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Expression of B7-H1 on Gastric Epithelial Cells: Its Potential Role in Regulating T Cells during *Helicobacter pylori* Infection[1]

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***Helicobacter pylori* infection is associated with gastritis, ulcers, and gastric cancer. The infection becomes chronic as the host response is unable to clear it. Gastric epithelial cells (GEC) play an important role during the host response, and their expression of class II MHC and costimulatory molecules such as CD80 and CD86 suggests their role in local Ag presentation. Although T cells are recruited to the infected gastric mucosa, they have been reported to be hyporesponsive. In this study, we detected the expression of B7-H1 (programmed death-1 ligand 1), a member of B7 family of proteins associated with T cell inhibition on GEC. Quantitative real-time RT-PCR revealed that B7-H1 expression increased significantly on GEC after *H. pylori* infection. Western blot analysis showed that B7-H1 expression was induced by various *H. pylori* strains and was independent of *H. pylori* virulence factors such as Cag, VacA, and Urease. The functional role of B7-H1 in the cross talk between GEC and T cells was assessed by coculturing GEC or *H. pylori*-infected GEC with $CD4^+$ T cells isolated from peripheral blood. Using blocking Abs to B7-H1 revealed that B7-H1 was involved in the suppression of T cell proliferation and IL-2 synthesis, and thus suggested a role for B7-H1 on the epithelium as a contributor in the chronicity of *H. pylori* infection. *The Journal of Immunology,* 2006, 176: 3000–3009.**

Helicobacter pylori infects the gastric mucosa of approximately half of the world's population. This Gram-negative bacterium is the major cause of acute and chronic gastroduodenal diseases, including duodenal and gastric ulcers, gastric cancer, and B cell lymphoma of mucosa-associated lymphoid tissue (1). Because of its carcinogenic potential, *H. pylori* is the first bacterial agent to be classified as a class I carcinogen by the World Health Organization (2). Colonization of the mucosa by *H. pylori* leads to local infiltration of neutrophils, macrophages (3), as well as T and B cells, some of which are specific for *H. pylori* Ags (4–6). In addition, circulating *H. pylori*-specific T cells (7, 8) and B cells (9) are also induced by the infection in most individuals. However, despite these immune responses, the host is unable to clear the bacterium from the mucosa. The infection normally persists for life, which has led to the suggestion that *H. pylori* may alter the normal host immune response.

Early reports have suggested that T cells exposed to *H. pylori*, both in vivo and in vitro, have impaired ability to proliferate (10, 11). Although the effect of *H. pylori* on epithelial cells has been investigated widely, there are limited studies describing a mechanism by which T cell responses are impaired. Impairment in the host's normal immune defenses may contribute to the chronicity of infection. Although the mechanisms influencing this impaired T cell response are not clear, various studies have addressed effects that *H. pylori* has on T cells. *H. pylori* can induce apoptosis in Fas-bearing T cells through the induction of Fas ligand expression (12). The VacA toxin was shown previously to impair class II MHC-dependent Ag presentation, and thus it was suggested that VacA interferes with protective immunity (13). In a recent report, *H. pylori* VacA was shown to efficiently block proliferation of T cells by inducing a G_1-S cell cycle arrest (14). Immunization inhibited the development of *H. pylori*-associated gastritis and induced T cell hyporesponsiveness, which might be mediated by the early induction of CTLA-4 following challenge (15, 16).

During *H. pylori* infection, gastric epithelial cells (GEC)[3] are thought to play a major role in the host response due to their strategic location between luminal Ag and resident intraepithelial and lamina propria T cells. In addition to their well-characterized contribution to the host response via the secretion of multiple cytokines, GEC may function as APCs due to their constitutive expression of class II MHC molecules, and this expression is increased during *H. pylori* infection (17). In support of this possibility, previous studies demonstrated CD80 (B7-1) and CD86 (B7-2) in gastric epithelium and up-regulation of CD86 expression following *H. pylori* infection (17). For efficient T cell activation, T cells require not only the TCR-mediated Ag-specific signal, but also costimulatory signals provided by APCs (18, 19). The B7 family of molecules provides signals that are critical for both stimulating and inhibiting T cell activation (20, 21). Engagement of CD28 by CD80 (B7-1) and CD86 (B7-2) stimulates and sustains T cell responses (22, 23), whereas engagement of CTLA-4 by the same ligands inhibits T cell responses (24). Recently, several new members of the B7 family have been identified. B7-H2 (also known as GL50, B7h, B7RP-1, and LICOS) has been identified as a ligand for the CD28 family member ICOS (25). Two additional B7 family members, programmed death-1 ligand 1 (B7-H1) (26) and PD-L2 (B7-DC) (27), bind to the receptor programmed death-1 (PD-1) (28), and their interaction down-regulates T cell

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[3] Abbreviations used in this paper: GEC, gastric epithelial cell; MFI, mean fluorescence intensity; PD-1, programmed death-1 receptor.

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activation. The receptor for B7-H1 and B7-DC is PD-1, a type I transmembrane receptor expressed on activated T and B cells. Like CTLA-4, PD-1 contains an ITIM in its cytoplasmic region and acts as a negative regulator of lymphocyte function. The literature suggests that there is another unidentified receptor for B7-H1 and B7-DC whose function is yet to be determined. Two other receptors of the B7 family are B7-H3 (29, 30) and B7-H4 (also known as B7S1 and B7x) (31); however, their receptors and functions are unclear. It is reported that mouse B7-H3 has a negative regulatory function in T cell-mediated immune responses both in vitro and in vivo (30).

Because the epithelium is ideally situated to interact with both *H. pylori* and mucosal T cells, and given that *H. pylori* induces multiple responses in the epithelium that include altered receptor expression and cytokine secretion, the objective of these studies was to determine whether *H. pylori* induces the expression of novel members of the B7 family that may have an inhibitory function on T cells. The expression of these coinhibitory molecules on the epithelium could, in turn, down-regulate T cell activation, which in turn could explain the noted T cell hyporesponsiveness in *H. pylori*-infected individuals. We observed that among B7-H1, B7-DC, and B7-H3, B7-H1 expression was significantly induced following *H. pylori* infection. B7-H1 is constitutively expressed on freshly isolated splenic T cells, B cells, macrophages, and dendritic cells, and is up-regulated on T cells, macrophages, and dendritic cells after activation (32). B7-H1 mRNA has been detected in nonlymphoid organs such as heart, placenta, lung, and pancreas in both human and mouse tissues (32) and nonlymphoid cells such as epithelial cells (33), endothelial cells (34), trophoblasts (32), and tumor cells (35), but its role in disease processes is poorly characterized.

The results presented in this study show that GEC express basal levels of B7-H1. However, the expression of B7-H1 by GEC was significantly increased when they were exposed to *H. pylori*, as determined by independent approaches that included real-time quantitative RT-PCR, Western blot analysis, and flow cytometry. More importantly, B7-H1 expressed by GEC inhibited T cell proliferation induced via the Ag receptor signaling apparatus (CD3), and this inhibition was reversed by blocking B7-H1 with specific Abs. To our knowledge, this is the first report in which *H. pylori* is shown to induce B7-H1 in infected GEC, and this expression inhibits T cell proliferation and IL-2 synthesis. These observations may offer an explanation for the low T cell responses in infected individuals and could also help explain the chronicity of this infection.

Materials and Methods

Cell lines

The N87, Kato III, MKN45, and AGS GEC, of human gastric adenocarcinoma origin, were obtained from the American Type Culture Collection. HS738-nontransformed gastric epithelial fetal cells were also obtained from American Type Culture Collection. All cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1 mM glutamine, penicillin (1 U/ml), and streptomycin (100 μg/ml) in a humidified 37°C, 5% CO_2 incubator.

Bacterial cultures

LC-11 (Cag A^+) *H. pylori* strain was originally isolated from the antral mucosa of a patient with duodenal ulcer, as previously described (36). The $VacA^-$ strain is a gift from T. Vanderbilt (Nashville, TN). $UreB^-$ mutant strain is from H. Mobley (University of Maryland School of Medicine, Baltimore, MD). The Cag^- mutant strain was obtained from Y. Yamaoka (Baylor College of Medicine, Houston, TX). All *H. pylori* strains were grown on blood-agar plates (BD Biosciences) at 37°C under microaerophilic conditions. After 48 h in blood agar plate, *H. pylori* strains were grown in *Brucella* broth for 24 h. The bacteria were washed with normal saline, and their concentration was measured by OD at the absorbance of 530 nm using a DU-65 spectrophotometer (Beckman Instruments). The bacteria were then adjusted to a concentration taking 1 OD equivalent to 2×10^8 bacteria/ml.

Abs and recombinant proteins

PE-conjugated anti-human B7-H1 and PE-conjugated anti-human PD-1 were purchased from eBioscience to stain B7-H1 and PD-1, respectively. The isotype control was PE-conjugated mouse IgG1 (BD Biosciences). For Western blot analysis, monoclonal anti-human B7-H1, B7-DC, and B7-H3 were purchased from R&D Systems. For functional studies, purified mouse anti-human monoclonal CD3 was purchased from BD Biosciences. Blocking Abs for B7-H1 in functional studies included the purified functional grade anti-human B7-H1 from eBioscience. The isotype control Ab used for functional studies was functional grade mouse IgG1 from eBioscience. Anti-human CD4-CyChrome-conjugated and anti-human CD69-FITC-conjugated Abs were purchased from BD Biosciences. Ab for FITC-conjugated epithelial cell Ag (BER EP4) was purchased from DakoCytomation. LPS from *Salmonella* was purchased from Sigma-Aldrich. Human rIL-4 and rTNF-α were purchased from R&D Systems. Human rIFN-γ (Roche) was used in 100 U/ml or as otherwise specified in dose responses. For treatment of GEC, the concentration of effectors is as follows: LPS (1 μg/ml), IL-4 (25 ng/ml), and TNF-α (40 ng/ml).

Infection of GEC with H. pylori

In some cases, GEC were treated with IFN-γ (100 U/ml) for 48 h, then washed to remove IFN-γ and incubated an additional day in regular medium without IFN-γ. Before infecting the GEC with *H. pylori*, GEC were washed and medium was replaced with antibiotic-free medium. The bacteria were resuspended in RPMI 1640 medium and used with a cell:bacteria ratio of 1:100.

Surface staining for flow cytometry

Before performing the surface staining for flow cytometry, the cells ($\sim 10^6$) were preincubated with human serum and horse serum in PBS for 30 min in ice. After washing, cells were stained with the respective conjugated Ab (1 μg) and also with the proper isotype control Ab (1 μg) for 45 min in ice. Cells were washed twice with PBS and fixed in 0.2 ml of paraformaldehyde (1% in PBS). Cells were analyzed by flow cytometry on a FACScan (BD Biosciences) instrument. The data were analyzed with the CellQuest program (BD Biosciences) and WinMDI software.

RNA isolation and RT-PCR

For each of the specified conditions, total RNA was isolated from GEC using Qiagen RNeasy mini kit. The RNA concentration was measured taking OD at 260 nm. For RT-PCR, SuperScript III First Strand Synthesis System (Invitrogen Life Technologies) was used in which first strand cDNA was synthesized from 5 μg of total RNA using oligo$(dT)_{20}$ as primers. Briefly, RNA, primer, and dNTPs were denatured at 65°C for 5 min and annealed on ice for 1 min. cDNA synthesis was done at 50°C for 50 min with 2 μl of 10× reverse transcriptase buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT, 1 μl of RNase OUT, and 1 μl of SuperScript III RT in a total 20-μl volume. Reaction was terminated at 85°C for 5 min. RNA was removed by adding 1 μl of RNaseH at 37°C for 20 min. A total of 2 μl of specific cDNA was used for PCR amplification using initial denaturation at 95°C for 1 min and then by using cycles in which denaturation was for 15 s at 95°C, annealing at 55°C for 15 s, and extension at 72°C for 30 s. Lastly, an extra 7-min extension was used. Only for B7-H3 annealing was performed at 60°C. Primer sequence for B7-H1 is left primer, 5′-GATACAAACTCAAAGAAGCAAAG-3′ and right primer, 5′-CAAAATAAATAGGAAAAACTCAT-3′. Primer sequence for B7-DC, left primer, 5′-GGCCTCGTTCCACATACCTCAAG-3′ and right primer, 5′-ATGAAAATGTGAAGCAGCCAAGT-3′; for B7-H3, left primer, 5′-GGCACAGCTCAACCTCATCT-3′ and right primer, 5′-TGATCTT TCTCCAGCACACG-3′. After PCR amplification, the PCR product was observed in a 1.5% agarose gel.

Real-time quantitative RT-PCR

This was done with the help of University of Texas Medical Branch Real Time PCR Core facilities directed by T. Ko. Briefly, 20× assay mix of primers and TaqMan MGB probes (FAM dye labeled) for the target genes and predeveloped 18S rRNA as endogenous control (VIC dye-labeled probe) TaqMan assay reagent (P/N 4319413E) from Applied Biosystems were used. The probe sequence for B7-H1 (accession NM_014143) is 5′-AGTCAATGCCCCATACAACAAAATC-3′. These assays are designed to span exon-exon junctions so as not to detect genomic DNA.

Relative quantification of gene expression

In separate tubes (singleplex), one-step RT-PCR was performed with 40 ng of RNA for both target genes and endogenous control. The reagent we used was TaqMan one-step RT-PCR master mix reagent kit (P/N 4309169; Applied Biosystems). The cycling parameters for one-step RT-PCR were as follows: reverse transcription 48°C for 30 min, AmpliTaq activation 95°C for 10 min, denaturation 95°C for 15 s, and annealing/extension 60°C for 1 min (repeat 40 times) on an ABI7000 real time cycler. Duplicate C_T values were analyzed in Microsoft Excel using the comparative C_T ($\Delta\Delta C_T$) method, as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalizing an endogenous reference (18S) and relative to a (untreated cells) control.

Collection of gastric biopsy tissue and staining with B7-H1 Ab

Biopsy specimens of the gastric antrum were obtained from consenting patients undergoing gastro-esophageal-duodenoscopy for various clinical indications in accordance with Institutional Review Board approved human subject protocols. Patients were considered infected if *H. pylori* was detected by rapid urease testing and by histopathology on biopsies. The biopsy tissue was placed in calcium- and magnesium-free HBSS, supplemented with 5% FCS, and transported immediately to the laboratory. The biopsy tissue was placed in dispase II solution (2.4 U/ml; Roche Applied Science) and agitated at 37°C for 45 min. The cells were pelleted by centrifugation, washed, and double stained with FITC-conjugated epithelial cell Ag and PE-conjugated B7-H1 Ab. Mean fluorescence intensity (MFI) value of B7-H1 was detected after gating positive cells with epithelial cell Ag. The graph was plotted taking four biopsies positive with *H. pylori* and four biopsies that were negative with *H. pylori*.

Western blot analysis

Proteins were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane (Bio-Rad), and blocked in TBST (20 mM Tris-HCl/500 mM NaCl (pH 7.5) with 0.05% Tween 20) containing 5% dry milk. The membranes were subsequently probed with respective primary Ab in TBST containing 5% dry milk. The binding of primary Abs was detected with HRP-conjugated secondary Ab (Amersham Biosciences). Subsequently, membranes were washed and incubated in ECL reagent (Amersham Biosciences).

T cell proliferation

$CD4^+$ T cells were isolated from peripheral blood by negative selection (Miltenyi Biotec) with magnetic beads using a mixture of Abs against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR-γδ, and glycophorin A. $CD4^+$ T cells were stimulated with a predetermined optimal concentration of anti-CD3 mAb (100 ng/ml)-coated Dynabeads (beads:T cell ratio 2:1). To determine the role of B7-H1 in GEC for T cell proliferation, GEC were treated with either IFN-γ, *H. pylori*, or both, when indicated. After the required treatment, GEC were irradiated at 14,000 rad, and 10^4 cells/well were cocultured with 10^5 $CD4^+$ T cells in 96-well flat-bottom plates. For blocking B7-H1, anti-B7-H1-blocking Ab (5 μg/ml; functional grade from eBioscience) was used for 1 h. As an isotype control, mouse IgG1 Ab (5 μg/ml; functional grade from eBioscience) was used. T cell proliferation was determined after 72 h by the addition of 1 μCi of [^{3}H]thymidine during the final 16 h of culture. The incorporation of [^{3}H]thymidine was determined in a Microbeta TriLux liquid scintillation counter (Wallac).

Cytokine assay

Culture supernatant from the coculture of GEC and T cells was collected at 48 and 72 h for IL-2 determination using an ELISA (R&D Systems), according to the manufacturer's instructions.

Statistical analysis

Results were expressed as the mean ± SD. Data were compared by Student's *t* test and considered significant if $p \leq 0.05$.

Results

Expression of B7-H1 and its effect on H. pylori *infection*

To determine whether GEC express coinhibitory members of the B7 family, we performed RT-PCR of a panel of GEC (KATO III, N87, AGS, and HS738) to detect mRNA corresponding to B7-H1 and B7-DC are ligands for the inhibitory TCR PD-1, and B7-H3 is a related member with a different receptor. The specificity of the PCR products was confirmed by restriction digestion (data not shown). Among the GEC examined, the basal level expression of B7 molecules was higher in AGS cells than the other GEC. To determine the effect of conditions present during infection on the expression of the three different B7 molecules by GEC, the cells were treated with IFN-γ, infected with *H. pylori*, or both. IFN-γ was shown previously to increase both the expression of class II MHC and the binding of *H. pylori* to GEC (37). Cells were treated with IFN-γ (100 U/ml) or culture medium for 48 h before they were exposed to *H. pylori* LC11 strain for 24 h. Interestingly, *H. pylori* alone increased B7-H1 mRNA expression in AGS (Fig. 1*A*), whereas B7-DC and B7-H3 expression remain unchanged (Fig. 1*B*). To determine whether B7-H1 is expressed in vivo, gastric biopsies from *H. pylori*-infected individuals were also examined for mRNA expression (Fig. 1*A*), and the B7-H1 mRNA detected while in uninfected individuals' B7-H1 mRNA was very low.

Expression of B7-H1 on gastric biopsies

To further confirm the expression of B7-H1 on gastric biopsies and its relation with *H. pylori* infection, cells isolated from biopsy samples were dual stained with epithelial Ag-specific Ab BerEP4 and B7-H1. From surface staining of B7-H1, after gating only the cells positive for epithelial Ag, a panel of four *H. pylori*-positive samples showed 3-fold more B7-H1 expression compared with a panel of four *H. pylori*-negative samples (Fig. 2).

Real-time RT-PCR to quantitate the expression level of B7-H1 following H. pylori *infection*

Because RT-PCR analysis suggested that B7-H1 mRNA expression in AGS cells increases after *H. pylori* infection, we wanted to confirm those observations by quantitative real-time RT-PCR analysis in various GEC lines. The expression of B7-H1 mRNA in

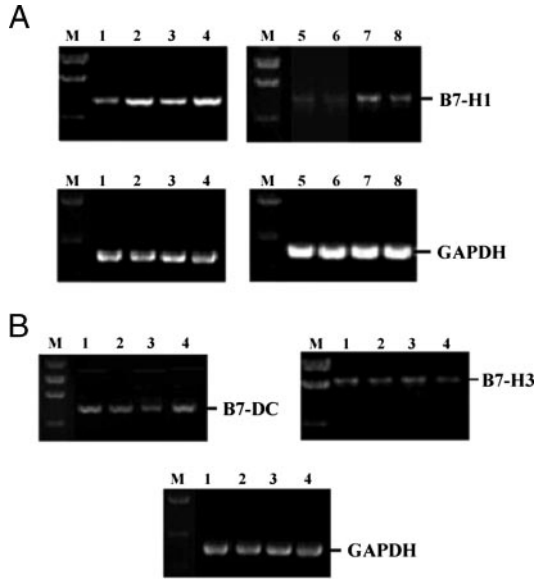

FIGURE 1. B7-H1, B7-DC, and B7-H3 expression by RT-PCR. *A*, B7-H1 expression on GEC and gastric biopsies. M, Molecular mass marker; *lane 1*, AGS untreated; *lane 2*, AGS *H. pylori* infected for 24 h; *lane 3*, AGS IFN-γ treated for 48 h; *lane 4*, IFN-γ-treated AGS infected with *H. pylori* for 24 h; *lanes 5* and *6*, gastric biopsy samples from patient negative to *H. pylori* infection; and *lanes 7* and *8*, gastric biopsy samples from *H. pylori*-infected patient. *Upper panel*, B7-H1 transcript (516 bp); *lower panel*, GAPDH used as an internal control. *B*, B7-DC (429 bp) and B7-H3 (644 bp) expression by RT-PCR in *upper panel*, and GAPDH as an internal control in *lower panel*. M, Molecular mass marker; *lane 1*, AGS untreated; *lane 2*, AGS *H. pylori* infected for 24 h; *lane 3*, AGS IFN-γ treated for 48 h; *lane 4*, IFN-γ-treated AGS infected with *H. pylori* for 24 h.

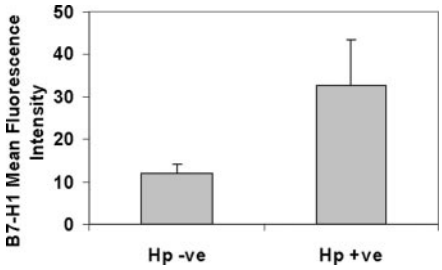

FIGURE 2. B7-H1 expression in gastric biopsies by surface staining increased in *H. pylori*-positive samples. After gating the positive cells with epithelial cell Ag, MFI value of B7-H1 was determined between *H. pylori*-positive and *H. pylori*-negative samples. Graph was plotted with average MFI value, taking four positive and four negative samples with $p < 0.05$.

AGS cells was rapidly induced to 9.5-fold after 24-h *H. pylori* treatment (Fig. 3*A*), whereas in N87 cells there was a 4-fold induction of B7-H1 mRNA following *H. pylori* treatment (Fig. 3*B*). To verify the real-time quantitative RT-PCR analysis of B7-H1 in a nontransformed cell line, we used HS738 GEC. In HS738 cells, the expression of B7-H1 was increased 3-fold compared with their untreated counterparts (Fig. 3*C*). When IFN-γ-treated cells were infected with *H. pylori*, B7-H1 expression was increased five times in AGS cells compared with IFN-γ-treated AGS cells that were not exposed to *H. pylori* (Fig. 3*A*). The corresponding induction of B7-H1 in IFN-γ-treated N87 cells that were exposed to *H. pylori* was three times higher than their counterparts not exposed to the bacteria (Fig. 3*B*). The same comparison between IFN-γ-treated and *H. pylori*-infected HS738 cells showed 8 times more B7-H1 expression compared with HS738 cells treated only with IFN-γ (Fig. 3*C*). There were also some differences in the B7-H1 expression between IFN-γ-treated N87 and AGS. Although AGS have a larger increase in the expression of B7-H1 mRNA in response to *H. pylori* when compared with uninfected AGS, the N87 and HS738 GEC cell lines had the highest increase when they were pretreated with IFN-γ before they were exposed to *H. pylori*. In the case of AGS cells, the cells pretreated with IFN-γ and infected with *H. pylori* appeared to have a lower level of B7-H1 mRNA than AGS cells treated with *H. pylori* alone, but the overall expression of B7-H1 mRNA is still higher than in noninfected cells.

Time kinetics of B7-H1 expression in GEC following H. pylori *infection*

Real-time quantitative RT-PCR results confirmed that B7-H1 expression was induced following *H. pylori* infection, but it was important to determine the kinetics of B7-H1 in response to *H. pylori* infection before performing functional assays. We performed quantitative real-time RT-PCR analysis for B7-H1 at different time points following *H. pylori* infection (Fig. 3*D*). We observed that HS738 cells infected with *H. pylori* had increased B7-H1 mRNA expression by 24 h postinfection. In IFN-γ-treated HS738 cells, the expression of B7-H1 mRNA increased almost 4-fold after 12 h of infection and showed an 8-fold increase after 24 h of *H. pylori* infection. Similar results were obtained with other cells with a considerable expression of B7-H1 by 24 h postinfection (data not shown).

B7-H1 protein expression on GEC is strain independent

Because *H. pylori* acts as an inducer of B7-H1 mRNA expression, we needed to confirm that the protein was also induced and that the induction was not strain specific. We thus performed Western blot analysis for B7-H1 protein on AGS cells that were treated with different strains of *H. pylori* to determine whether B7-H1 expression is strain specific or it is a consistent response to *H. pylori*. We exposed AGS cells to LC11, 26695, 60190, and 43504 strains and assessed B7-H1 protein expression by Western blot analysis. All strains examined induced B7-H1 expression (Fig. 4*A*). The Western blot showed the molecular mass of B7-H1 was ~65 kDa in SDS-PAGE under reducing conditions, but the predicted molecular mass of the core polypeptide expected from the amino acid sequence of B7-H1 is 33,275 Da. Studies of murine B7-H1 reported a molecular mass of 43 kDa after immunoprecipitation of B7-H1-transfected murine cells (38) and suggested probable glycosylation of B7-H1. The molecular mass that we observed could

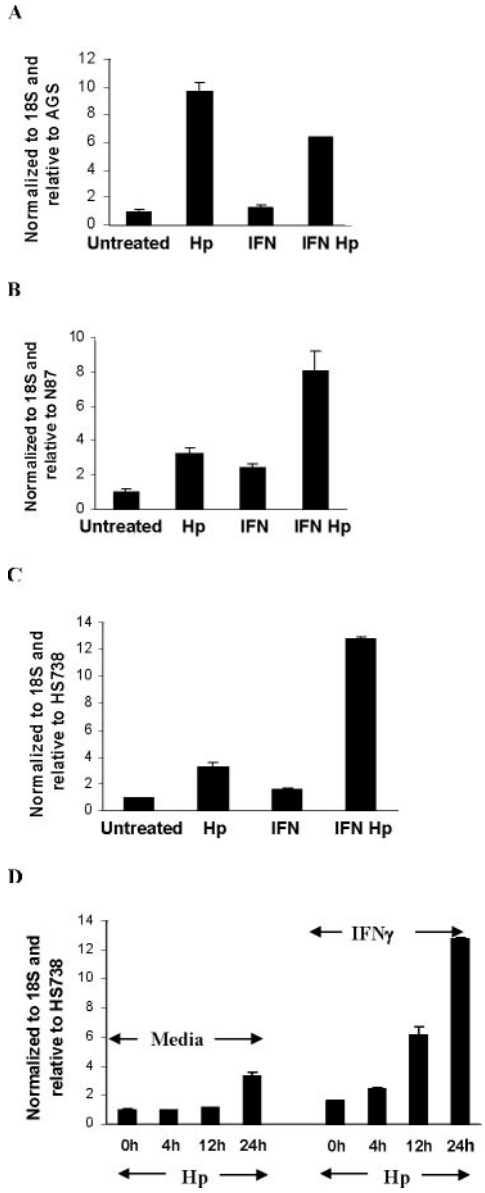

FIGURE 3. B7-H1 expression on GEC after *H. pylori* infection. Quantitative real-time RT-PCR was done in AGS (*A*), N87 (*B*), and HS738 (*C*). RNA was isolated from untreated GEC, 24-h *H. pylori*-infected GEC (Hp), 48-h IFN-γ (100 U/ml)-treated GEC (IFN), and 24-h *H. pylori*-infected and IFN-γ-pretreated GEC (IFN Hp). *D*, Time kinetics of B7-H1 expression was detected in HS738 cells by quantitative real-time RT-PCR. In each case, HS738 were infected with *H. pylori* for the specified time. IFN-γ pretreatment was done for 48 h. *A–D*, TaqMan one-step RT-PCR was done. SD was determined from triplicate set of samples in each day of experiment after repeating three different days ($n = 9$). Those datasets were selected in which $p < 0.05$.

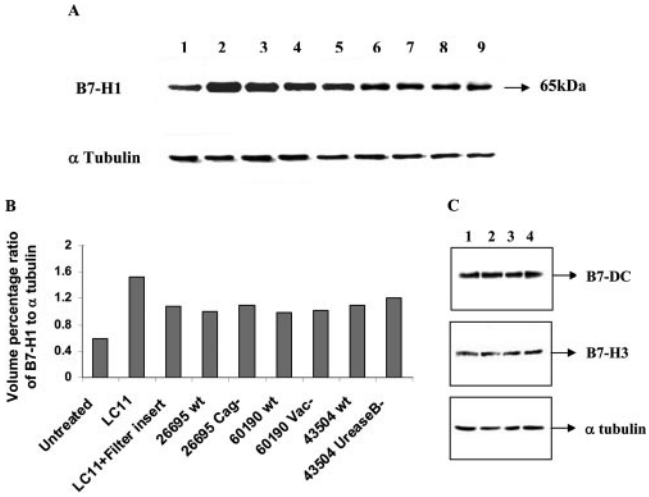

FIGURE 4. Effect of different *H. pylori* strains and virulence factors on B7-H1 expression from GEC. *A*, Western blot of B7-H1 expression from AGS cells using mouse anti-human B7-H1 mAb. *Lane 1*, AGS untreated; *lane 2*, AGS infected with *H. pylori* LC11 strain (CagA$^+$) for 24 h; *lane 3*, AGS with LC11 separated by filter insert (0.2 μM) for 24 h; *lane 4*, AGS infected with *H. pylori* 26695 wild-type (wt) strain (Cag$^+$) for 24 h; *lane 5*, AGS infected with Cag$^-$ strain of 26695 for 24 h; *lane 6*, AGS infected with wt strain 60190 for 24 h; *lane 7*, AGS infected with Vac$^-$ strain of 60190 for 24 h; *lane 8*, AGS infected with wt strain 43504 for 24 h; and *lane 9*, AGS infected with UreaseB$^-$ strain of 43504 for 24 h. For equal loading, total protein concentration in cell lysate was measured, checked in another gel, and maintained as 50 μg/lane. α-Tubulin was used as an internal control. *B*, Densitometric scan was done using Bio-Rad's Quantity One software. Percentage of adjacent volume was determined for each band of B7-H1 and α-tubulin. Ratio of percentage of adjacent volume was plotted. *C*, Western blot of B7-DC (72-kDa) and B7-H3 (75-kDa) expression from AGS cells. *Lane 1*, AGS untreated; *lane 2*, AGS *H. pylori* infected for 24 h; *lane 3*, AGS IFN-γ treated for 48 h; and *lane 4*, IFN-γ-treated AGS infected with *H. pylori* for 24 h.

be attributed to posttranslational modifications such as glycosylation. Also, GEC appear to glycosylate proteins in a different manner than hemopoietic cells, as previously reported for class II MHC molecules (39). The possibility of dimerization was excluded as we observed the protein in SDS-PAGE under reducing conditions. Densitometric scan using α-tubulin as an internal standard showed that B7-H1 protein expression increased ~2.5-fold in cells exposed to the LC11 strain and near 2-fold with all other strains (Fig. 4*B*). B7-H1 expression by GEC was also increased when *H. pylori* were separated from the GEC by inserts (pore size = 0.2 μm) to avoid direct contact, which suggested that secreted products from *H. pylori* can induce the B7-H1 expression. Because the Cag pathogenicity island has been implicated in gastric epithelial responses (40), such as cytokine secretion, we examined a Cag$^-$ isogenic mutant of 26695 strain on B7-H1 expression. There were no significant differences in the induction of B7-H1 expression by AGS cells exposed to the isogenic Cag$^-$ mutant of the parental 26695 strain (Fig. 4*A*), which suggests that the effect of *H. pylori* on epithelial expression of B7-H1 is cag independent. Because VacA (vacuolating cytotoxin) is an important secretory toxin (41) from *H. pylori* that has a direct role in pathogenesis, we examined its potential role in B7-H1 induction. We used a wild-type and a VacA$^-$ of 60190 strains for B7-H1 expression, but no marked difference was observed between the wild-type and the VacA$^-$ strain of *H. pylori* (Fig. 4*A*). The same result was also obtained with urease B$^-$ strain of *H. pylori* (Fig. 4*A*). Urease is another virulence factor of *H. pylori* that has been shown to stimulate apoptosis of epithelial cells (42), NO release (43), and cytokine secretion in monocytes (44), and its role in B7-H1 induction was assessed using UreB$^-$ mutant. The absence of urease did not affect B7-H1 induction by *H. pylori*. Western blot analysis of B7-DC and B7-H3 confirmed that their expression was unaffected by *H. pylori* (Fig. 4*C*), which confirmed the results obtained by RT-PCR analysis.

Soluble mediators of T cell origin induce B7-H1 expression on GEC

Cytokines regulate the expression of numerous immunoregulatory molecules, both temporally and quantitatively, thus allowing for fine tuning of the immune response. IFN-γ is an important cytokine in the gastric mucosa of *H. pylori*-infected patients (45). In RT-PCR analysis (Figs. 1*A* and 3), we observed that IFN-γ acts as an inducer of B7-H1 expression on GEC. We observed the expression of B7-H1 protein in response to IFN-γ was increased on N87 cells in a dose-dependent manner (1–1000 U/ml for 48 h) (Fig. 5*A*). The presence of this surface ligand was examined by surface staining a set of GEC (N87, Kato III, AGS, and MKN45 cells) that were untreated and after IFN-γ treatment. Expression was noticeably increased in N87 and Kato III cells after 48 h of IFN-γ (100 U/ml) treatment. The increase was more readily observed than other cell lines whose basal level of expression of B7-H1 was much higher, as is the case for AGS and MKN45 cells (Fig. 5*B*). B7-H1 was highly expressed in the nontransformed primary HS738 cell without any treatment (Fig. 5*B*).

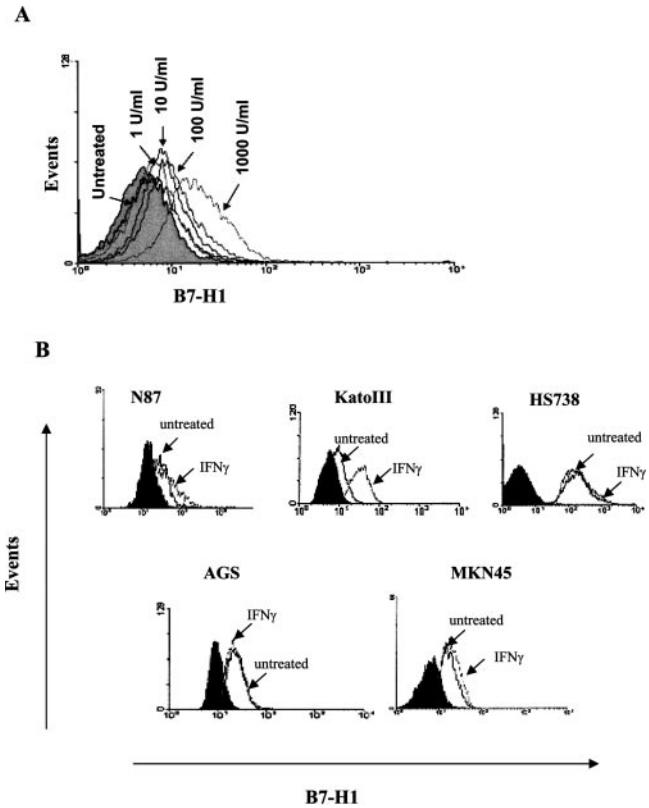

FIGURE 5. Effect of IFN-γ on B7-H1 expression from GEC. *A*, Dose-response effect of IFN-γ-induced B7-H1 expression on N87 cells after 48 h. Filled curve represents staining by the isotype control. The other curves represent the various doses of IFN-γ, as indicated by the arrows. *B*, B7-H1 expression by surface staining in flow cytometry for untreated and 48-h IFN-γ-treated GEC. IFN-γ concentration was used at 100 U/ml. Filled curve represents staining by the isotype control.

Because products of Th1 cells as well as microbial products can enhance B7-H1 expression on different macrophage populations and B7-H1 and B7-DC are differentially regulated by Th1 and Th2 cells (46), the effect that some of these mediators might have on B7-H1 expression by GEC was examined. *Salmonella* LPS (1 μg/ml) was used as a stimulus for different time points, and cells were harvested and stained for B7-H1 expression. The concentration of LPS is in the higher range, as a lower concentration did not affect B7-H1 expression. IL-4 (25 ng/ml) was also used to examine the role of this Th2 cytokine in B7-H1 expression. Because TNF is a predominant cytokine produced in the gastric mucosa of patients with *H. pylori* infection (47), the role of TNF-α (40 ng/ml) on B7-H1 induction was determined in AGS cells. LPS and IL-4 had little effect on B7-H1 expression by AGS cells, but TNF-α had a marked effect on the expression of B7-H1 in a time-dependent manner (Fig. 6*A*). Similar results were obtained with N87 cells (data not shown). Other cytokines produced during inflammatory responses, such as GM-CSF, IL-1α (200 U/ml), and IL-8 (10–1000 pg/ml), were also examined as potential inducer of B7-H1, but none of these cytokines had an effect after 48 h of treatment (data not shown). The dose used for IL-1α and LPS was in the highest range, as we wanted to determine whether they could have an effect on B7-H1 expression.

B7-H1 expression changes in a biphasic manner with TNF-α, as noted following surface staining and analysis by flow cytometry. Therefore, changes in B7-H1 expression in response to TNF-α induction were confirmed by real-time quantitative RT-PCR (Fig. 6*B*) and Western blot (Fig. 6*C*). AGS cells treated with TNF-α for 2 and 4 h showed increased expression of B7-H1 compared with untreated cells. B7-H1 expression started to decrease after 8 h and again increased by 48 h, as determined by Western blot (Fig. 6*D*) and real-time RT-PCR.

Functional analysis of B7-H1 on GEC

Induction of B7-H1 expression in *H. pylori*-infected GEC may be of importance in the inhibition of T cell responses during *H. pylori* infection. Thus, it was important to assess the functional role of B7-H1 in GEC and its correlation with *H. pylori* infection. Before addressing the function of B7-H1 on GEC, the binding of ligand with the soluble form of the receptor PD-1-Fc was confirmed by staining the GEC with biotinylated PD-1-Fc, followed by streptavidin-PE (data not shown). The importance of this was to ensure that any differential glycosylation of B7-H1 on GEC would not result in impaired interactions with its receptor on T cells.

For determining the functional role of B7-H1 on GEC in correlation to *H. pylori* infection, we performed functional assays with *H. pylori*-infected GEC. We chose the conditions of GEC in which real-time quantitative RT-PCR showed maximum expression of B7-H1 from GEC. For that reason, we selected AGS and *H. pylori*-infected AGS, but in case of HS738, we compared IFN-γ-pretreated *H. pylori*-infected HS738 with only IFN-γ-treated cells. For functional studies, we isolated $CD4^+$ T cells from peripheral blood by negative selections. To correlate the effect of B7-H1 expression on *H. pylori*-infected GEC, and its possible effect on T cells, cocultures of $CD4^+$ T cells with *H. pylori*-infected GEC were established. $CD4^+$ T cells were activated for all the time periods of culture examined with suboptimal concentrations of anti-CD3 (100 ng/ml), after selecting that concentration as a best condition in our system after testing amounts between 30 and 500 ng/ml. The literature suggests that PD-1 expression on activated T cells is highest when there is a weak TCR signal, so that weakly activated T cells are most readily down-regulated by PD-L engagement (33). In addition, B7-H1:PD-1 interactions can counterbalance CD3-mediated activation when CD28-mediated costimulation is suboptimal (33). For the specified anti-CD3 concentration (100 ng/ml), PD-1 receptor expression on $CD4^+$ T cells was checked by FACS analysis (data not shown).

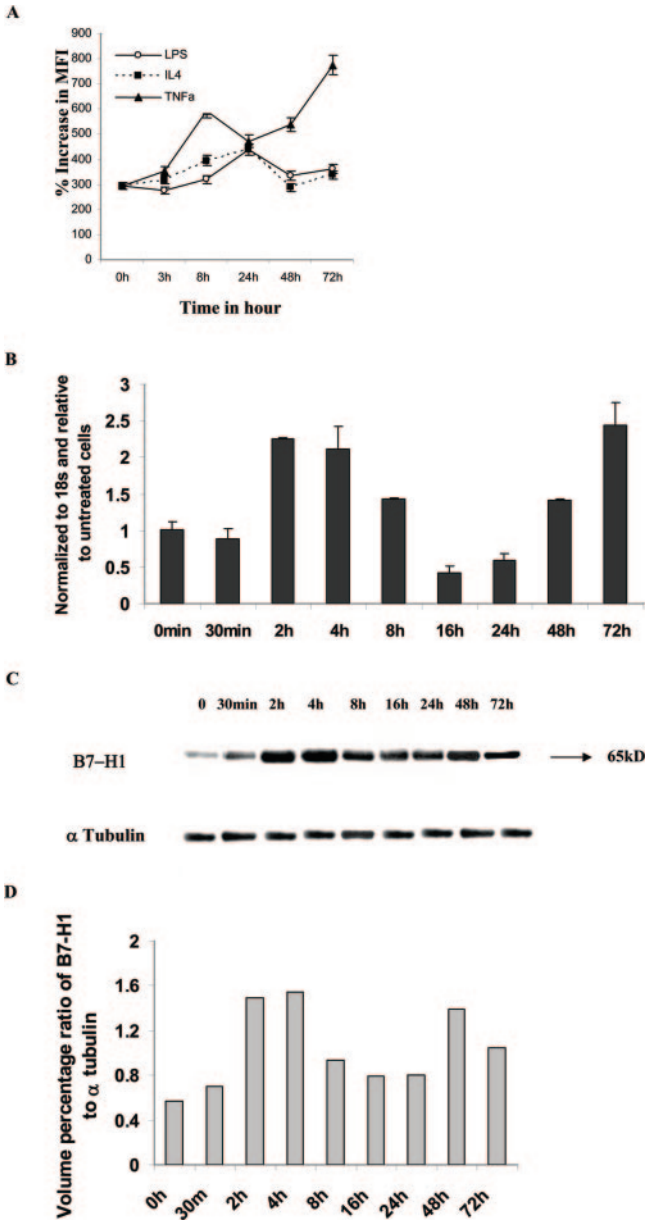

FIGURE 6. Effect of different inducers on B7-H1 expression from GEC. *A*, Time course of B7-H1 expression on AGS cells using different inducers. AGS cells were treated either with rIL-4 (25 ng/ml), TNF-α (40 ng/ml), or LPS (1 μg/ml) for the indicated time period. B7-H1 expression was assessed by flow cytometry, and the corresponding increase in MFI was compared with respective isotype control and was plotted at each time point. SD was determined between triplicate sets of samples, and each experiment was done three times. *B*, Real-time quantitative RT-PCR for AGS cells for B7-H1 expression after TNF-α treatment. SD was determined from triplicate set of samples in each day of experiment after repeating 3 different days ($n = 9$). *C*, Western blot of B7-H1 expression with TNF-α in a time-dependent manner in AGS cells using mouse anti-human B7-H1 mAb. For equal loading, total protein concentration in cell lysate was measured, checked in another gel, and maintained as 50 μg/lane. α-Tubulin was used as an internal control. *D*, Densitometric scan was done using Bio-Rad's Quantity One software. Percentage of adjacent volume was determined for each band of B7-H1 and α-tubulin. Ratio of percentage of adjacent volume was plotted for different time points of TNF-α treatment.

After irradiating the GEC, they were blocked with B7-H1-blocking Ab at an optimal concentration (5 μg/ml). As an isotype control, mouse IgG1 Ab was used in the same concentration. CD4$^+$ T cells were activated with a suboptimal concentration of anti-CD3 Ab (100 ng/ml). CD4$^+$ T cell proliferation was measured after coculture with *H. pylori*-infected GEC in the presence of either anti-B7-H1-blocking Ab or isotype control Ab. CD4$^+$ T cell proliferation decreased after coculturing with IFN-γ-treated HS738 cells and HS738 cells that were IFN-γ pretreated and infected with *H. pylori*, and the level of proliferation increased after blocking the B7-H1 ligand using blocking Ab (Fig. 7*A*). Similar results were obtained with AGS cells. The fold change of T cell proliferation was determined after blocking B7-H1 in different cell types compared with the isotype control for each cell type (Fig. 7*B*). Addition of anti-B7-H1 Ab enhanced the T cell proliferative response. CD4$^+$ T cells cultured with either AGS or IFN-γ-treated HS738 had higher (20–50%) proliferation rates after blocking B7-H1 on GEC (Fig. 7*B*). Similar results were obtained when CD4$^+$ T cells were cocultured with IFN-γ-treated N87 cells in which B7-H1 was blocked (data not shown). Interestingly, when *H. pylori*-infected GEC were used, the T cell response was increased 170–220% ($p < 0.01$) when B7-H1 was blocked (Fig. 7*B*). Although the levels of proliferative responses were individually variable and the exact value of cpm for T cell proliferation varies from donor to donor, the effect of blocking B7-H1 on GEC consistently increased T cell responses. In addition to anti-CD3, we used superantigen (*Staphylococcus* enterotoxin A) to preactivate CD4$^+$ T cells, and the results were consistent with those using anti-CD3, in which proliferation of T cells cocultured with GEC was higher when B7-H1 was blocked (data not shown).

To correlate the data obtained by CD4$^+$ T cell proliferation with IL-2 synthesis, supernatants of cocultures were collected from 48 and 72 h for IL-2 determination by ELISA (Fig. 8*A*). The same samples showed increased IL-2 levels after blocking B7-H1 on GEC. CD4$^+$ T cells that were cocultured with AGS (*H. pylori*

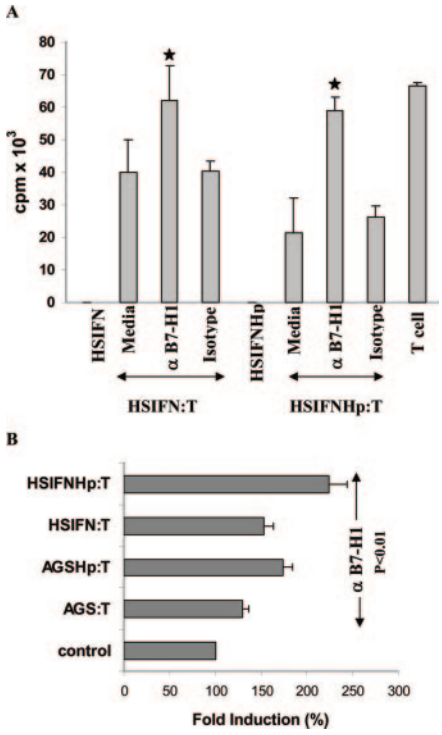

FIGURE 7. Proliferative response of CD4$^+$ T cell after B7-H1 blocking on GEC in coculture. Allogenic T cells (1×10^5) were cocultured with GEC (10^4) cells after irradiation at 14,000 rad. To activate CD4$^+$ T cells, anti-CD3 Ab was used in a suboptimal concentration of 100 ng/ml in total time period of coculture. Anti-B7-H1 and isotype mouse IgG were used at a final concentration of 5 μg/ml from eBioscience. *A*, T cell proliferation of HS738 cell was shown after 72 h of coculture. [^{3}H]Thymidine was added for the final 16 h of coculture, and the incorporated radioactivity was measured. Only one representative experiment from three independent experiments and four different donors was chosen in *A*. Datasets were selected with $p < 0.05$. Star indicates $p < 0.01$. The symbol denotes: HSIFN, HS738 treated with IFN-γ (100 U/ml) for 48 h; HSIFNHp, HS738 pretreated with IFN-γ (100 U/ml) for 48 h and infected with *H. pylori* LC11 strain for 24 h; αB7-H1, HS738 cells were blocked with anti B7-H1-blocking Ab for 1 h; isotype, HS738 incubated with mouse IgG isotype for 1 h as a control. Forty-eight-hour IFN-γ-treated HS738 (HSIFN:T) and 24-h *H. pylori*-infected 48-h IFN-γ-pretreated HS738 (HSIFNHp:T) were shown in figure. *B*, Fold change of T cell proliferation was shown in cocultures of T cells with AGS (AGS:T), AGS 24-h *H. pylori*-infected (AGSHp:T), 48-h IFN-γ-treated HS738 (HSIFN:T), and 24-h *H. pylori*-infected 48-h IFN-γ-pretreated HS738 (HSIFNHp:T). In all four sets, $p < 0.01$. Fold induction of proliferation was determined when the response of isotype control in each specified condition was determined and taken as 100%.

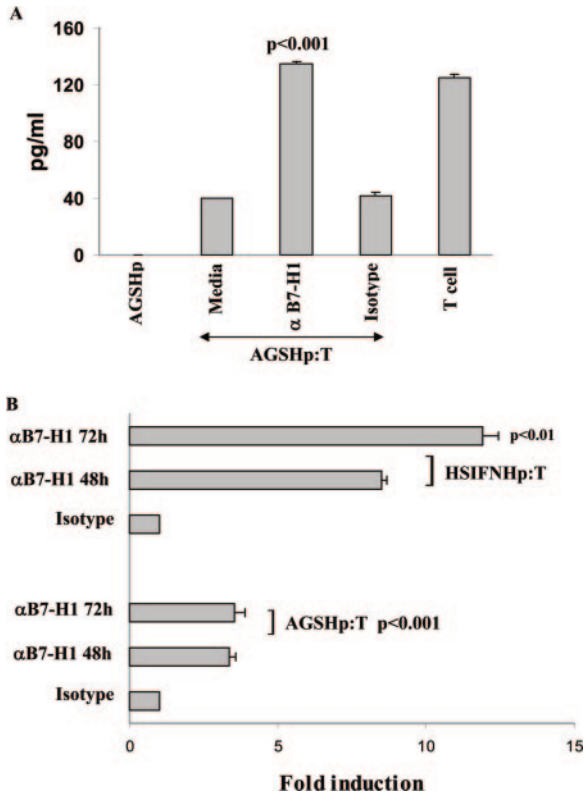

FIGURE 8. IL-2 production of CD4$^+$ T cell after B7-H1 blocking on GEC in coculture. A total of 100 μl of supernatant was used in each experiment collected from GEC:T cell coculture (10^4:10^5). *A*, IL-2 level was determined by ELISA in triplicate set of each experiment. One representative experiment was shown from 48-h collected supernatant of CD4$^+$ T cells with AGS cells infected with *H. pylori* LC11 strain for 24 h (AGSHp). Only those datasets were selected in which $p < 0.05$. αB7-H1: B7-H1-blocking Ab 5 μg/ml from eBioscience for 1 h. Isotype: isotype control mouse IgG1 5 μg/ml from eBioscience. *B*, Fold induction of IL-2 synthesis after blocking B7-H1 (αB7-H1) on GEC. Supernatant was collected from 48 and 72 h of coculture of GEC with CD4$^+$ T cells. Twenty-four-hour *H. pylori* LC11 strain-infected AGS (AGSHp:T) and 48-h IFN-γ (100 U/ml)-pretreated and *H. pylori* LC11 strain-infected HS738 (HSIFNHp:T) were cocultured with CD4$^+$ T cells. To determine the fold induction, isotype control Ab at each specified condition was taken as 1. Actual value of IL-2 is in the range of 40–500 pg/ml. IL-2 in HSIFNHp:T set after blocking B7-H1 has p value <0.01, and in AGSHp:T the p value <0.001.

infected) produced ~3.5-fold more IL-2 at 48 and 72 h after blocking B7-H1, whereas $CD4^+$ T cells cocultured with IFN-γ-pretreated and *H. pylori*-infected HS738 produced 8.5-fold more IL-2 at 48 h and ~12-fold more IL-2 after 72 h compared with similar cocultures treated with isotype control (Fig. 8*B*). Similar results were obtained when *H. pylori*-infected N87 cells were used as feeder cells (data not shown).

As a third independent method to examine T cell activation in the presence of GEC and to establish the functional role of B7-H1 on GEC, the expression of the activation marker CD69 was also determined in $CD4^+$ T cells cocultured with GEC. CD69 expression by $CD4^+$ T cells was measured at 16 and 48 h, and maximum expression was noted at 48 h. To determine CD69 expression, T cells were stained with anti-CD4 CyChrome and anti-CD69 FITC to gate only $CD4^+$ T cells. CD69 expression by $CD4^+$ T cells increased after blocking B7-H1 on GEC in the cocultures of $CD4^+$ T cells with either *H. pylori*-infected AGS or IFN-γ-pretreated, *H. pylori*-infected HS738. The MFI of CD69 in $CD4^+$ T cells in AGSHp:T was increased to 87.7 ± 12.8 after blocking B7-H1 compared with the MFI of their counterparts in the isotype control (70.9 ± 10.05), and in HSIFNHp:T the MFI of the $CD4^+$ T cells increased to 103.4 ± 7.78 after blocking B7-H1 compared with isotype control-treated cells in which MFI was 84.3 ± 5.94. The AGSHp:T condition means 24-h *H. pylori* LC11 strain-infected AGS cells cocultured with $CD4^+$ T cells, whereas the HSIFNHp:T condition reflects 48-h IFN-γ (100 U/ml)-pretreated and 24-h *H. pylori* LC11 strain-infected HS738 cells, cocultured with $CD4^+$ T cells. These data suggest that B7-H1 signaling by GEC affects the level of T cell cytokine expression and surface expression of the activation marker CD69.

Discussion

H. pylori infection is one of the most common infections of humans because >50% of the global population carries this bacterium in the stomach, and in some individuals infection leads to serious diseases. Despite the inflammatory and immune responses that are elicited by *H. pylori* infection, it remains chronic. Various studies have suggested that T cell functions are affected during infection with *H. pylori*. Early studies to assess T cell function in response to *H. pylori* suggested that T cells in infected individuals were hyporesponsive compared with noninfected persons. Gastric T cells from infected individuals were suppressed in their proliferation and IFN-γ production (7). The mechanisms responsible for the low response were not clear.

Various independent studies have presented evidence to suggest that *H. pylori* has the ability to evade or hijack host immune defense mechanisms. One of the earlier mechanisms reported was the ability of the vacuolatin toxin, VacA, to disrupt endosomal functions and thus alter Ag processing and presentation (13, 48). More recent studies have shown that VacA also has the ability to directly interfere with T cell activation by two different mechanisms, both of which result in altered intracellular signaling processes (49). One of the pathogenic aspects of *H. pylori* is its ability to induce apoptosis of host cells. The epithelium has been shown to be the target of this type of damage induced by the bacteria (37, 50–53). Interestingly, one of the mechanisms responsible for this pathogenic process is the engagement of molecules that are central in the process of Ag presentation to $CD4^+$ T cells (37, 42). *H. pylori* cross-links class II MHC molecules on the host epithelium via urease and elicits epithelial cell apoptosis (37, 42). Induction of apoptosis of immune cells is undoubtedly an important evasion mechanism used by some pathogens to be able to persist in the host. Recent studies have shown that *H. pylori* also elicits apoptosis of macrophages (54, 55). Not only does *H. pylori* induce apoptosis of APCs, but other studies have shown that it can also negatively select against T cells via the induction of Fas ligand and Fas receptor expression on T cells, which upon their interaction results in apoptosis (12).

As our objective was to explore the underlying mechanism of chronicity of infection while addressing potential mechanisms to explain T cell hyporesponsiveness during *H. pylori* infection, the presence of the immunoinhibitory molecule B7-H1 was studied on GEC following *H. pylori* infection. In a comparative analysis of B7-H1, B7-DC, and B7-H3 expression, B7-H1 was the most prominent coinhibitory molecule of B7 group whose expression was induced following *H. pylori* infection. Most interestingly, epithelial cells in gastric biopsies infected with *H. pylori* showed higher B7-H1 expression compared with uninfected samples. The results presented in this study show a novel mechanism of immune avoidance used by *H. pylori*, which involves the induction of coinhibitory molecule expression on GEC by the bacterium. Our studies showed that the expression of B7-H1 increases in the gastric mucosa during infection with *H. pylori* as determined by real-time quantitative RT-PCR using RNA from GEC either *H. pylori* infected or uninfected. GEC lines were examined by multiple independent methods, to determine the expression of B7-H1 at the mRNA as well as the protein levels. GEC were found to constitutively express B7-H1, and the level of expression increased significantly during infection. T cells cocultured with these GEC had a lower proliferation index, IL-2 secretion, and CD69 expression in response to activation via CD3. However, blockage of B7-H1 with specific anti-B7-H1 Ab restored the responses to levels close to those of T cells not cocultured with GEC. Although an additional member of these novel coinhibitory receptors, B7-DC (PD-L2), shares the same receptor, PD-1, on T cells and consequently has the same effects, its expression on GEC was not affected by *H. pylori*, and blocking studies with PD-1-Fc did not increase T cell responses more than blocking with anti-B7-H1 Abs alone. A direct measure of the role of B7-DC in the observed T cell inhibition in cocultures with GEC is not currently possible because the corresponding blocking Abs are not available.

IFN-γ is produced within the infected gastric mucosa, and IFN-γ alone induced B7-H1 expression in GEC, as has been reported for conventional APCs (33). In two of the GEC lines, N87 and HS738, IFN-γ and *H. pylori* appeared to synergize in the induction of B7-H1 expression, while one cell line, AGS, only responded to the bacteria. This may reflect a defect in the responsiveness of this cell line to IFN-γ, because the expression of other markers (i.e., class II MHC) is not induced in this cell line by IFN-γ. The observed synergy in the response to IFN-γ and *H. pylori* may be a result of the increased expression of a receptor used by *H. pylori* that in turn leads to enhanced intracellular signaling that results in increased B7-H1 expression.

The expression by APCs of this and other recently described coinhibitory receptors may be to limit or regulate continuous T cell activation and limit potential immunopathology. *H. pylori* seem to incorporate this regulatory mechanism in the armamentarium used to evade clearance by the host immune response. An interesting possibility is that this mechanism could also down-regulate immune surveillance mechanisms needed to clear transformed cells that arise within the site of infection. Interestingly, the apoptotic effect of B7-H1 was suggested to be mediated receptors other than PD-1. Although the *H. pylori* virulence factor responsible for the induction of B7-H1 expression is not characterized, this may represent an important target in vaccine development efforts against this prevalent and significant human pathogen. Manipulation of

B7-H1 expression in the gastric mucosa may provide unique opportunities for designing new therapies in diseases associated with *H. pylori* infection.

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Disclosures

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