Expression of the Programmed Death Ligand 1, B7-H1, on Gastric Epithelial Cells after *Helicobacter pylori* Exposure Promotes Development of CD4+ CD25+ FoxP3+ Regulatory T Cells[▽]

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During *Helicobacter pylori* infection, T cells are recruited to the gastric mucosa, but the host T-cell response is not sufficient to clear the infection. Some of the recruited T cells respond in a polarized manner to a Th1 response, while others become anergic. We have previously shown that T-cell anergy may be induced during infection by the interaction of T cells with B7-H1, which is up-regulated on the gastric epithelium during *H. pylori* infection. Recently, regulatory T (Treg) cells with a CD4+ CD25high FoxP3+ phenotype were found at an increased frequency in the gastric mucosa of biopsy specimens from *H. pylori*-infected patients. While Treg cells are important in maintaining tolerance, they can also suppress immune responses during infection. In this study, we examined the induction of the Treg phenotype when naïve T cells were incubated with gastric epithelial cells exposed to *H. pylori*. The frequency of this phenotype was markedly decreased when B7-H1 was blocked with monoclonal antibodies or its expression was blocked with small interfering RNA. The functional role of these Treg cells was assessed in proliferation assays when the cells were cocultured with activated T cells, which effectively decreased proliferation of the cells.

Helicobacter pylori is possibly the world's second most common human pathogen. This gram-negative spiral bacterium is responsible for 90% of gastric and duodenal ulcers and has also been associated with gastric adenocarcinoma (9). Infection with the pathogen often becomes chronic and is accompanied by gastritis. Colonization of the gastric mucosa leads to an influx of host macrophages, neutrophils, B cells, and T cells. Although some of these cells are specific for *H. pylori* (6, 18) the responses induced are not sufficient to clear the infection. In fact, untreated infections generally persist for life, which suggests that *H. pylori* avoids or suppresses normal host responses. Recent studies have suggested that immune-regulatory mechanisms may be induced during infection that lead to persistent infection by limiting damage to the host but result in low-level chronic inflammation.

T cells are generally hyporesponsive during *H. pylori* infection, and the existent response is polarized toward a Th1 response (2, 25). These cells have been shown to produce gamma interferon, which may play an important role in the proinflammatory responses induced during infection. Additionally, studies of infected patient biopsy specimens have also shown the presence of transforming growth factor β (TGF-β) (13, 14), which has a suppressive effect on other T cells. Several studies have shown that T cells exposed to *H. pylori* exhibit impaired proliferation rates (11). Also, *H. pylori* virulence factors, such as the vacuolating toxin (VacA), have been suggested to suppress T cells. When mixed with T-cell lines, VacA was shown to inhibit interleukin 2 (IL-2) production, arrest cell cycle pro-

gression, and down-regulate surface expression of IL-2 receptors, all of which are required for T-cell proliferation and viability (4). Another study also showed that the CagA virulence factor may inhibit T-cell proliferation (19).

The inability of the host response to eradicate the infection may also be attributed to the depressed T-cell response during *H. pylori* infection. Although gastric epithelial cells (GEC) have been shown to express the class II major histocompatibility complex and to function as antigen-presenting cells (3, 10), we have recently shown that the coinhibitory molecule, the programmed death ligand 1, or B7-H1, is up-regulated on GEC during exposure to *H. pylori* both in vitro and in vivo (5). Since binding of B7-H1 to the programmed death 1 receptor on T cells causes them to undergo anergy or apoptosis (23, 27), B7-H1 may play a key role in the chronicity of those infections and associated inflammatory responses. *H. pylori*-exposed GEC incubated with isolated CD4+ T cells resulted in decreased proliferation and IL-2 production, but this inhibition was abrogated by anti-B7-H1 blocking antibodies (5). These findings suggest that B7-H1 plays an important role in T-cell inhibition during *H. pylori* infection.

Recently, CD4+ CD25high FoxP3+ regulatory T (Treg) cells have been detected in the gastric mucosa of *H. pylori*-infected biopsy specimens (16), but their origin is not known. Current knowledge of Treg cells suggests that they may play a crucial role in suppressing the overall T-cell response to infection and cancer. Cells of this phenotype may inhibit T-cell activation by producing IL-10 and TGF-β. In *H. pylori*-infected individuals, Treg cells have been shown to impair the response of memory T cells in the periphery (17). The depletion of Treg cells from the memory population led to an increase in proliferation in response to *H. pylori* antigens, while addition of Treg cells to the memory T cells suppressed the *H. pylori*-specific responses. Similarly, in infected mice, depleting the Treg cells led to

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increased pathology (20, 21). Since Treg cells may play an important role in the impaired T-cell responses during *H. pylori* infection, and we and others have shown that B7-H1 also plays a role in T-cell suppression or generation of Treg cells (5, 12, 30), we sought to determine the role of B7-H1 on GEC in the development of $CD4^+$ $CD25^{high}$ $FoxP3^+$ Treg cells during *H. pylori* infection. In this study, we show that coculture of naïve $CD4^+$ T cells with *H. pylori*-exposed GEC generates $CD4^+$ $CD25^{high}$ $FoxP3^+$ T cells. Blocking B7-H1 on *H. pylori*-exposed GEC with monoclonal antibodies or specific knockdown with small interfering RNA (siRNA) prevents the formation of the $CD4^+$ $CD25^{high}$ $FoxP3^+$ phenotype within those cocultures. T cells with this phenotype were also shown to produce TGF-β and IL-10 and to have the ability to suppress T effector (Teff) cell proliferation.

MATERIALS AND METHODS

Cell lines. The GEC line AGS and the nontransformed fetal gastric cell line HS-738 were obtained from the American Tissue Culture Collection (ATCC) (Manassas, VA). AGS cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 1 mM glutamine. The HS-738 cells were maintained in Dulbecco's minimal essential medium with high glucose, 10% fetal calf serum, and 1 mM glutamine.

Bacterial cultures. *H. pylori* strain 26695 was obtained from Yoshio Yamaoka at Baylor College of Medicine. The bacteria were grown on blood agar plates (Becton Dickinson, San Jose, CA) at 37°C under microaerophilic conditions and used as previously described (8).

siRNA transfection. GEC were transfected with siRNA for B7-H1 by using the basic nucleofection kit for epithelial cells (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's instructions with a cocktail of 1 μg of siRNA numbers 134191, 20742, and 134192 or a negative control siRNA (Ambion, Austin, TX). For AGS cells, program B-023 was used as recommended by Amaxa's Cell Database, while program T-20 was used for HS-738 cells for optimal cell viability. Knockdown of expression of B7-H1 was verified by staining the cells with anti-B7-H1–phycoerythrin (PE)-conjugated monoclonal antibodies (eBiosciences, San Diego, CA) and analyzing them by flow cytometry compared to cells stained with isotype control.

GEC exposure to *H. pylori*. AGS and HS-738 cells were incubated at a 100:1 bacteria/cell ratio with *H. pylori* for 24 h. The cells were then trypsinized, washed twice with phosphate-buffered saline, gamma irradiated at 14,000 rad, and plated at 1×10^5 cells per well in a 24-well plate for 24 h before the addition of T cells.

T-cell isolation and incubation with GEC. Heparinized venous blood was obtained from a healthy adult volunteer negative for *H. pylori*, using an Institutional Review Board approved protocol (no. 94-102). Peripheral blood mononuclear cells (PBMC) were prepared from collected blood by density gradient centrifugation over Ficoll-Paque Plus (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instructions. Naïve $CD4^+$ T cells were isolated from the PBMC using the naïve-T-cell isolation kit for negative selection (Miltenyi Biotech Inc., CA) with magnetic beads labeled with monoclonal antibodies for CD45RO, CD8, CD14, CD16, CD19, CD36, CD56, CD123, T-cell receptor γ/ζ, and glycophorin A. Negative selection was performed to prevent the accidental activation of signals in the cells that were used in the cocultures described below. Approximately 3×10^7 to 4×10^7 naïve T cells were isolated from each donor per experiment. The naïve $CD4^+$ T cells were stained with anti-human CD25-PEcy5 (BD Biosciences) and anti-human FoxP3 (eBiosciences) monoclonal antibodies as negative controls for the starting population. For coculture with GEC, 1×10^6 naïve T cells were added to each well of GEC in RPMI as described above for a 10:1 T-cell/GEC ratio and incubated for 7 days at 37°C with 5% CO_2. For blocking, anti-B7-H1 blocking antibody (5 μg/ml; functional grade from eBiosciences) was incubated with the cells for 1 h before the addition of T cells. As an isotype control, mouse immunoglobulin G1 antibody (5 μg/ml; functional grade from eBioscience) was used. B7-H1 expression was also blocked in other cells by siRNA.

Flow cytometry. T cells from coculture assays were stained for CD4, CD25, and FoxP3 for analysis by flow cytometry. Mouse anti-human CD4-fluorescein isothiocyanate (FITC) or -PE (BD Biosciences) and CD25-FITC antibodies (BD Biosciences) were incubated with the cells for 1 h at 4°C. After being washed, the cells were permeabilized with a Fixation and Permeabilization Kit from eBiosciences (San Diego, CA) according to the manufacturer's instructions. The

cells were then incubated with mouse anti-human FoxP3-PE-conjugated antibodies (eBiosciences) for 1 h at 4°C. Some samples were single and triple stained with isotype control antibodies, as well. After being washed two more times, the cells were analyzed by flow cytometry on a FACScan cytometer (Becton Dickinson) using Cell Quest software. Gates were set up to eliminate dead cells and cellular debris based on controls.

IL-10 and TGF-β ELISA. Supernatants were collected from coculture wells after 7 days and used to quantitate the production of IL-10 and TGF-β by enzyme-linked immunosorbent assays (ELISAs) (BD Biosciences), according to the manufacturer's instructions.

Real-time PCR. Total cellular RNA was isolated from T cells with an RNeasy RNA isolation kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The sample concentration was measured with a spectrophotometer at 260 nm, and the RNA quality was determined on a 1% agarose gel. Real-time PCR was performed according to the Applied Biosystems's two-step real-time PCR protocol (Applied Biosystems, Foster City, CA). All reagents were purchased from Applied Biosystems. The reverse transcription (RT) reaction mixture included 2.5 μM random hexamers, 500 μM deoxynucleoside triphosphates, 0.4 U/μl of the RNase inhibitors, 5.5 mM $MgCl_2$, MultiScribe Reverse Transcriptase (3.125 U/μl) and its buffer, 1 μg of cellular RNA. The RT mixture was adjusted to a final volume of 50 μl by using RNase- and DNase-free H_2O (Sigma). The RT step was performed using a GeneAmp PCR system 9700 thermocycler (Perkin-Elmer Applied Biosystems) according to the following protocol: 10 min at 25°C, 60 min at 37°C, 5 min at 95°C. The cDNA samples obtained were stored at −20°C (if necessary) and used for the PCR step. The PCR mixture was prepared using TaqMan Universal PCR Master Mix (Applied Biosystems). The assays-on-demand gene expression assay mix (Applied Biosystem) for human 18S rRNA, FoxP3, IL-10, TGF-β1, TGF-β2, or TGF-β3 (a 20× mixture of unlabeled PCR primers and TaqMan MGB probe, 6-carboxyfluorescein dye labeled) and 2 μl of cDNA were added to the PCR mixture. The reaction was carried out in a 20-μl final volume using a GeneAmp 5700 Sequence Detection System (Applied Biosystems) according to the following protocol: 2 min at 50°C, 10 min at 95°C (1 cycle), 15 s at 95°C, and 1 min at 60°C (40 cycles). The negative controls were included in the RT real-time two-step reaction. The endpoint used in real-time PCR quantification, the threshold cycle (C_T), was defined as the PCR cycle number that crossed the signal threshold. C_T values ranged from 0 to 40, with the latter number assumed to represent no product formation. Quantification of cytokine gene expression was performed using the comparative C_T method (Sequence Detector User Bulletin 2; Applied Biosystems) and was reported as the difference relative to the human housekeeping gene, 18S rRNA. In order to calculate the change (*n*-fold increase or decrease), the C_T value of 18S rRNA was subtracted from the C_T value of the target cytokine gene to yield the ΔC_T. Change in the expression of the normalized target gene as a result of experimental conditions was expressed as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ was equal to ΔC_T experimental samples minus the ΔC_T biological control.

T-cell suppression assays. $CD4^+$ T cells recovered from the cocultures with GEC were washed twice with Hanks solution without Ca^{2+} and Mg^{2+} (Cellgro, Herndon, VA) and resuspended in RPMI at a concentration of 10^7 cells/ml. The population of $CD4^+$ $CD25^{high}$ cells was sorted by staining them with monoclonal antibodies against CD4 and CD25 (BD Biosciences) and was sorted into $CD4^+$ $CD25^{bright}$ and $CD4^+$ $CD25^-$ cells using the BD FACSAria cell-sorting system (Becton Dickinson, Heidelberg, Germany). The purity of the $CD4^+$ $CD25^{high}$ T cells after being sorted was >98% (as determined by flow cytometry). For T-cell suppression assays, 10^5 naïve $CD4^+$ T cells isolated from PBMC, as described above, were activated by using a T-cell Activation/Expansion kit containing beads with anti-CD2, anti-CD3, and anti-CD28 antibodies (Miltenyi Biotec) according to the manufacturer's instructions. The activated T cells were cultured in the absence or presence of increasing numbers of newly generated $CD4^+$ $CD25^{bright}$ T cells at Treg/Teff cell ratios of 1:20 to 1:120. The cells were cultured for 72 h at 37°C in 5% CO_2 using 96-well flat-bottom plates. One microcurie of [^{3}H]thymidine was added 18 h prior to cell harvesting. At the end of the cultures, cells were harvested onto glass fiber filter mats and [^{3}H]thymidine incorporation (cpm) was determined using a liquid scintillation counter (Perkin Elmer, Wellesley, MA). The direct suppressive effect of $CD4^+$ $CD25^{high}$ T cells was evaluated by measuring [^{3}H]thymidine incorporation. The results were expressed in cpm as the mean cpm ± standard error (SE) of triplicate cultures.

Statistical analysis. Results are expressed as the mean ± SE of the mean (SEM). Data were analyzed using one-way analysis of variance. A P value of < 0.05 was considered significant.
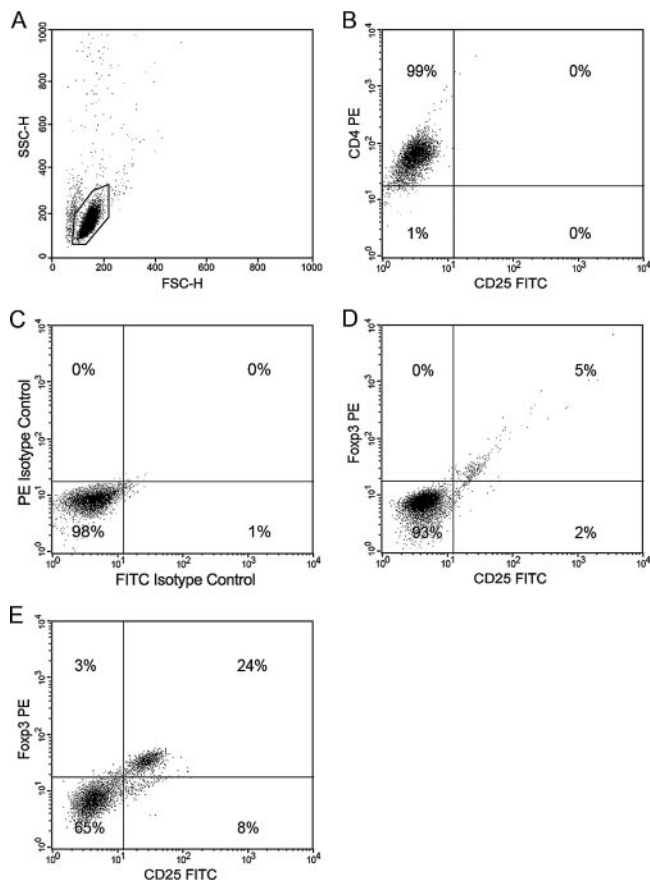
FIG. 1. CD4$^+$ CD25^{high} FoxP3$^+$ T cells are induced from naïve T cells after incubation with *H. pylori*-exposed GEC. Shown are (A) naïve T cells incubated with HS-738 cells for forward and side scatter gates, (B) CD4 and CD25 staining of freshly isolated naïve T cells, (C) FITC and PE isotype controls, (D) naïve T cells incubated with HS-738 cells plus isotype control for 7 days and stained with anti-CD25 FITC and FoxP3 PE monoclonal antibodies, and (E) naïve T cells incubated with *H. pylori*-exposed HS-738 cells plus isotype control for 7 days and stained with anti-CD25 FITC and FoxP3 PE monoclonal antibodies. Representative results of five experiments are shown.

FIG. 2. CD4$^+$ CD25^{high} FoxP3$^+$ T cells induced from naïve T cells from four donors after incubation with *H. pylori*-exposed GEC. The average percentages of cells of the Treg phenotype induced from naïve T cells isolated from four donors negative for *H. pylori* from all flow cytometry experiments are shown. Naïve T cells from each donor were incubated with both AGS and HS-738 cells. $n = 4$ for each donor. The error bars indicate SEM.

RESULTS

GEC exposed to *H. pylori* induce CD4$^+$ CD25^{high} FoxP3$^+$ Treg cells. Since Treg cells have recently been identified in the mucosa of *H. pylori*-infected patients (16), we sought to determine the role the gastric epithelium plays in the induction of the Treg phenotype (CD4$^+$ CD25^{high} FoxP3$^+$). To this end, the AGS cell line and HS-738 normal, nontransformed GEC were treated with medium or exposed to *H. pylori* (100:1 *H. pylori*/cell ratio) for 24 h. After 24 h, the cells were washed extensively, irradiated, and incubated with isolated naïve T cells isolated from four donors negative for *H. pylori*. The naïve T cells were incubated with GEC at a T cell/GEC ratio of 10:1 for 7 days, which was determined to be the best time for induction of Treg cells while maintaining viability of the T-cell population in culture. At 7 days, T cells were harvested and stained for CD4, CD25, and FoxP3 with monoclonal antibodies for flow cytometry. Samples were gated to remove dead cells (Fig. 1A). Figure 1B shows a sample from the beginning naïve cell population stained for CD4-PE and CD25-FITC, indicat-
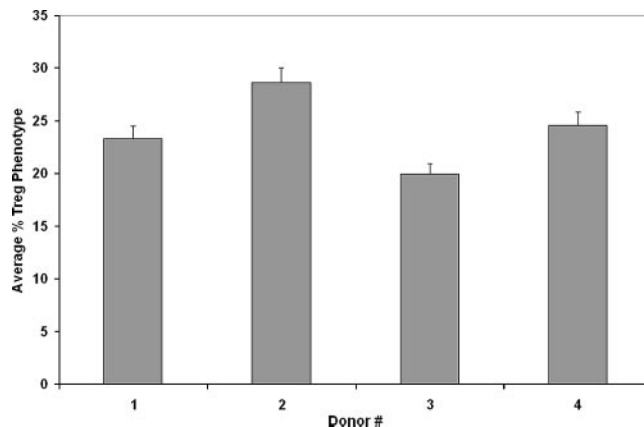
ing the purity of naïve cells and their expression of CD4 only, while Fig. 1C shows the naïve population stained for CD25 and FoxP3, indicating there were no cells staining positive for FoxP3 in the beginning population. As seen in Fig. 1D, compared to the isotype control sample, the cells staining positive for CD25 and FoxP3 harvested from cultures with HS-738 cells were minimal (5%). In contrast, cells that had been exposed to *H. pylori* prior to exposure to naïve T cells induced 24% of the cells with the Treg phenotype (Fig. 1 E). Similar results were seen with T cells harvested from cultures with AGS cells, as shown in Fig. 2, where the average percentage of CD4$^+$ CD25$^+$ FoxP3$^+$ cells for each donor for both GEC is shown. Since *H. pylori* had been irradiated and the majority washed away, these results suggest that the epithelial cells can induce naïve T cells to be become Treg cells after exposure to *H. pylori*.

B7-H1 expressed by GEC after *H. pylori* exposure induces the development of CD4$^+$ CD25^{high} FoxP3$^+$ T cells. We have previously shown that B7-H1 expression is up-regulated on GEC after *H. pylori* exposure and that it may play a role in decreasing overall T-cell responses (5). A separate study suggested that B7-H1 could influence the induction of Treg cells (12), and multiple studies of cancer suggest that B7-H1 expression and the presence of Treg cells in the tissue are increased. Hence, we sought to determine if the expression of B7-H1 on GEC affects the induction of the Treg phenotype, which has been shown to be present in the gastric mucosa of *H. pylori*-infected individuals (16). In order to investigate this, B7-H1 was blocked with monoclonal antibodies on *H. pylori*-exposed GEC before the addition of naïve T cells for 7 days of incubation with GEC. As seen in Fig. 3A, HS-738 cells blocked with anti-B7-H1 after *H. pylori* exposure reduced the numbers of Treg cells present to only 8% compared to 24% for GEC incubated with isotype control, as seen in Fig. 1.

The induction of the Treg phenotype and the role B7-H1 plays in this mechanism was verified by real-time PCR. RNA was isolated from T cells in coculture assays with GEC incu-
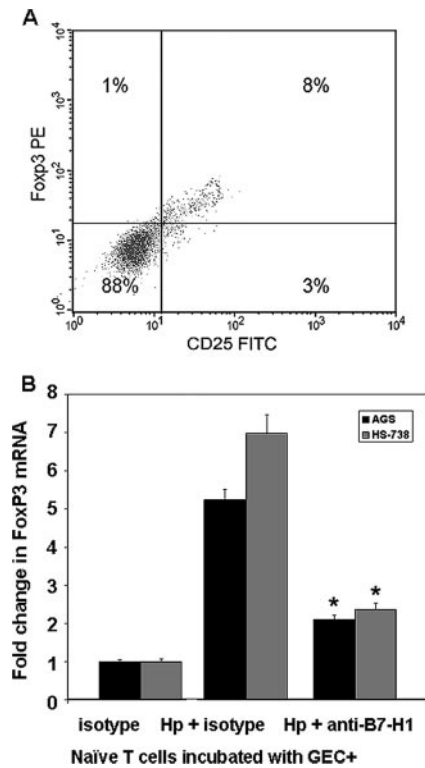
FIG. 3. B7-H1 on GEC induces the development of T cells to the Treg cell phenotype after *H. pylori* exposure. (A) Naïve T cells incubated with HS-738 cells in the presence of anti-B7-H1 are decreased in staining for CD25 and FoxP3 compared to those incubated with isotype control (Fig. 1E). Representative results of five experiments are shown. (B) Real-time PCR for FoxP3 mRNA isolated from T cells incubated with GEC exposed to *H. pylori* results in less FoxP3 mRNA when GEC are preincubated with anti-B7-H1 than with isotype control. The results indicate the change over the isotype control relative to the 18S housekeeping gene. The means and SEM are shown as the results of duplicates in four experiments; $n = 8$.
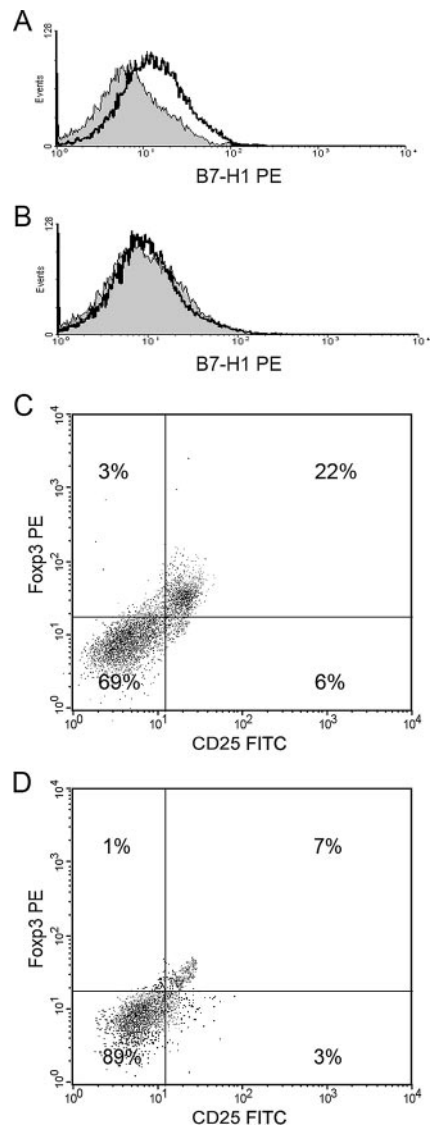

FIG. 4. B7-H1 siRNA decreases the development of T cells to the Treg cell phenotype. AGS cells previously exposed to *H. pylori* were transfected with (A) control siRNA or (B) a cocktail of B7-H1 siRNA and stained for B7-H1, indicating that B7-H1 expression is blocked compared to the control. Naïve T cells incubated for 7 days with AGS cells previously exposed to *H. pylori* transfected with (C) control siRNA or (D) a cocktail of B7-H1 siRNA were stained with anti-CD25–FITC, and anti-FoxP3–PE antibodies. Representative results of four experiments are shown.

bated with isotype control alone, isotype control and *H. pylori*, or anti-B7-H1 and *H. pylori* and analyzed for FoxP3 message. The results indicated that naïve T cells incubated with HS-738 or AGS cells previously exposed to *H. pylori* yielded an approximately four- to sixfold increase in FoxP3 mRNA compared to T cells cocultured with untreated GEC in the presence of isotype control (Fig. 3B). However, when *H. pylori*-exposed cells were incubated with anti-B7-H1 prior to the addition of naïve T cells, the level of FoxP3 mRNA was minimally increased over untreated cells.

In order to further confirm that B7-H1 expressed by GEC plays a role in the induction of the Treg phenotype, a cocktail of siRNAs for B7-H1 or negative control siRNAs were incorporated into GEC previously infected with *H. pylori*, and the cells were rested in culture for 48 h. In order to verify that the siRNA was able to block the expression of B7-H1, samples of the transfected cells were then stained for B7-H1 with monoclonal antibodies and analyzed for expression by flow cytometry. Figure 4A and B shows that expression of B7-H1 was almost completely abolished by this procedure compared to cells transfected with siRNA control. When these cells were exposed to *H. pylori* and then put into coculture with naïve T

cells, flow cytometric analysis for CD25 and FoxP3 revealed results similar to those of antibody blocking of B7-H1 (Fig. 3) compared to cells with control siRNA (Fig. 4C and D). Both blocking B7-H1 with monoclonal antibodies and blocking expression by utilizing siRNA to knock down B7-H1 expression in cells exposed to *H. pylori* clearly demonstrate the importance of this receptor in the induction of Treg cells. Since the cells treated with blocking anti-B7-H1 or siRNA were also exposed to *H. pylori* but failed to induce the putative Treg phenotype in the cocultured naïve T cells, the potential role of residual bacteria or bacterial components in this process was negated.
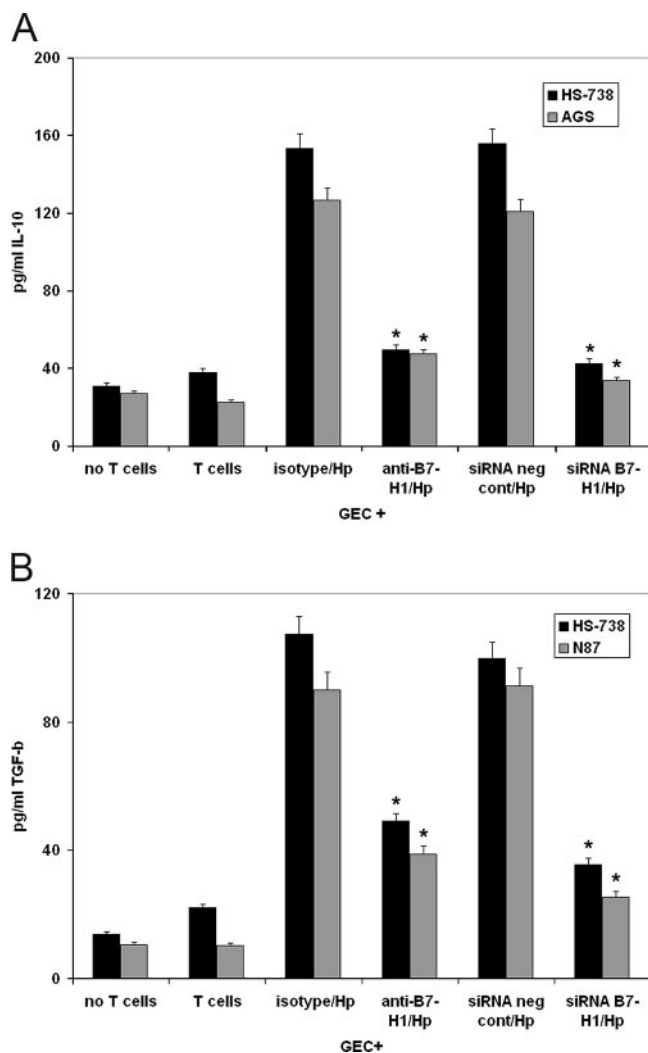
FIG. 5. T cells cocultured with *H. pylori*-exposed AGS cells produce IL-10 and TGF-β. Naïve T cells incubated with *H. pylori*-exposed GEC produce (A) IL-10 and (B) TGF-β, as shown after 7 days in coculture by ELISA with supernatants. Cytokine levels are decreased in the presence of anti-B7-H1 or B7-H1 siRNA. The means and SEM are shown as the results of duplicates in four experiments; $n = 8$ (*, $P < 0.05$).

IL-10 and TGF-β are produced during CD4$^+$ T-cell coculture with *H. pylori*-exposed GEC but are decreased by blocking B7-H1. Treg cells have been shown to produce high levels of IL-10 and TGF-β (24). In order to investigate the role of the Treg cells that develop during *H. pylori* infection and to assess their potential for inhibition of other T cells, levels of these cytokines were measured in supernatants of coculture experiments. GEC were irradiated prior to the addition of naïve T cells to ensure that only T cells were producing cytokines. ELISAs indicated that IL-10 and TGF-β were produced minimally by T cells in culture with GEC for 7 days or by *H. pylori*-exposed GEC alone, but when naïve T cells were in cultures with GEC that had been previously exposed to *H. pylori*, production of both IL-10 and TGF-β was detected (Fig. 5A and B). However, when B7-H1 was blocked by monoclonal antibodies or its expression was blocked by siRNA, the levels of IL-10 and TGF-β were decreased to minimal levels, similar

to those observed when naïve T cells were cocultured with GEC that were not exposed to *H. pylori*. These results provide further evidence for the presence of Treg cell development in cocultures of naïve T cells with *H. pylori*-exposed GEC. Furthermore, the presence of these cytokines suggests these Treg cells play a role in inhibiting the T-cell response during *H. pylori* infection.

CD4$^+$ CD25^{high} FoxP3$^+$ T cells induced by *H. pylori*-exposed GEC inhibit activated-T-cell proliferation. Since Treg cells are known to inhibit Teff cell responses, T cells expressing the Treg phenotype from coculture with *H. pylori*-exposed GEC were assessed for the ability to inhibit activated T cells. After 7 days of incubation with GEC or GEC preincubated with *H. pylori*, T cells from these cultures were sorted into CD4$^+$ CD25^{high} and CD4$^+$ CD25$^-$ populations. These populations were incubated for 4 days with naïve T cells from the same donor activated with anti-CD2, anti-CD3, and anti-CD28 in various ratios of Treg to Teff cells, and [³H]thymidine uptake was measured for T-cell proliferation. Figure 6A illustrates that CD4$^+$ CD25^{high} T cells from *H. pylori*-infected AGS cell cocultures incubated with activated Teff cells significantly reduced their thymidine incorporation. In contrast, CD4$^+$ CD25$^-$ T cells did not affect the proliferation of activated Teff cells. Thymidine incorporation decreased as the number of CD4$^+$ CD25^{high} cells increased. The suppressive effects of Treg cells generated from *H. pylori*-exposed cells is further shown in Fig. 6B, which shows the mean percent suppression of cells isolated from several donors in Treg/Teff cell ratios of 1:20. The mean percent suppression for Treg cells isolated from *H. pylori*-exposed GEC was approximately 40%, based on three experiments ($n = 9$). These results were similar to the level (percentage) of suppression seen with CD4$^+$ CD25^{high} T cells isolated from PBMC from the same donors. The ability of the Treg cells induced from naïve-T-cell incubation with the *H. pylori*-infected epithelial cells to inhibit activated T cells suggests a crucial role for these cells in the hyporesponsiveness of T cells during *H. pylori* infection.

CD4$^+$ CD25^{high} T cells produce increased FoxP3, IL-10, and TGF-β mRNA levels. Sorted CD25^{high} and CD25$^-$ T cells from *H. pylori*-infected coculture assays with GEC were further investigated for levels of FoxP3, IL-10, and TGF-β mRNA by real-time PCR to assess if differences in the suppressive abilities of these populations were correlated with the expression of these Treg markers. CD25^{high} T cells produced high levels of FoxP3 mRNA compared to CD25$^-$ T cells, suggesting that CD25^{high} Treg cells are the main FoxP3 expressers (Fig. 7A). However, T cells negative for CD25 had approximately twofold induction of FoxP3 mRNA, suggesting the presence of a small population of CD25$^-$ FoxP3$^+$ T cells. Similar results were seen with Treg cells sorted from PBMC.

The mRNA levels of T-cell-inhibitory cytokines were also assessed by real-time PCR for CD25^{high} and CD25$^-$ T-cell populations and compared to naïve T cells. For IL-10, T cells expressing high levels of CD25 exhibited approximately fivefold-higher mRNA levels, while CD25-negative cells produced levels only twofold higher than those in naïve T cells (Fig. 7B). The mRNA levels for the three isoforms of human TGF-β were also investigated, and the cells expressing high levels of CD25 expressed higher levels of all three than the CD25-negative cells. TGF-β1 mRNA was present in high expressers of CD25 at levels three- to fourfold higher than in naïve T cells
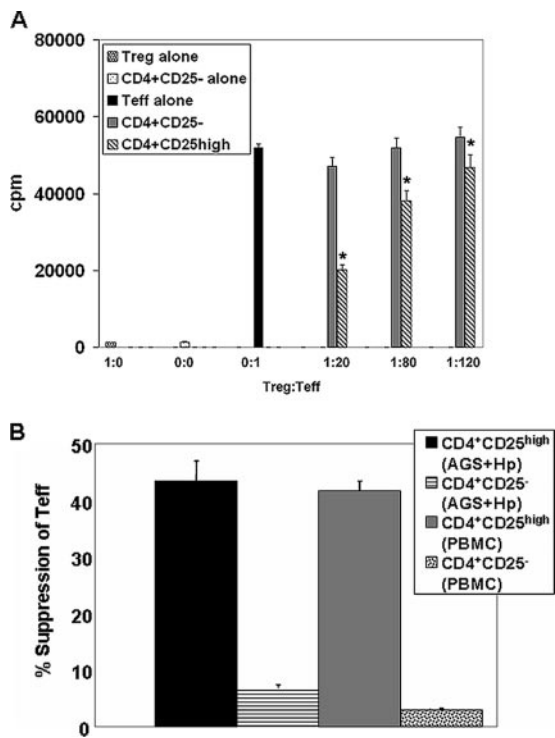
FIG. 6. CD4$^+$ CD25^{high} T cells induced by coculture with *H. pylori*-exposed GEC suppress the proliferation of activated CD4$^+$ T cells. CD4$^+$ CD25^{high} T cells induced by coculturing of naïve T cells with AGS cells previously exposed to *H. pylori* for 7 days were sorted by FACSAria sorter, and CD4$^+$ CD25^{high} or CD4$^+$ CD25$^-$ T cells were cocultured with CD2/CD3/CD28-activated CD45RA$^+$ CD4$^+$ CD25$^-$ Teff cells for 4 days in various Treg/Teff cell ratios. Eighteen hours prior to the end of coculture, the cells were pulsed with [methyl-^{3}H]thymidine. (A) Mean cpm + SE of triplicate cultures of CD4$^+$ T cells isolated from one donor are shown in a representative of three experiments in which CD4$^+$ CD25^{high} cells suppressed the proliferation of activated CD4$^+$ T cells while CD4$^+$ CD25$^-$ cells did not. (B) The percent suppression of Teff cells is shown, which represents the decrease in the proliferative response (cpm) of CD4$^+$ Teff cells compared to activated Teff cells alone. The values represent the mean percent suppression for three independent healthy donors. The means are shown as the results of triplicates in three experiments; $n = 9$ ($*$, $P < 0.05$).

FIG. 7. CD4$^+$ CD25^{high} T cells express high FoxP3, IL-10, and TGF-β mRNA levels. Real-time PCR for (A) FoxP3 and (B) IL-10 and TGF-β isoforms with RNA isolated from CD4$^+$ CD25^{high} or CD4$^+$ CD25$^-$ T cells sorted from populations incubated with *H. pylori*-infected cells and compared to that of naïve T cells from the same donor illustrates that the CD25^{high} population of T cells produced more RNA for these genes than the CD25$^-$ population. The mean (+SEM) increase over naïve T cells is shown for triplicates.

from the same donors (Fig. 7C), while CD25-negative cells did not exhibit an increase in TGF-β1 mRNA. TGF-β2 mRNA was increased by sixfold for high expressers of CD25, while the CD25-negative cells exhibited a twofold increase. TGF-β3 mRNA resulted in only minimal increases. These results indicate that Treg cells expressing high levels of CD25 also express dramatically higher levels of FoxP3, IL-10, TGF-β1, and TGF-β2 than CD25-negative cells in this system. Although CD25$^-$ cells do not produce dramatically increased levels of IL-10 and TGF-β2 mRNA, they do have twofold-higher levels than naïve T cells, which may suggest that they could exert suppressive effects in this system.

DISCUSSION

The host immune response during *H. pylori* infection is not sufficient to clear infection. Interestingly, T cells from infected individuals are hyporesponsive and are polarized toward a Th1
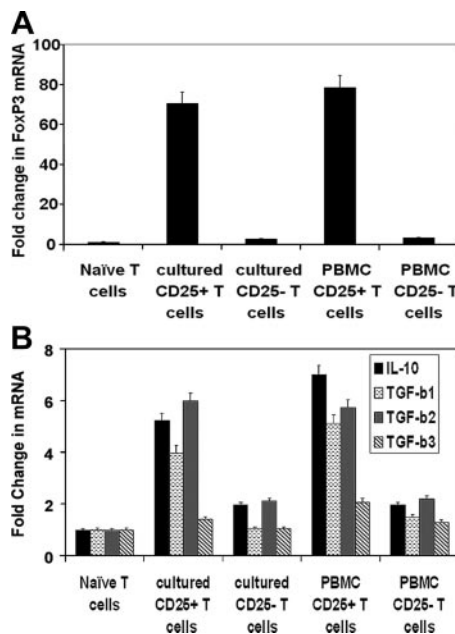
phenotype. *H. pylori* has been shown to directly affect the T-cell response by multiple mechanisms. For example, *H. pylori* can induce T-cell apoptosis by up-regulating Fas ligand on Fas-expressing T cells (29). Also, the VacA virulence factor has been shown to down-regulate IL-2 receptors on T cells, inhibit production of IL-2, and decrease cell viability (4). We have shown that *H. pylori* may inhibit T-cell responses in an indirect way by up-regulating B7-H1 expression on GEC, both transformed and nontransformed, which in turn inhibited T-cell proliferation in coculture experiments (5).

Recently, yet another mechanism of T-cell inhibition during infection has been discovered. The presence of Treg cells has been revealed in the gastric mucosa of *H. pylori*-infected patients (16). In another recent study, CD4$^+$ FoxP3$^+$ T cells were detected in *H. pylori*-infected patients with gastric adenocarcinoma (7). More specifically, higher levels of these cells were present in tumor tissue than in tumor-free gastric tissue. Together, these observations could contribute not only to the chronicity of *H. pylori* infection, but also to the development of associated neoplasia. Although recent studies have reported the presence of Treg cells in *H. pylori*-infected gastric mucosa, the recruitment or development of these T cells has not been characterized. Here, we have shown that naïve T cells cultured with *H. pylori*-infected GEC can develop into cells with the Treg phenotype. The formation of these cells was dependent upon B7-H1 expressed on GEC, which we previously showed was dramatically up-regulated by exposure both in vivo and in vitro to multiple strains of *H. pylori* (5) and which has been similarly shown in other studies to be induced by microbial

products or Th1 cells (15). However, it is unlikely that residual *H. pylori*-derived products influenced the expansion of cells with a Treg phenotype, since cocultures of GEC were extensively washed and the presence of anti-B7-H1 antibodies prevented their expansion. The increased expression of B7-H1 on epithelium appears to be complex and can be induced by gamma interferon and tumor necrosis factor alpha to some extent. The induction of cells of the Treg phenotype by B7-H1 may very well play an important role in the T-cell suppression associated with *H. pylori* infection, as well as other infections that result in an up-regulation of B7-H1. These cells have already been shown to play crucial inhibitory roles in cancer and parasitic infections (1, 26).

Since IL-10 and TGF-β are produced by some types of Treg cells and have essential T-cell-inhibitory functions, their expression during *H. pylori* infection by induced Treg cells may play an important role in the inhibition of Teff cells. In this study, the T-cell population expressing high levels of CD25 also expressed high levels of FoxP3 mRNA, along with IL-10 and TGF-β, particularly TGF-β2. In contrast, the CD25⁻ sorted population expressed very little FoxP3 mRNA but also exhibited small increases in IL-10 and TGF-β levels, suggesting the presence of a small population of T-regulatory type 1 (Tr1) cells. Although several studies have suggested that Tr1 cells are more important in inhibitory cytokine production than CD25-expressing Treg cells (22, 28), we found that both cell types produced increased mRNA for IL-10 and TGF-β, with CD25-expressing Treg cells producing much higher levels.

As for functionality, the cells expressing high levels of CD25 were able to decrease the proliferation of activated T cells from the same donors at much higher levels than the CD25⁻ cells. The high expressers of CD25 were also sorted from the PBMC of the same donors to compare functionality with those induced by *H. pylori*-infected GEC. The results from this population indicated that the induced Treg cells had functionality similar to that of the cells from PBMC. It should be noted that the numbers of Treg cells used here in functional assays were similar to or below the numbers reported to be present in patient samples of *H. pylori*-infected gastric mucosa (16) and still had a suppressive effect on the proliferation of CD4⁺ Teff cells. The studies presented here show how *H. pylori* may use the epithelium to promote the development of Treg cells. These Treg cells, in turn, play a key role in the suppression of CD4⁺ Teff cells that are recruited to the gastric mucosa during infection. While this may represent an important mechanism that facilitates the persistence of *H. pylori* in the host, perhaps these cells may also facilitate immune avoidance of any cancer cells that develop within the infected stomach. Thus, the presence of CD4⁺ CD25^high FoxP3⁺ Treg cells during *H. pylori* infection may be crucial in down-regulating the T-cell response and failure to clear infection. These Treg cells may play a crucial role in promoting chronic infections, and perhaps eventual carcinogenesis.

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