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*J Immunol* 2007; 179:5433-5440; doi: 10.4049/jimmunol.179.8.5433

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Helicobacter pylori Vacuolating Cytotoxin Inhibits Activation-Induced Proliferation of Human T and B Lymphocyte Subsets

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Helicobacter pylori are Gram-negative bacteria that persistently colonize the human gastric mucosa despite the recruitment of immune cells. The H. pylori vacuolating cytotoxin (VacA) recently has been shown to inhibit stimulation-induced proliferation of primary human CD4+ T cells. In this study, we investigated effects of VacA on the proliferation of various other types of primary human immune cells. Intoxication of PBMC with VacA inhibited the stimulation-induced proliferation of CD4+ T cells, CD8+ T cells, and B cells. VacA also inhibited the proliferation of purified primary human CD4+ T cells that were stimulated by dendritic cells. VacA inhibited both T cell-induced and PMA/anti-IgM-induced proliferation of purified B cells. Intoxication with VacA did not alter the magnitude of calcium flux that occurred upon stimulation of CD4+ T cells or B cells, indicating that VacA does not alter early signaling events required for activation and proliferation. VacA reduced the mitochondrial membrane potential of CD4+ T cells, but did not reduce the mitochondrial membrane potential of B cells. We propose that the immunomodulatory actions of VacA on T and B lymphocytes, the major effectors of the adaptive immune response, may contribute to the ability of H. pylori to establish a persistent infection in the human gastric mucosa. The Journal of Immunology, 2007, 179: 5433–5440.

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Although effects of VacA on primary human CD4+ T cells have been documented (24–26), possible effects of VacA on other types of primary human immune cells have not yet been investigated in detail. In the current study, we demonstrate that VacA can suppress the proliferation of primary human CD8+ T cells and CD4+ Th cells. We show that VacA inhibits proliferation of Th cells regardless whether the cells are stimulated by TCR/CD28 Abs, PMA/ionomycin, or APCs. Moreover, we show that VacA can act directly on B cells and thereby inhibit B cell proliferation. We propose that VacA may enhance the capacity of H. pylori to establish a persistent infection by suppressing the activity of CD4+ and CD8+ T cells, as well as B cells, in the gastric mucosa.

Materials and Methods

Purification of VacA

H. pylori strains were grown as described previously (17, 27). H. pylori 60190 is a wild-type strain and strain AV452 secretes a mutant form of VacA (VacAΔ–27) that is defective in membrane channel formation (27). Oligomeric forms of VacA were purified from broth culture supernatants of H. pylori as described previously (17). All experiments were performed using acid-activated preparations of VacA or acidified buffer control (PBS), unless stated otherwise (16). The final VacA concentration was 10−20 μg/ml for all experiments, as indicated.

Cell culture and primary cell isolation

Blood was obtained from healthy human adult volunteer donors, in accordance with procedures approved by the Vanderbilt University Medical Center Institutional Review Board. PBMC were isolated from blood using a Ficoll-Hypaque gradient. Resting (i.e., nonproliferating) CD4+ and CD8+ human T cells were purified as previously described (28). B cells were purified using a CD19 AutoMACS sort (Miltenyi Biotec) using positive selection or the EasySep Human B cell enrichment kit (StemCell Technologies), as described by the manufacturer. The purified cells were 90–99% pure as assessed by staining and flow cytometric analysis.

Stimulation of primary human PBMC and T cells

For proliferation assays, cells were labeled with CFSE (Moebus Probes) and proliferation of the cells was monitored by CFSE partitioning 5 days poststimulation, as previously described (24). Cell populations were analyzed with a FACSCalibur flow cytometer (BD Biosciences), using anti-CD3-Cy5.5, anti-CD4-biotin, anti-CD8-biotin, anti-CD19-biotin Abs and streptavidin-allophycocyanin. The proliferation index was calculated as the sum of the cells in all generations divided by the estimated number of original parent cells. The estimated number of original parent cells was calculated by dividing the final number of cells in each generation by the squared generation number. Forward and side scatter profiles as well as MFI profiles were analyzed using the FlowJo software (Tree Star) program. T cells were stimulated with anti-CD3 and anti-CD28, and B cells were stimulated with PMA (50 ng/ml) and anti-IgM in medium supplemented with IL-2 as described. The PMA/ionomycin remained present for the duration of the experiment. As controls to inhibit TCR/CD28 stimulation or IL-2-driven stimulation, T cells were treated with cyclosporine A (10 μM; Alexis Biochemicals) or rapamycin (300 ng/ml; Alexis Biochemicals), respectively. Proliferation of T cells and PBMC was monitored by CFSE partitioning 5 days poststimulation, as described.

DC-induced activation of CD4+ T cells was accomplished by mixing CFSE-labeled CD4+ T cells with DC (10:1 ratio) in a 96-well plate, followed by stimulation with staphylococcal enterotoxin B (SEB, 10 ng/ml; Sigma-Aldrich). T cell proliferation was monitored by CFSE partitioning 5 days poststimulation as described.

Stimulation of primary human B cells

T cell-induced B cell proliferation was accomplished by treating purified primary human CD4+ T cells with 50 μg/ml mitomycin C (Sigma-Aldrich) for 30 min at 37°C. T cells were then washed twice to remove the mitomycin C. The mitomycin C-treated T cells were then mixed with CFSE-labeled B cells (2:1 ratio). The cell mixtures were stimulated with TCR/CD28 Abs and expanded in the presence of IL-2 as described. B cell proliferation was monitored by CFSE partitioning 5 days poststimulation as described.

Calcium mobilization and mitochondrial membrane potential

Calcium flux was measured using the Fluo-4 NW Calcium Assay kit from Invitrogen Life Technologies/Molecular Probes. Briefly a stock solution of the Fluo-4 dye was prepared by resuspending the powdered dye in 10 ml of the provided assay buffer and 100 μl of 250 mM probe stock solution. T cells or B cells (200,000 cells) were pretreated with VacA, and were then pelleted and resuspended in 200 μl of the Fluo-4 solution for 30 min at 37°C in 5% CO2, followed by incubation for 30 min at room temperature before analysis by FACS. Cells were analyzed on a FACSCalibur for 20 s to establish a baseline, and the cells were then briefly removed from the FACSCalibur machine. B cells were stimulated with anti-IgM (10 μg/ml) and anti-CD40 (10 μg/ml). The cells were vortexed and quickly placed back on the FACSCalibur machine and analyzed for 60 s. The cells were removed again, 10 μg/ml goat-anti-mouse Ab was added to cross-link bound primary Abs (anti-IgM and anti-CD40), and the cells were analyzed for an additional 70 s. T cells were analyzed in a similar manner, following stimulation with anti-CD3 and anti-CD28. As a positive control, cells were removed after the analysis, treated with 100 ng/ml ionomycin, and analyzed by FACS for an additional 50 s. Cells were analyzed by monitoring fluorescence of the Fluo-4 dye in the FL1 channel (520 nm) over time.

The effect of VacA intoxication on mitochondrial membrane potential was done as previously described (25). Briefly, the Mito Flow reagent (Cell Signaling Technology) was used according to the manufacturer’s instructions. Activated T or B cells (1 × 105 cells) were used for the Mito Flow assays. The Mito Flow data were collected by FACS and analyzed using flow cytometry (FACSCalibur; BD Biosciences). Live cells were gated based on forward and side scatter profiles and analyzed using the FlowJo (Tree Star) program. T cells were stimulated with anti-CD3 and anti-CD28, and P cells were stimulated with PMA (50 ng/ml) and anti-IgM in medium supplemented with recombinant human IL-2 (200 U/ml; Chiron) as described.

Results

VacA inhibits stimulation-induced proliferation of primary human PBMC

We showed recently that VacA can inhibit the activation-induced proliferation of purified primary human CD4+ Th cells (24). To investigate whether VacA can inhibit the activation-induced proliferation of other primary human immune cells, we first isolated human PBMC from the blood of healthy adult donors and labeled the cells with CFSE. Labeled cells were then intoxicated with wild-type VacA. As a control, cells were also treated with a VacA mutant protein (VacAΔ6–27) that fails to form membrane channels (24, 27), or with control buffer. Cells were then stimulated...
with anti-CD3/CD28 Abs (TCR stimulation) or with a combination of chemical agonists (PMA and ionomycin) and cultured for 5 days. Intoxication of PBMC with wild-type VacA resulted in inhibition of PBMC proliferation, with varying degrees of inhibition detected depending on the activation stimulus (Fig. 1). In contrast, PBMC intoxicated with VacAΔ6–27 proliferated similarly to cells treated with the buffer control (Fig. 1). These data demonstrate that VacA is able to inhibit the stimulation-induced proliferation of PBMCs.

To investigate which subsets of cells within primary human PBMC are affected by VacA, resting PBMCs from healthy adult donors were labeled with CFSE and intoxicated with acid-activated wild-type VacA, a mutant VacA protein (VacAΔ6–27), or control buffer. Cells were then activated via TCR/CD28 stimulation and cultured for five days. The proliferation of individual cell types was determined by gating on CD3+/CD4+ (CD4+ T cells), CD3+/CD8+ (CD8+ T cells), and CD19+ (B cells) (top). Data represent proliferation indices of the cells (bottom). Results represent one of three independent experiments, each done at least in triplicate, using different adult blood donors. *, p < 0.05 compared with PBS-treated cells, analyzed by two-tailed t test.
was monitored by cell surface Ab staining and multiparametric flow cytometric analyses. Wild-type VacA, but not the control buffer or the mutant VacAΔ6–27, inhibited the proliferation of CD4\(^+\) T cells, CD8\(^+\) T cells, or B cells within the PBMC population (Fig. 2A). As an alternate method for stimulating cell proliferation, the PBMC were stimulated with PMA/ionomycin instead of anti-CD3/CD28 Abs. Similar to the effect observed following VacA treatment of TCR/CD28-stimulated cells (Fig. 2A), VacA also inhibited the proliferation of CD4\(^+\) T cells, CD8\(^+\) T cells, and B cells within the PBMC population when stimulated with PMA/ionomycin (Fig. 2B).

**VacA inhibits superantigen-induced proliferation of T cells**

These experiments demonstrated that VacA can inhibit proliferation of PBMC when the cells are stimulated in vitro using cross-linking Abs (i.e., anti-CD3/CD28 Abs) or chemical agonists (PMA/ionomycin) (Fig. 2). To investigate VacA-mediated inhibition of human T cell proliferation in a more physiologically relevant context, we determined whether VacA is capable of inhibiting the proliferation of T cells stimulated by APCs. We first investigated whether VacA can inhibit the stimulation-induced proliferation of T cells induced by superantigen SEB. In the presence of professional APC, SEB induces polyclonal stimulation of primary human T cells, resulting in T cell proliferation and cytokine production (29–31). PBMC from healthy adult donors were labeled with CFSE as before. Cells were then intoxicated with wild-type VacA, the mutant VacAΔ6–27, or control buffer, stimulated with SEB, and cultured for 5 days before FACS analysis. Similar to cells treated with control buffer, cells intoxicated with the mutant VacAΔ6–27 proliferated in a SEB-dependent manner (Fig. 3A). In contrast, SEB-induced proliferation of PBMC was inhibited by wild-type VacA (Fig. 3A). Furthermore, VacA inhibited SEB-mediated proliferation of both CD4\(^+\) and CD8\(^+\) T cells within the PBMC population (Fig. 3B).

**VacA inhibits DC-induced proliferation of primary human CD4\(^+\) T cells**

To begin dissecting how VacA inhibits SEB-mediated proliferation of T cells, we investigated whether VacA could inhibit the ability of purified primary human myeloid DC to activate primary human Th cells. CFSE-labeled resting CD4\(^+\) T cells were mixed with DC and then intoxicated with VacA. The cell mixture was stimulated with SEB and cultured for 5 days before FACS analysis (Fig. 4A). Similar to the observed effect of VacA on proliferation of SEB-stimulated PBMC (Fig. 3), VacA inhibited the proliferation of DC-stimulated Th cells (Fig. 4A).

The ability of VacA to inhibit SEB-induced proliferation of Th lymphocytes could potentially be due to 1) a direct VacA effect on proliferating lymphocyte populations, 2) an inhibitory effect of VacA on DC, or 3) effects of VacA on both Th lymphocytes and DC. To differentiate between these possibilities, we individually treated purified CFSE-labeled CD4\(^+\) T cells or purified primary human DC with wild-type VacA, mutant VacAΔ6–27, or control buffer (Fig. 4B, top). After treatment, cells were washed to remove unbound additives. VacA-intoxicated CFSE-labeled Th cells were cocultured with untreated DC, and VacA-intoxicated DC were cocultured with untreated CFSE-labeled Th cells. The cocultures were stimulated with SEB, expanded for 5 days, and proliferation of the CFSE-labeled Th cells was assessed by FACS analysis. Pretreatment of DC with wild-type VacA did not inhibit Th cell proliferation (Fig. 4B, bottom). In contrast, when the CD4\(^+\) T cells were pretreated with VacA, their proliferation was inhibited (Fig. 4B, bottom). These data demonstrate that the observed VacA-mediated inhibition is due to a direct effect of VacA on T cells, and not due to an inhibitory effect of VacA on DC.

**VacA inhibits T cell-induced proliferation of primary human B cells**

As shown in Fig. 2, VacA inhibited the proliferation of B cells within the PBMC population when cells were stimulated with TCR/CD28 Abs or with PMA/ionomycin. In the experiments shown in Fig. 2, B cells were activated indirectly by CD3/CD28-activated Th cells. Thus, inhibition of B cell proliferation by VacA could either be due to the suppression of Th cell activation or through direct effects of VacA on B cells. To differentiate between these possibilities, we performed experiments using purified B cells and purified CD4\(^+\) T cells. We labeled purified B cells with CFSE and treated the purified Th cells with mitomycin C, a drug that inhibits Th cells proliferation without adversely affecting the up-regulation of surface activation markers (such as CD40L) and cytokine secretion (32). CFSE-labeled B cells and mitomycin C-treated Th cells were mixed, treated with wild-type VacA, mutant VacAΔ6–27, or control buffer, and the cocultures were activated using TCR/CD28 Abs (Fig. 5A). Intoxication of the cocultures with wild-type VacA or treatment with cyclosporine A, a drug that inhibits T and B cell activation, each resulted in inhibition of B cell proliferation (Fig. 5A). We then repeated these experiments using a protocol in which either mitomycin C-treated T cells or CFSE-labeled B cells were treated separately with the different additives before coculture (Fig. 5B). After treatment, cells were washed to remove unbound additives, mixed, and stimulated using TCR/CD28 Abs. Pretreatment of the primary human Th cells with VacA did not inhibit the ability of these
cells to induce B cell proliferation (Fig. 5B). In contrast, pre-treatment of Th cells with cyclosporine A inhibited their ability to induce B cell proliferation (Fig. 5B). When CFSE-labeled B cells were pretreated with wild-type VacA, inhibition of B cell proliferation was observed (Fig. 5B). These data suggest that VacA directly targets primary human B cells and inhibits their proliferation.

VacA directly inhibits proliferation of primary human B cells

Further studies were then undertaken to investigate in more detail whether VacA can directly target B cells. We tested the ability of several agents (i.e., PMA/ionomycin, PMA/anti-IgM, anti-IgM/anti-CD40) to stimulate in vitro growth of primary human B cells. Stimulation with PMA/anti-IgM resulted in a low level of B cell proliferation, whereas the other agents had no detectable effect (data not shown and Fig. 6A). Intoxication of primary human B cells with wild-type VacA, but not the mutant VacAΔ6–27 or control buffer, markedly inhibited PMA/anti-IgM-induced B cell proliferation (Fig. 6A). These data provided further evidence that VacA can directly inhibit the proliferation of primary human B cells.

To obtain insight into the mechanism by which VacA inhibits B cell proliferation, we first investigated whether VacA interfered with early events that occur upon B cell activation. As an initial
approach, we investigated whether VacA had any effect on calcium fluxes that are induced upon stimulation of lymphocytes. Calcium is known to play a crucial role in lymphocyte activation, primarily due to its role in the activation of the NFAT. Intoxication of primary human B and Th cells with VacA did not result in a significant change in the magnitude of the calcium fluxes upon stimulation (Fig. 6B). These data provide further evidence indicating that VacA does not have an effect on early signaling events required for lymphocyte activation and proliferation (25).

We recently showed that VacA causes mitochondrial membrane depolarization in primary human Th cells (25). Therefore, we investigated whether VacA has a similar effect on primary human B cells. As expected, intoxication of primary human Th cells resulted in a significant increase in the percentage of Th cells with a low mitochondrial membrane potential (Fig. 6C). In contrast, VacA intoxication of B cells did not result in a reduction in membrane potential, and actually increased the mitochondrial membrane potential (Fig. 6C). These data suggest that there are differences in the susceptibility of T cells and B cells to VacA-induced mitochondrial membrane depolarization.

**Discussion**

In this study, we show that VacA can inhibit the activation-induced proliferation of multiple types of primary human immune cells, including CD4+ and CD8+ T cells, as well as B cells (Fig. 1). The inhibitory effect of VacA on lymphocytes was observed when cells were stimulated with either cross-linking Abs or chemical agonists (i.e., TCR/CD28 Abs or PMA/ionomycin) (Figs. 1 and 2). In addition, we show that VacA inhibits proliferation of CD4+ T cells that are stimulated by APC.

Our data demonstrate that VacA inhibits Th cell-mediated B cell proliferation when the B cells are individually pretreated with VacA, but not when the Th cells are pretreated with VacA (Fig. 5). Thus, VacA intoxication of primary human CD4+ T cells does not perturb the ability of Th cells to activate B cells, a process that requires T cells to become fully activated. In contrast, treatment of primary human Th cells with cyclosporine A, an NFAT-activation inhibitor, potently inhibited T cell-mediated B cell proliferation. These data demonstrate that, in contrast to the effects of VacA on Jurkat cells (21, 23), VacA does not interfere with TCR-mediated activation of primary human T cells (24). Furthermore, we show that VacA inhibits PMA/anti-IgM-induced proliferation of primary human B cells, which provides further evidence that the inhibitory effects of VacA on B cell proliferation are due to direct effects of VacA on B cells (Fig. 6). To our knowledge, an inhibitory effect of VacA on stimulation-induced B cell proliferation has not been described previously.

Another important finding in the current study is that VacA can inhibit the proliferation of primary human Th cells that are activated by APC and a superantigen (Fig. 4). Our data indicate that inhibition of APC/superantigen-induced Th cell proliferation is due to the ability of VacA to target Th cells, rather than APC (i.e., DCs) (Fig. 4). Intoxication of DC with VacA did not inhibit superantigen-induced Th cell proliferation, suggesting that VacA does not alter the expression of cell surface proteins or cytokine signals derived from DC, which are required for T cell activation and proliferation (Fig. 4). In a previous study, VacA was shown to interfere with the process of Ag presentation in B cells by specifically inhibiting the Ii-dependent pathway of Ag presentation mediated by newly synthesized MHC class II molecules (33). This

**FIGURE 6.** Effects of VacA on PMI/anti-IgM-induced B cell proliferation, calcium mobilization, and mitochondrial membrane potential. A, Resting CD19+ B cells were labeled with CFSE and pretreated with control buffer, acid-activated wild-type VacA (WT-VacA, 10 μg/ml), or a mutant VacA (VacAΔ6–27, 10 μg/ml) for 4 h and then stimulated with anti-IgM and PMA. Cells were then expanded for 5 days in the presence of IL-2, harvested, and analyzed for CFSE content by flow cytometry. Numbers indicate proliferation indices ± SD for each histogram. B, Resting CD19+ B cells or CD4+ T cells were pretreated with control buffer, acid-activated wild-type VacA, or the mutant VacAΔ6–27 as described and then resuspended in 200 μl of the Fluo-4 solution for 30 min at 37°C in 5% CO₂, followed by incubation for 30 min at room temperature before analysis by FACS. Cells were stimulated as described in Materials and Methods. Calcium fluxes were analyzed by monitoring fluorescence of the Fluo-4 dye in the FL1 channel (520 nm) over time. The y-axis depicts the level of stimulation-induced calcium flux detected in VacA-treated cells compared with that detected in buffer-treated control cells. A value of 100% indicates the level of calcium flux detected in stimulated control cells. C, Resting CD19+ B cells and CD4+ T cells were pretreated with the different additives and then stimulated as described in the Methods. Cells were cultured for 5 days and the mitochondrial membrane potential of the cultured cells was assayed as described in Materials and Methods (left). Data representative of the percentage of Th cells with a low mitochondrial (Mito) membrane potential (right) are shown. Data are representative results from one of two independent experiments, each done in triplicate, using different adult blood donors. *, p < 0.05 compared with PBS-treated controls, as analyzed by two-tailed t test.
previously observed effect of VacA on Ag presentation is consistent with the ability of VacA to alter endocytic trafficking (5, 34). In the current study, the absence of detectable effects of VacA on DC can be explained by the fact that superantigen presentation requires MHC class II expression but does not require Ag processing.

Despite the presence of numerous B cell and plasma cells in the gastric mucosa of infected individuals, H. pylori are able to persistently colonize the human stomach. One possible consequence of the VacA-mediated inhibition of B cell proliferation observed in this study may be a reduction in secretory IgA synthesis in the stomach. In support of this hypothesis, plasma cells containing intracytoplasmic crystalline Ig inclusions have been detected in gastric biopsy specimens from patients with H. pylori-associated gastritis (35). The formation of crystalline inclusions in plasma cells is thought to reflect altered production, storage, or secretion of Ig. Inhibited production or secretion of Ig could be a factor that contributes to the persistence of H. pylori in the stomach.

Because VacA inhibits activation-induced proliferation of both T and B lymphocytes, it seems possible that a similar mechanism of action might account for the effects of the toxin on the two cell types. We recently showed that VacA does not inhibit the activation of important signal transduction components, including the signal transducer and activator of transcription STAT3 and STAT5, ERK, and p38 MAPK, in primary human T cells (25). In contrast, we showed that VacA inhibits the activation of regulatory proteins required for G1 cell cycle transition (25). In the current study, we show that VacA does not have a detectable effect on stimulation-induced calcium fluxes in either T cells or B cells (Fig. 6B). This finding, together with the observation that VacA inhibits proliferation regardless whether the cells are stimulated with TCR/CD28 Abs or with PMA/ionomycin, suggests that VacA has an effect on a cellular process downstream of receptor signaling in both T and B lymphocytes. Interestingly, we demonstrate in the current study that VacA causes a reduction in the mitochondrial membrane potential of T cells but not B cells (Fig. 6C). This observed difference in the susceptibility of T cells and B cells to VacA-induced mitochondrial depolarization suggests that there may be differences in the VacA target sites in these two cell types. Membrane channel formation by VacA seems to be required for inhibiting the proliferation of both B and T cells. Specifically, based on the observation that VacAΔΔe–27 is defective in formation of membrane channels (27) and fails to inhibit proliferation of T cells or B cells (24, 25), it can be surmised that membrane channel formation by VacA has an important mechanistic role in the process by which VacA inhibits lymphocytes proliferation. Patch clamp studies indicate that VacA can form membrane channels in the plasma membrane of epithelial cells (36), and there is evidence that VacA also may form channels in the membranes of endosomes and mitochondria (5, 34, 37). Further investigation will be required to determine the relevant site of VacA channel formation in Th and B cells and to more completely elucidate the specific mechanism by which VacA exerts its inhibitory activities against these primary human lymphocytes.

Gastric biopsies from H. pylori-infected humans consistently demonstrate infiltration of T lymphocytes, plasma cells, DCs, mononuclear phagocytes, B cells, and neutrophils into the lamina propria (38–40). Despite the development of humoral and cellular immune responses and a gastric mucosal inflammatory response, H. pylori are able to evade the immune response and establish a life-long persistent infection (4). VacA is potentially an important bacterial factor that contributes to H. pylori persistence. Although it is not known what concentrations of VacA are present in the gastric mucosa of H. pylori-infected humans, we speculate that the local VacA concentration adjacent to sites of H. pylori colonization could be relatively high, and that VacA may target several types of immune cells, including B and T lymphocytes, that are recruited to such sites. We propose that VacA might inhibit the clonal expansion of infiltrating T cells, as well as B cells, that have been activated by H. pylori Ags. Effects of VacA on proliferation of T cells and B cells may effectively attenuate the host immune response, and thereby facilitate the establishment of a life-long persistent H. pylori infection.

Acknowledgments

We thank Beverly Hosse for assistance with VacA preparation and Michelle Tseng for assistance with the purification of the primary human cells used in this study.

Disclosures

The authors have no financial conflict of interest.

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