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

Soumita Das,* Giovanni Suarez,* Ellen J. Beswick,* Johanna C. Sierra,* David Y. Graham,‡ and Victor E. Reyes2*†

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.

H. 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 G1-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) 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 responses.

‡ Department of Pediatrics and † Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555; and ‡ Department of Medicine, Michael E. DeBakey Veterans Affairs Medical Center and Baylor College of Medicine, Houston, TX 77030

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Address correspondence and reprint requests to Dr. Victor E. Reyes, Children’s Hospital, Room 2.300, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555. E-mail address: vreyes@utmb.edu

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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 \textit{H. pylori} and mucosal T cells, and given that \textit{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 \textit{H. pylori} induces the expression of novel members of the B7 family that may have an inhibitory function on T cells. The expression of this co-inhibitory molecules on the epithelium could, in turn, down-regulate T cell activation, which in turn could explain the noted T cell hyporesponsiveness in \textit{H. pylori}-infected individuals. We observed that among B7-H1, B7-DC, and B7-H3, B7-H1 expression was significantly induced following \textit{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 non-lymphoid 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 \textit{H. pylori}, as determined by independent approaches that included real-time quantitative RT-PCR, Western blot analysis, and flow cytometry. More importantly, B7-H1 expression 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 \textit{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

\textbf{Cell lines}

The N87, Kato III, MKN45, and AGS GEC, of human gastric adenocarcinoma origin, were obtained from the 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% CO₂ incubator.

\textbf{Bacterial cultures}

LC-11 (Cag A⁺) \textit{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). \textit{UreB} mutation strain is from H. Mobley (University of Maryland School of Medicine, Baltimore, MD). The \textit{Cag}⁺ mutant strain was obtained from Y. Yamaoka (Baylor College of Medicine, Houston, TX). All \textit{H. pylori} strains were grown on blood-agar plates (BD Biosciences) at 37°C under microaerophilic conditions. After 48 h in blood agar plate, \textit{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 × 10⁶ bacteria/ml.

\textbf{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 DakoCytona.

\textbf{Infection of GEC with \textit{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 \textit{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.

\textbf{Surface staining for flow cytometry}

Before performing the surface staining for flow cytometry, the cells (~10⁶) were preincubated with human serum and horse serum in PBS for 30 min in ice. After washing, cells were stained with the respective monoclonal Abs. To our knowledge, this is the first report in which \textit{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.

\textbf{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)₁₅ 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. 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.

\textbf{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 reverse transcription buffer, 4 µl of 25 mM dNTP mixture, 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 1 min. 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'-GATACAAACTTCAAGAAGCAAG-3’ and right primer, 5’-CAAAATATAGGAAAAACTCTAT-3’. Primer sequence for B7-DC, left primer, 5’-GCCCCTGTTCCACATACCTCAAG-3’ and right primer, 5’-ATGAAAATGTGAAAGCCACGACTGTT-3’; for B7-H3, left primer, 5’-GCCACACGTCCTAACACTCATCT-3’ and right primer, 5’-TGATCTTCTCCAGCACAGC-3’. After PCR amplification, the PCR product was observed in a 1.5% agarose gel.
Expression of B7-H1 on GEC

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 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. 1A), whereas B7-DC and B7-H3 expression remain unchanged (Fig. 1B). To determine whether B7-H1 is expressed in vivo, gastric biopsies from H. pylori-infected individuals were also examined for mRNA expression (Fig. 1A), 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...
MFI value, taking four positive and four negative samples with \( p \) different strains of analysis for B7-H1 protein on AGS cells that were treated with induction was not strain specific. We thus performed Western blot we needed to confirm that the protein was also induced and that the case of AGS cells, the cells pretreated with IFN-\(/\)H9253 positive and \( H. \) pylori pretreated with IFN-\(/\)HS738 GEC cell lines had the highest increase when they were \( H. \) pylori Because \( B7-H1 \) protein expression on GEC is strain independent postinfection (data not shown).

Other cells with a considerable expression of \( B7-H1 \) by 24 h \( H. \) pylori 24 h of infection and showed an 8-fold increase after 53,275 Da. Studies of murine \( B7-H1 \) expressed 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 9,5-fold after 24-h \( H. \) pylori treatment (Fig. 3A), whereas in N87 cells there was a 4-fold induction of \( B7-H1 \) mRNA following \( H. \) pylori treatment (Fig. 3B). 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. 3C). When IFN-\( \gamma \)-treated cells were infected with \( H. \) pylori, \( B7-H1 \) expression was increased five times in AGS cells compared with IFN-\( \gamma \)-treated AGS cells that were not exposed to \( H. \) pylori (Fig. 3A). The corresponding induction of \( B7-H1 \) in IFN-\( \gamma \)-treated N87 cells that were exposed to \( H. \) pylori was three times higher than their counterparts not exposed to the bacteria (Fig. 3B). The same comparison between IFN-\( \gamma \)-treated and \( H. \) pylori-infected HS738 cells showed 8 times more \( B7-H1 \) expression compared with HS738 cells treated only with IFN-\( \gamma \) (Fig. 3C). There were also some differences in the \( B7-H1 \) expression between IFN-\( \gamma \)-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-\( \gamma \) before they were exposed to \( H. \) pylori. In the case of AGS cells, the cells pretreated with IFN-\( \gamma \) 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. 3D). We observed that HS738 cells infected with \( H. \) pylori had increased \( B7-H1 \) mRNA expression by 24 h postinfection. In IFN-\( \gamma \)-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 \( B7-H1 \) expres-

![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 \( H. \) pylor

![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-\( \gamma \) (100 U/ml)-treated GEC (IFN), and 24-h \( H. \) pylori-infected and IFN-\( \gamma \)-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-\( \gamma \) 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.](http://www.jimmunol.org/content/3003/1/3003/F1.large.jpg)
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. 4B). 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-lacZ isogenic mutant of 26695 strain on B7-H1 expression. There were no significant differences in the induction of B7-H1 expression by GES cells exposed to the isogenic Cag− mutant of the parental 26695 strain (Fig. 4A), 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), 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. 4C), 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. 1A 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. 5A). 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. 5B). B7-H1 was highly expressed in the nontransformed primary HS738 cell without any treatment (Fig. 5B).

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. 6A). 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. 6B) and Western blot (Fig. 6C). 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. 6D) 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 costimu-
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. 7A). 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. 7B). 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. 7B). 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. 7B). 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. 8A). The same samples showed increased IL-2 levels after blocking B7-H1 on GEC. CD4+ T cells that were cocultured with AGS (H. pylori LC11 strain-infected AGS (AGS:Hp:T)) and 48-h IFN-γ-treated HS738 (HSIFNHp:T) were shown in figure. B, Fold change of T cell proliferation was determined and taken as 100%.

**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 IgG1 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. [3H]Thymidine was added for the final 16 h of coculture. Anti-B7-H1-blocking Ab 5 μg/ml from eBioscience for 1 h. Isotype: isotype mouse IgG1 5 μg/ml from eBioscience. B, Fold induction of T cell proliferation with blocking B7-H1 on GEC. A, T cell proliferation of 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; ab7-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.

**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 (10^5:10^4). 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. ab7-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 (ab7-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 (AGS:Hp:T) and 48-h IFN-γ (100 U/ml)-pretreated and H. pylori LC11 strain-infected HS738 (HSIFN:Hp: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 AGS:Hp:T the p value <0.001.
infected) produced ~3.5-fold more IL-2 at 48 and 72 h after block-
ing B7-H1, whereas CD4^+ T cells cocultured with IFN-γ-pre-
treated and \textit{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. 8B). Similar results were obtained when \textit{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 expres-

sion 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 \textit{H. pylori}-infected AGS or IFN-γ-pretreated, \textit{H. pylori}-infected HS738. The MFI of CD69 in CD4^+ T cells in AGS/H11001 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:H11001 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 AGS/H11001 T condition means 24-h \textit{H. pylori} LC11 strain-infected AGS cells cocultured with CD4^+ T cells, whereas the HSIFNHp:H11001 T condition reflects 48-h IFN-γ (100 U/ml)-pretreated and 24-h \textit{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**

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

Various independent studies have presented evidence to suggest that \textit{H. pylori} has the ability to evade or hijack host immune de-
fense mechanisms. One of the earlier mechanisms reported was the ability of the vacuolatin toxin, VacA, to disrupt endosomal func-
tions 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 \textit{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 patho-
genic process is the engagement of molecules that are central in the process of Ag presentation to CD4^+ T cells (37, 42). \textit{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 \textit{H. pylori} also elicits apoptosis of macrophages (54, 55). Not only does \textit{H. pylori} induce apo-

ptosis of APCs, but other studies have shown that it can also neg-
atively 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 hyposresponsiveness during \textit{H. pylori} infection, the presence of the immunoinhibitory molecule B7-H1 was studied on GEC following \textit{H. pylori} infection. In a comparative analysis of B7-H1, B7-DC, and B7-H3 expression, B7-H1 was the most prom-
inent co-inhibitory molecule of B7 group whose expression was induced following \textit{H. pylori} infection. Most interestingly, epithe-

dial cells in gastric biopsies infected with \textit{H. pylori} showed higher B7-H1 expression compared with uninfected samples. The results presented in this study show a novel mechanism of immune avoidance use by \textit{H. pylori}, which involves the induction of coinhibit-

ory molecule expression on GEC by the bacterium. Our studies showed that the expression of B7-H1 increases in the gastric mu-
cosa during infection with \textit{H. pylori} as determined by real-time quantitative RT-PCR using RNA from GEC either \textit{H. pylori} infected or uninfected. GEC lines were examined by multiple inde-
pendent methods, to determine the expression of B7-H1 at the mRNA as well as the protein levels. GEC were found to constit-

tively 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 addi-
tional 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 \textit{H. pylori}, and blockading 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 corre-
sponding 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 re-
ported for conventional APCs (33). In two of the GEC lines, N87 and HS738, IFN-γ and \textit{H. pylori} appeared to synergize in the in-
duction of B7-H1 expression, while one cell line, AGS, only re-

sponded to the bacteria. This may reflect a defect in the respon-
siveness 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 \textit{H. pylori} may be a result of the increased expression of a receptor used by \textit{H. pylori} that in turn leads to enhanced intracellular sig-
naling 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. \textit{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 im-

mune 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 \textit{H. pylori} virulence factor responsible for the induction of B7-H1 expression is not characterized, this may rep-
resent 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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