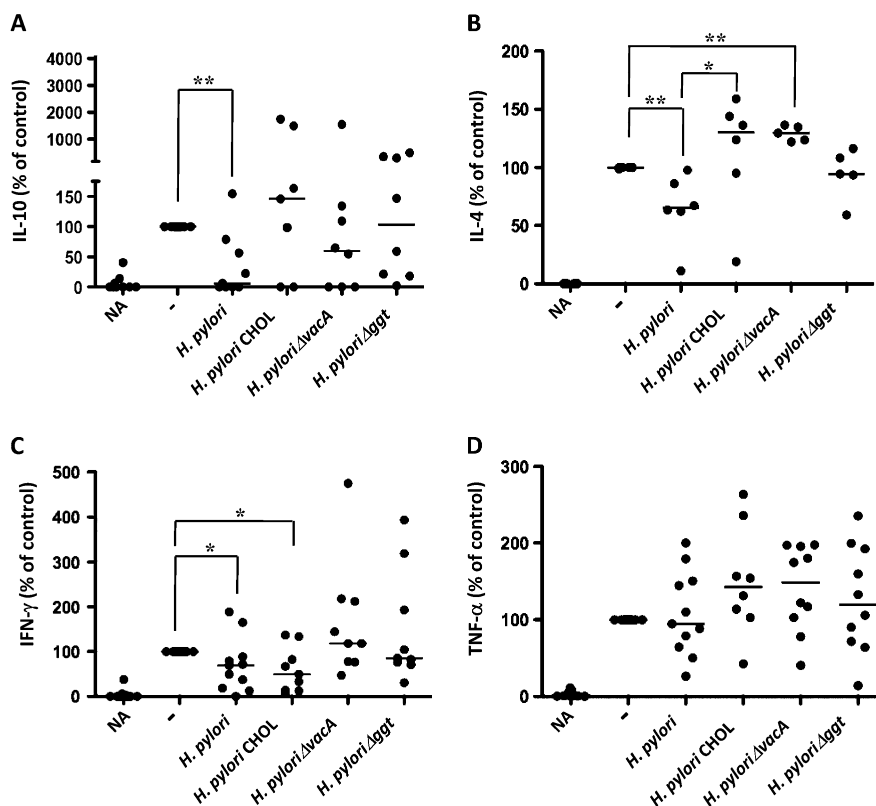

Figure 3. Interdependent effects of *Helicobacter pylori* virulence factors on T-helper 1 and T-helper 2 type cytokine release in activated human T cells. Human $CD4^+$ T cells were incubated with *H. pylori*, cholesterol-coated *H. pylori* (*H. pylori* CHOL), mutant *H. pylori* lacking γ-glutamyl transpeptidase (*H. pylori* Δ*ggt*), or mutant *H. pylori* lacking vacuolating exotoxin (*H. pylori* Δ*vacA*) at a multiplicity of infection of 50 for 2 hours and then activated with anti-CD3 and anti-CD28. Supernatants were harvested after 24 hours, and the concentration of interleukin 10 (IL-10) (*A*), interleukin 4 (IL-4) (*B*), interferon γ (IFN-γ) (*C*), or tumor necrosis factor α (TNF-α) (*D*) was evaluated by enzyme-linked immunosorbent assay. Data are the median percentage relative to uninfected cells. NA, not activated. $^*P < .05$ (Mann–Whitney *U* test). ** $P < .007$

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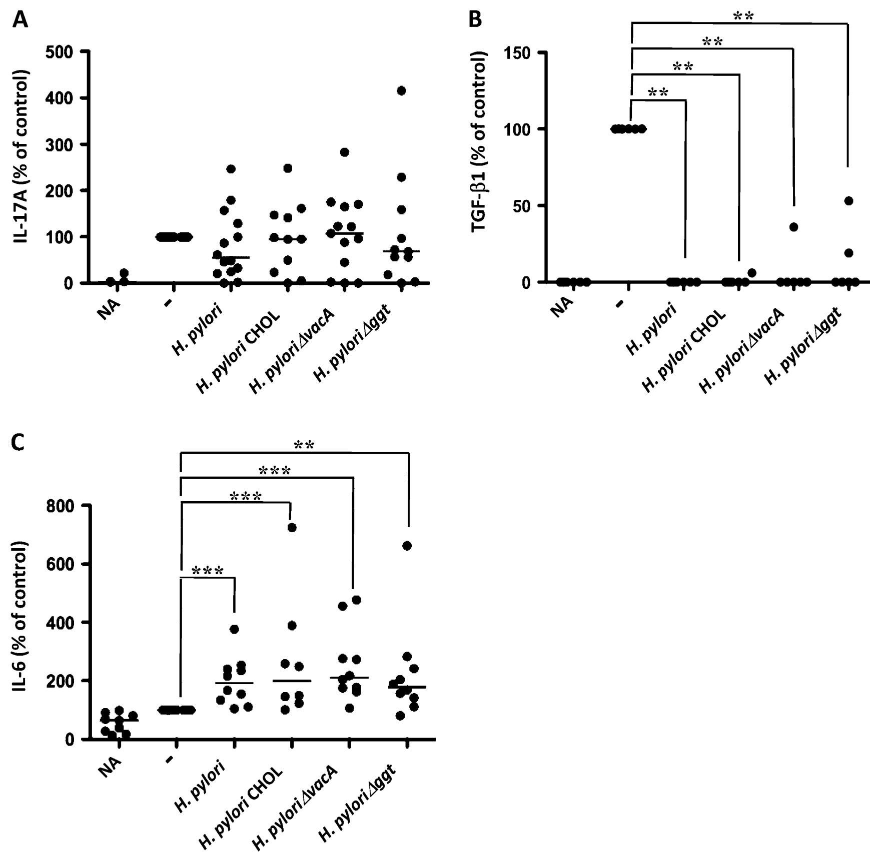

Figure 4. Vacuolating exotoxin (VacA), γ-glutamyl transpeptidase (GGT), and cholesterol independent modulation of T-helper 17 related cytokine release in human CD4$^+$ T cells. Human CD4$^+$ T cells were incubated with *Helicobacter pylori,* cholesterol-coated *H. pylori* (*H. pylori* CHOL), a mutant strain lacking GGT (*H. pylori* Δ*ggt*), or a mutant strain lacking VacA (*H. pylori* Δ*vacA*) at a multiplicity of infection of 50 for 2 hours and then activated with anti-CD3 and anti-CD28. Supernatants were harvested after 24 hours, and the concentration of interleukin 17a (IL-17A) (*A*), transforming growth factor β1 (TGF-β1) (*B*), or interleukin 6 (IL-6) (*C*) was evaluated by enzyme-linked immunosorbent assay. Data are the median percentage relative to uninfected cells. NA, not activated. *$P < .05$ (Mann–Whitney *U* test). ** $P < .005$, *** $P < .001$

has been shown to inhibit IL-2 production in human [8] but not murine T cells [28] that lack a cognate receptor [29]. Although the cholesterol/cholesterol α-glucosyl content of the bacteria had a profound effect on antigen presentation and T-cell responses in mice [9], its effect on human cells remained to be determined. Here, we demonstrate that bacterial cholesterol/cholesterol α-glucoside content constitute a third molecular component that synergises and/or modifies the activity of VacA and GGT to modulate human CD4$^+$ T-cell responses.

Previous studies of *H. pylori* factors that inhibited T-cell responses have focused on secreted molecules, such as VacA and GGT, reasoning these may preferentially be encountered by T cells in the inflamed gastric tissue. However, *H. pylori* is also present deeper within infected tissues [30], and similar to other gram-negative bacteria, it sheds outer membrane vesicles. These vesicles are considered to be pathologically relevant mimicks of the bacterial membrane, carrying virulence factors [31] that can act at a distance [32, 33]. Thus, investigating the direct interaction of *H. pylori* with T cells at low MOIs using whole bacteria, as reported here, likely reflects physiological conditions.

Conditioned culture supernatants containing GGT or recombinant GGT have been shown to abolish T-cell proliferation [6], in a mechanism dependent on GGT enzymatic activity and, potentially, the modulation of the GTPase Ras signaling pathway, leading to cell cycle arrest. However, the process remains ill defined and may also be linked to the antiproliferative role proposed for endogenous human GGT expressed by activated T cells [34]. Our results confirmed and extended previous findings that GGT blocks proliferation but has little or no significant modulating activity on additional response parameters such as secretion of pro- and anti-inflammatory cytokines. Furthermore, using whole mutant bacteria, we detected an additional inhibitory effect of GGT on CD25 expression. Most importantly, our findings indicate that the dramatic GGT-specific antiproliferative activity was subject to modulation by the bacterial cholesterol/cholesterol α-glucoside content, in particular at lower, likely more physiologically relevant MOIs. Cholesterol glucosides are thought to be characteristic of *H. pylori* because they are rarely found in other bacteria [35]. It has been proposed

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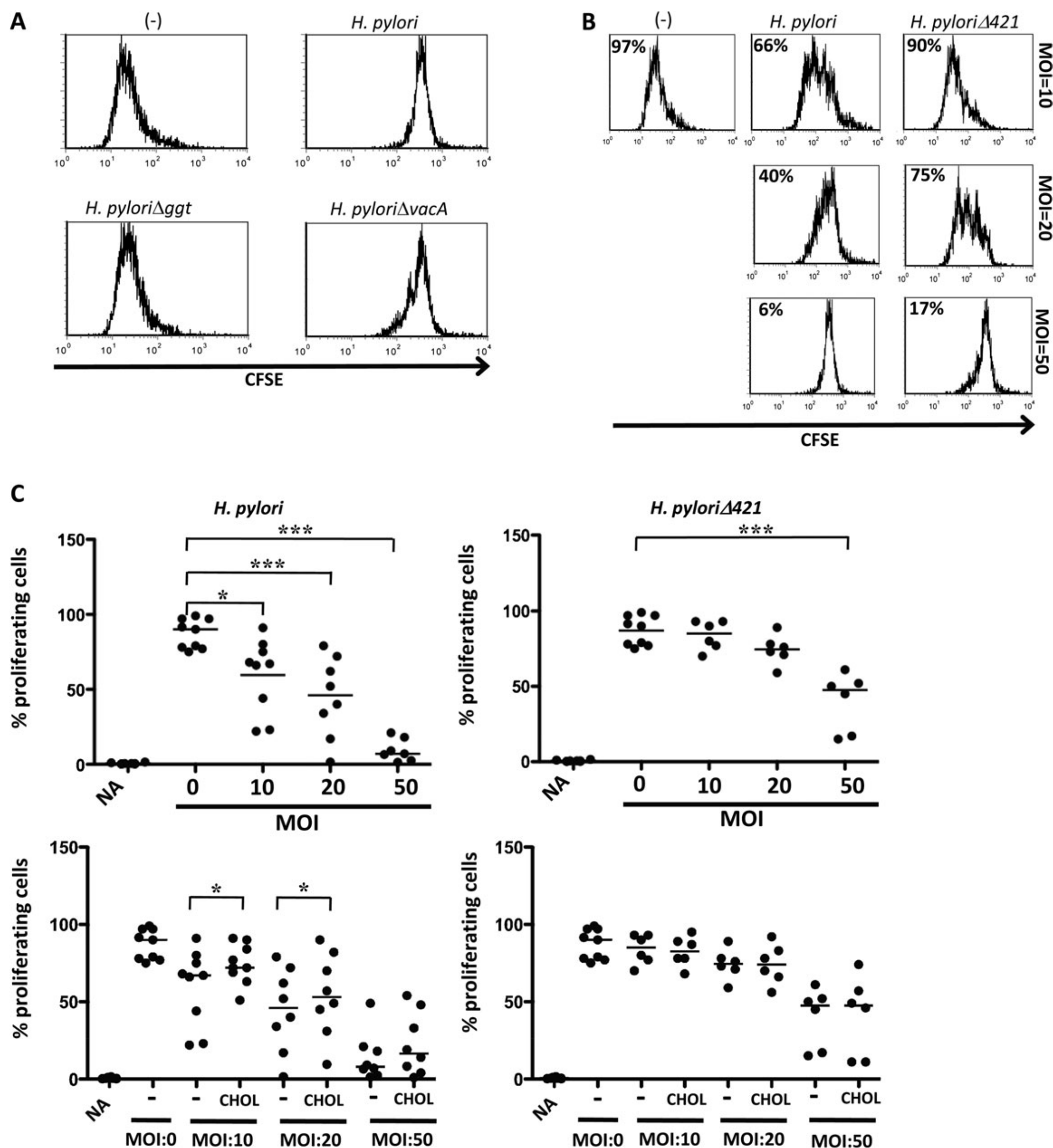

Figure 5. Inhibition of T-cell proliferation by cholesterol α-glucosyltransferase from *Helicobacter pylori*. *A,* Carboxyfluorescein succinimidyl ester (CFSE)–stained human $CD4^+$ T cells were incubated with wild-type *H. pylori,* a mutant strain lacking γ-glutamyl transpeptidase (*H. pylori* Δ*ggt*), or a mutant strain lacking vacuolating exotoxin (*H. pylori* Δ*vacA*) at a multiplicity of infection (MOI) of 50 for 2 hours and then activated with anti-CD3 and anti-CD28 for 7 days. Shown is a representative experiment of $n = 9$. *B,* CFSE-stained human $CD4^+$ T cells were incubated with wild-type *H. pylori* or a mutant strain lacking cholesterol α-glucosyl transferase (*H. pylori* Δ*421*) at MOI of 10, 20, or 50 for 2 hours and then activated with anti-CD3 and anti-CD28 for 7 days. Shown is the percentage of proliferating cells for a representative experiment of $n = 9$. *C,* CFSE-stained human $CD4^+$ T cells were incubated with wild-tye *H. pylori* or *H. pylori* Δ*421* uncoated (*upper panels*) or coated with cholesterol (CHOL) (*lower panels*) at MOI of 10, 20, or 50 for 2 hours and then activated with anti-CD3 and anti-CD28 for 7 days. NA, not activated. Data are expressed as a percentage of proliferating cells. Upper panels, $^*P < .05$, $^{***}P < .001$ (analysis of variance Tukey posttest); lower panels, $^*P < .05$ (Wilcoxon test).

that *H. pylori* controls the availability of cholesterol glucosilated phospholipids in the cell wall according to environmental changes [35]. Dendritic cell responses are modulated by *H. pylori* through phase variation of lipopolysaccharides [36]. Here, we show the first evidence that *H. pylori* directly manipulates host T-cell responses through changes in cholesterol/cholesterol α-glucoside content.

Purified VacA also inhibits $CD4^+$ T-cell responses after polyclonal in vitro stimulation. This effect depends on the VacA allele present because only some forms bind to the leukocyte-specific receptor CD18 [29]. At high concentrations, VacA inhibited proliferation and expression of early surface activation markers such as CD25, as well as IL-2 production. Similar to cyclosporine or FK506, VacA inhibits the calcium-dependent activation of

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Table 2. *Helicobacter pylori* Shapes Human CD4$^+$ T-Cell Responses

	Regulation of cytokine production										
					Pro-inflammatory (type I)			Anti-inflammatory (type II)			
	T-cell activation				Th1		Th17	Th2			Treg
Strain	Prolif.	CD69	CD25	IL-2	IFN-γ	TNF-α	IL-17A	IL-4	IL-6	IL-10	TGF-β
H. pylori	Down	Down	Down	Down	Down	None	None	Down	Up	Down	Down
H. pylori Δ*vacA*	Down	None	None	None	None	None	None	None	Up	None	Down
H. pylori Δ*ggt*	None	Down	None	Down	None	None	None	Down	Up	None	Down
H. pylori CHOL	Down	Down	Down	None	Down	None	None	None	Up	None	Down

Regulation of cytokine production compared with that in activated CD4$^+$ T cells in the absence of *H. pylori*. "None" means no change was observed compared with activated CD4$^+$ T cells in the absence of *H. pylori*. For CD25, only the data obtained after 7 days of co-incubation between CD4$^+$ T cells and *H. pylori* were considered for this table. Abbreviations: *H. pylori* CHOL, cholesterol-coated *H. pylori; H. pylori* Δ*ggt*, mutant strain lacking γ-glutamyl transpeptidase; *H. pylori* Δ*vacA*, mutant strain lacking vacuolating exotoxin; IFN-γ, interferon γ; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IL-10, interleukin 10; IL-17A, interleukin 17A; Prolif., percentage of proliferating cells; TGF-β, transforming growth factor β; Th1, T-helper 1; Th2, T-helper 2; Th17, T-helper 17; TNF-α, tumor necrosis factor α; Treg, T-regulatory.

nuclear factor of activated T cells, a transcription factor that regulates the *IL2* gene [37]. In our study, comparison of VacA-deficient mutants with parental bacteria expressing a CD18-binding VacA allele did not affect proliferation but the presence of VacA did result in reduced expression of CD25, CD69, and IL-2. In addition, we observed VacA-specific inhibition of IL-4, IL-10, and IFN-γ production. With the exception of CD69, the extent of these inhibitory effects of VacA was again modulated by the bacterial cholesterol content.

IL-4 and IFN-γ are often studied as surrogate markers of type 1 and type 2 T cell responses. Mucosal inflammation in mice is exacerbated in the absence of IL-4 [38] but correlates with Th1 T-cell signatures [21]. Furthermore, higher levels of IL-4 are produced by gastric epithelial cells from healthy individuals than those from *H. pylori*–infected patients [20], and IFN-γ–producing T cells are involved in gastric epithelial cell cytotoxicity [22]. Our data indicate that *H. pylori* interferes with IL-4 and IFN-γ through the concerted action of VacA, GGT, and cholesterol/cholesterol α-glucosides. Recent studies suggested that Th17 cells and Treg cells also play important roles in *H. pylori* infection and immunity [13, 39, 40]. IL-17 was shown to be expressed at a higher level in the gastric mucosa and lamina propria of *H. pylori*–infected patients [13], and *H. pylori* gastritis was associated with increased gastric mucosal production of IL-6 [41]. Moreover, levels of IL-6 in *H. pylori*–infected patients with early-stage gastric cancer decreased significantly after cure [24]. Therefore, we evaluated the effects of *H. pylori* on TGF-β, IL-6, and IL-17 levels. In our experiments, exposure of activated CD4$^+$ T cells to *H. pylori* did not change IL-17A expression but enhanced IL-6 production, whereas TGF-β levels became undetectable. Surprisingly, neither VacA, GGT, nor cholesterol/cholesterol α-glucoside levels had any influence on these changes. Thus, we propose that as yet unknown *H. pylori* factors interfere with these arms of the T-cell response.

In summary, our data suggest that *H. pylori* shapes rather than simply suppresses or evades CD4$^+$ T-cell–mediated responses in order to persist within its host. The results of the present study establish that in addition to VacA and GGT, cholesterol/cholesterol α-glucosides and as yet unknown factors target specific aspects of T-cell responses (summarized in Table 2). The findings are consistent with previous observations that *H. pylori* supports T-cell–dependent inflammation by maintaining high levels of TNF-α and IL-17A while suppressing IL-10 and TGF-β. In addition, up-regulation of IL-6 and down-regulation of IL-4 by *H. pylori* may be linked to the development of gastric cancer [24, 42, 43], heart disease [44, 45], and the inverse association between *H. pylori* infection and asthma [46–48]. Thus, the interplay between *H. pylori* and human CD4$^+$ T cells appears to be a key event in the establishment of a chronic infection that affects one-half of the world's population and is linked to a number of comorbidities [1].

Notes

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