Inhibition of primary human T cell proliferation by *Helicobacter pylori* vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion

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Recent evidence indicates that the secreted *Helicobacter pylori* vacuolating toxin (VacA) inhibits the activation of T cells. VacA blocks IL-2 secretion in transformed T cell lines by suppressing the activation of nuclear factor of activated T cells (NFAT). In this study, we investigated the effects of VacA on primary human CD4$^+$ T cells. VacA inhibited the proliferation of primary human T cells activated through the T cell receptor (TCR) and CD28. VacA-treated Jurkat T cells secreted markedly diminished levels of IL-2 compared with untreated cells, whereas VacA-treated primary human T cells continued to secrete high levels of IL-2. Further experiments indicated that the VacA-induced inhibition of primary human T cell proliferation was not attributable to VacA effects on NFAT activation or IL-2 secretion. We show here that VacA suppresses IL-2-induced cell-cycle progression and proliferation of primary human T cells without affecting IL-2-dependent survival. Through the analysis of a panel of mutant VacA proteins, we demonstrate that VacA-mediated inhibition of T cell proliferation requires an intact N-terminal hydrophobic region necessary for the formation of anion-selective membrane channels. Remarkably, we demonstrate that one of these mutant VacA proteins [VacA-Δ(6–27)] abrogates the immunosuppressive actions of wild-type VacA in a dominant-negative fashion. We suggest that VacA may inhibit the clonal expansion of T cells that have already been activated by *H. pylori* antigens, thereby allowing *H. pylori* to evade the adaptive immune response and establish chronic infection.

Helicobacter pylori is a Gram-negative spiral-shaped microaerophilic bacterium that colonizes the gastric mucosa of >50% of the human population (1, 2). Infection with this bacterium is consistently associated with gastric mucosal inflammation and is a risk factor for the development of peptic ulcer disease, distal gastric adenocarcinoma, and gastric lymphoma (1, 2).

Most *H. pylori* strains secrete a vacuolating toxin (VacA) into the extracellular space (3, 4). Epidemiological studies and experiments using animal models have suggested that VacA is an important *H. pylori* virulence factor in the pathogenesis of peptic ulceration and gastric cancer (5–9). Incubation of VacA with cultured mammalian cells results in multiple effects, including formation of intracellular vacuoles, depolarization of the cellular membrane potential, permeabilization of epithelial monolayers, apoptosis, detachment of epithelial cells from the basement membrane, and interference with the process of class II antigen presentation (3, 4). Many of these effects depend on the capacity of VacA to form anion-selective membrane channels (10–14). VacA also has been reported to alter the expression of syntaxin 7 (15) and to induce the activation of p38-mediated signaling pathways (16, 17).

H. pylori can persistently colonize the human gastric mucosa for decades despite the development of gastric mucosal inflammation and specific antibody production. Several lines of evidence indicate that CD4$^+$ T cells are critical for protection against *H. pylori* infection (18–21). Thus, it seems possible that immune evasion strategies of *H. pylori* may involve the inhibition or modulation of T cell immunity. Indeed, two reports have recently demonstrated that VacA inhibits activation of Jurkat T cells (a human T cell lymphoma/leukemia cell line) as well as human peripheral blood lymphocytes (17, 22). Studies in Jurkat T cells indicate that VacA blocks activation of the nuclear factor of activated T cells (NFAT), a key transcription factor required for optimal T cell activation (17, 22). The process by which VacA inhibits NFAT activation in Jurkat T cells is reportedly similar to the actions of the immunosuppressive drugs cyclosporine A and FK506, which inactivate the NFAT phosphatase calcineurin (22). However, the process by which VacA inhibits activation of primary human CD4$^+$ T cells has not yet been studied in detail.

In this report, we show that VacA inhibits the proliferation of primary human CD4$^+$ T cells and demonstrate that this inhibitory effect on proliferation is not attributable to VacA effects on NFAT activation or IL-2 expression. In addition, we show that VacA suppresses IL-2-induced cell cycle progression without affecting IL-2-dependent survival. We also show that VacA-mediated inhibition of primary T cell proliferation depends on an intact VacA N-terminal hydrophobic domain required for membrane channel formation, and that a mutant toxin lacking this domain blocks the T cell-suppressive action of wild-type VacA in a dominant-negative manner. We propose that these effects of VacA on T cells contribute to the capacity of *H. pylori* to evade the adaptive immune response and establish persistent infection.

Materials and Methods

Purification of VacA. *H. pylori* strains (wild-type strain 60190 and isogenic mutant strains) were grown as described (11, 12). Oligomeric forms of VacA were purified from broth culture supernatants of *H. pylori* as described (23). All experiments were performed by using acid-activated preparations of VacA (24, 25) or acidified buffer control (PBS), unless stated otherwise. The final VacA concentration was 10 μg/ml for all experiments, unless stated otherwise. For the dominant-negative assays, wild-type VacA was mixed with varying concentrations of VacA mutant toxins, and the mixtures were acid-activated before addition of these samples to cells (11).

Primary Human T Cell Purification and Carboxy Fluorescein Diacetate Succinimide Ester (CFSE) Labeling. Resting CD4$^+$ human T cells were purified from healthy adult donors as described (26). The purified cells were 99% CD3$^+$CD4$^+$ as assessed by staining and

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Abbreviations: VacA, vacuolating toxin; NFAT, nuclear factor of activated T cells; CFSE, carboxy fluorescein diacetate succinimide ester; PMA, phorbol myristate acetate; CBA, cytometric bead array; PI, propidium iodide; T_h, T helper.

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flow cytometric analysis. Cell proliferation was monitored by labeling T cells with 5 μM CFSE (Molecular Probes) before stimulation with α-CD3/α-CD28 antibodies.

Activation of Primary Human T Cells. Activation of T cells was accomplished by using α-CD3 (OKT3, American Type Culture Collection) and α-CD28 antibodies (BD Biosciences, Franklin Lakes, NJ) (hereafter termed TCR/CD28 stimulation) as described (26). Cells were removed from the activation signals after 48 h and expanded in media supplemented with recombinant human IL-2 (Chiron, 200 units/ml) and cultured as described (26). Jurkat T cells were TCR/CD28-stimulated as described above or with phorbol myristate acetate (PMA, 50 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma), and maintained in RPMI media 1640 containing 10% FCS. To inhibit TCR/CD28 stimulation or IL-2-driven stimulation, T cells were treated with cyclosporine A (50 nM, Alexis Biochemicals, Lausen, Switzerland), FK506 (100 nM, Alexis Biochemicals), or rapamycin (200 ng/ml, Alexis Biochemicals), respectively.

Fluorescence-Activated Cell Sorter Analysis and IL-2 Detection. IL-2 receptor (CD25) surface expression was detected by staining with phycoerythrin-conjugated anti-human CD25 (BD Biosciences), as described (26). IL-2 secretion into culture supernatants was determined by using cytometric bead array (CBA) according to the manufacturer's instructions (BD Biosciences) and analyzed by using CBA six-bead analysis software (BD Biosciences). Samples were analyzed on a FACSCalibur (Becton Dickinson) four-color cytometer by using the CELLQUEST program. Live cells were gated based on forward- and side-scatter properties, and analysis was performed by using CELLQUEST software (BD Biosciences), as described (26).

Generation and Use of NFAT Reporter Primary Human T Cells. To generate a primary human $CD4^+$ T cell line that expressed an NFAT transcriptional reporter, three tandem copies of the NFAT-binding site of the human IL-2 promoter were subcloned upstream of the enhanced GFP gene (Clontech) to direct its transcription (27). This NFAT-GFP expression cassette was then cloned into a lentiviral vector in reverse orientation, and VSV-G pseudotyped viruses were generated via cotransfection of 293 T cells, as described (26). Activated primary $CD4^+$ T cells were transduced with these pseudotyped viruses at a suboptimal multiplicity of infection. After 7 days, cells constitutively expressing GFP were removed by fluorescence-activated cell sorting, and GFP-negative cells were TCR/CD28-stimulated for 24 h. NFAT-GFP T cells that up-regulated GFP on activation were positively sorted by flow cytometry and further expanded in IL-2-supplemented media. After enrichment, at least 10% of the NFAT-GFP T cell population displayed GFP-inducible expression on TCR stimulation.

Cell Cycle Analysis of T Cell Proliferation. Day 4 TCR/CD28-stimulated T cells were washed to remove exogenous IL-2 and maintained in IL-2-free medium for 24 h to synchronize cells at phase G_1 of the cell cycle. During this period, the cells were treated with VacA or other additives. After incubation in IL-2-containing medium for the indicated times, cells were subjected to propidium iodide (PI) staining and analyzed by flow cytometry as described (28). To evaluate the kinetics of DNA replication, T cells were washed to remove IL-2 and treated with VacA for 8 h. After VacA treatment, cells were incubated for 24 h in IL-2-containing medium. Then 10 μM BrdUrd was added to the cultures, and cells were harvested at different time points for BrdUrd antibody staining with a commercially available kit (BD Biosciences), according to the manufacturer's instructions.

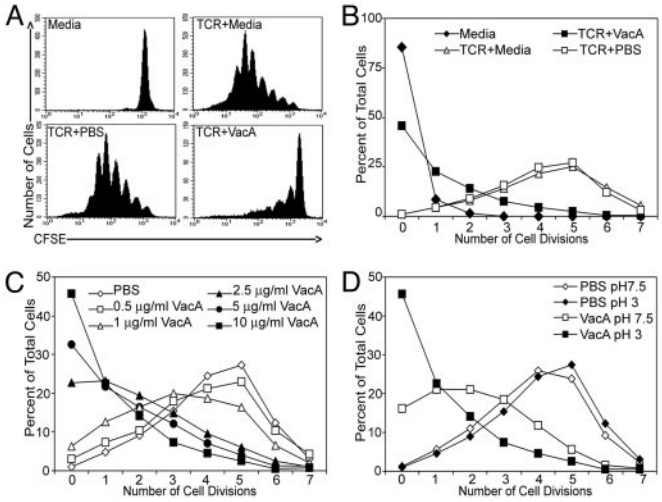

Fig. 1. VacA inhibits activation-induced proliferation of primary human $CD4^+$ T_h cells. (*A*) Purified primary human T_h cells were labeled with CFSE and treated with acid-activated VacA (10 μg/ml), acidified-PBS (PBS), or medium alone for 1 h, followed by TCR/CD28 stimulation for 48 h, as described in *Materials and Methods*. Control cells were treated with medium alone, without TCR/CD28 stimulation. Activated T cells were expanded in IL-2-containing media, and T cell proliferation was analyzed at day 5 postactivation by flow cytometry. (*B*) Graphic representation of the histograms shown in *A*. (*C*) Dose-response analysis of VacA effects on primary human $CD4^+$ T cell proliferation. T_h cells were CFSE-labeled and treated with different concentrations of acid-activated (pH 3) VacA for 1 h. Cells were then stimulated and analyzed as in *A*. (*D*) Effects of acid-activated VacA (pH 3) and nonacid-activated VacA (pH 7.5) on T cell proliferation. T_h cells were CFSE-labeled and treated with acid-activated or nonactivated VacA (10 μg/ml), as described above. All of the results are representative of three experiments using cells from different donors and different toxin preparations.

Results

VacA Inhibits Activation-Induced Proliferation of Primary Human $CD4^+$ T Cells. Previous reports have indicated that VacA inhibits activation of T cells (17, 22). These studies clearly demonstrated that the suppressive effect of VacA on a transformed T cell line (Jurkat T cells) is due to interference with NFAT activation, resulting in the inhibition of IL-2 secretion. Although several experiments were performed on peripheral blood lymphocytes, the mode of VacA action on purified primary human T cells was not assessed (17, 22). To investigate whether VacA inhibits the activation of primary T cells in a manner similar to its effect on transformed cells, we purified $CD4^+$ primary human T helper (T_h) cells from peripheral blood mononuclear cells of healthy individuals and labeled them with CFSE, a cell-permeable dye that allows for the quantification of cell division within a population. CFSE-labeled T_h cells were pretreated with medium, PBS, or VacA, followed by TCR stimulation using α-CD3 and -CD28 antibodies (TCR/CD28 stimulation) for 48 h. After TCR/CD28 stimulation, cells were expanded in IL-2-containing media for an additional 3 days. As expected, T_h cells pretreated with medium or PBS rapidly proliferated, resulting in up to seven divisions when analyzed at 5 days post-TCR/CD28 stimulation (Fig. 1 *A* and *B*). In contrast, treatment with VacA potently inhibited the proliferation of T_h cells (Fig. 1 *A* and *B*) in a dose-dependent manner (Fig. 1*C*). Incubation of purified VacA at acid pH (pH $\leq$4.5; a process termed acid activation) markedly enhances the capacity of the toxin to undergo internalization and cause vacuolating cytotoxic effects in mammalian cells (24, 25). Accordingly, acid activation markedly enhanced the capacity of VacA to inhibit T_h cell proliferation (Fig. 1*D*).

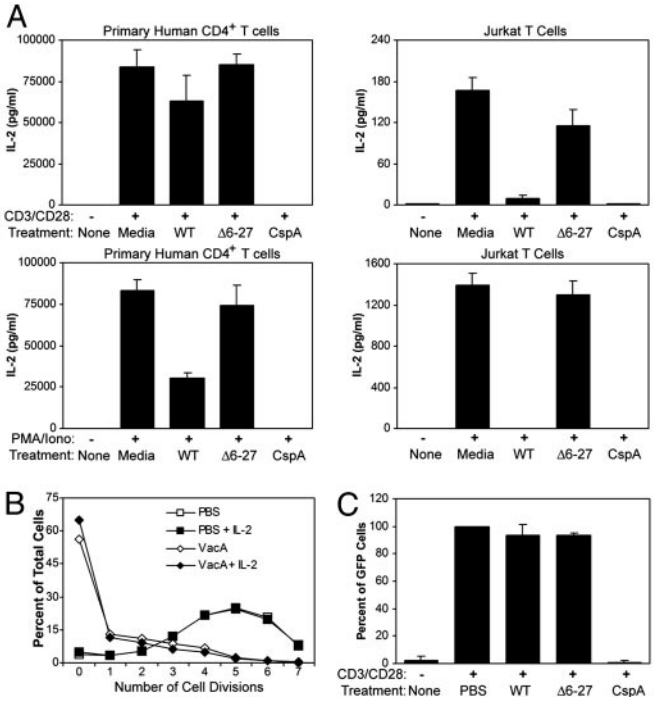

Fig. 2. VacA inhibits activation-induced proliferation of primary human T_h cells independent of effects on IL-2 secretion and NFAT activation. (*A*) Purified primary human T_h cells or Jurkat T cells were pretreated with medium alone, wild-type (WT) VacA, VacA-Δ(6–27), or cyclosporine A (CspA) for 1 h, followed by TCR/CD28 stimulation (CD3/CD28; *Upper*) or stimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) (*Lower*), as indicated. IL-2 secretion was measured at 24 h after stimulation by using a CBA, as described in *Materials and Methods*. Results represent the mean ± SD from triplicate samples. (*B*) Purified primary human T_h cells were CFSE-labeled and pretreated with wild-type VacA or PBS in the presence or absence of supplemental IL-2 (200 units/ml) for 1 h as indicated. Cells were then TCR/CD28 stimulated in the presence or absence of supplemental IL-2 for 48 h, expanded in IL-2-supplemented media, and subjected to flow cytometric analysis at day 5 posttreatment. (*C*) Primary human $CD4^+$ T cells stably transduced with a GFP reporter under the control of NFAT (NFAT-GFP T_h cells; see *Materials and Methods*) were pretreated with different additives as in *A* for 1 h before TCR/CD28 stimulation (CD3/CD28). GFP expression was assessed by flow cytometric analysis 24 h after stimulation. Results represent the mean ± SD from triplicate samples and are expressed as the percentage of cells demonstrating inducible expression of GFP, relative to the PBS-treated cells. CspA, cyclosporine A (50 nM); WT, wild-type VacA toxin (10 μg/ml); and Δ6–27, VacA-Δ(6–27) mutant toxin (10 μg/ml).

Effects of VacA on IL-2 Secretion in Jurkat T Cells and Primary Human T Cells. It has been reported recently that VacA blocks the secretion of IL-2 by mitogen-stimulated Jurkat T cells (22). Therefore, we investigated whether VacA also inhibits IL-2 secretion in primary T_h cells. In agreement with a previous report (22), we found that wild-type VacA potently suppressed IL-2 secretion in Jurkat T cells, very similar to the immunosuppressive drugs cyclosporine A and FK506, regardless of whether the cells were TCR/CD28-stimulated or stimulated with PMA and ionomycin (Fig. 2*A* and data not shown). Notably, a VacA mutant toxin deficient in vacuolating cytotoxic activity [VacA-Δ(6–27)] (11) (Table 1, which is published as supporting information on the PNAS web site) did not inhibit IL-2 secretion in Jurkat T cells (Fig. 2*A*). In contrast to the marked inhibitory effect of wild-type VacA on IL-2 secretion by Jurkat T cells, VacA had only a modest effect on IL-2 secretion by primary human T_h cells (Fig. 2*A*). To investigate whether VacA might selectively inhibit IL-2 secretion in naïve or memory T cell subsets, we compared levels of IL-2 secreted by fluorescence-activated cell-sorted naïve ($CD45RA^+/CD45RO^-$) and memory ($CD45RA^-/CD45RO^+$) $CD4^+$ T cells after treatment with VacA and TCR/CD28 stimulation. VacA treatment resulted in no detectable reduction of IL-2 secretion by either naïve or memory T cell subsets, whereas cyclosporine A treatment effectively inhibited IL-2 secretion by both $CD4^+$ subsets (data not shown). TCR stimulation of T cells induces the expression of both IL-2 and the high-affinity IL-2 receptor α-chain (CD25). Therefore, we next investigated whether VacA treatment inhibited the surface expression of CD25. Primary human T_h cells were pretreated as described above, and CD25 expression was analyzed both at 24 h and 5 days after TCR stimulation by flow cytometric analysis. No significant difference was detected in the CD25 expression of VacA-treated primary human T cells and cells treated with PBS or medium (data not shown). To investigate whether the lack of proliferation of VacA-treated primary T_h cells was due to insufficient IL-2 levels in the medium (Fig. 2), we further supplemented these cultures with saturating concentrations of exogenous recombinant IL-2 (200 units/ml) during TCR/CD28 stimulation. VacA potently inhibited the proliferation of primary T_h cells, even in the presence of excess exogenous IL-2 (Fig. 2*B*). Thus, we conclude that the VacA-mediated inhibition of primary human T_h cell proliferation cannot be attributed to a reduction in IL-2 secretion.

VacA Inhibits Primary Human T Cell Proliferation Through an NFAT-Independent Mechanism. VacA has been shown to inhibit NFAT activation in Jurkat T cells, resulting in a loss of IL-2 secretion (17, 22). However, because VacA has only a modest effect on IL-2 secretion in primary T cells (Fig. 2*A*), we hypothesized that VacA-mediated inhibition of primary human T cell proliferation may occur via an NFAT-independent mechanism. To test this hypothesis, we developed a primary human T_h cell stably transduced with a lentiviral vector in which the NFAT-binding site of the IL-2 promoter drives the expression of the GFP (NFAT-GFP T cells). NFAT-GFP T cells were treated with wild-type VacA, PBS, cyclosporine A, FK506, or VacA-Δ(6–27) for 1 h before TCR/CD28 stimulation. GFP expression was then analyzed 24 h poststimulation via flow cytometric analysis. As expected, GFP expression was induced upon TCR/CD28 stimulation of NFAT-GFP T cells (Fig. 2*C*). In the presence of cyclosporine A or FK506, which are potent inhibitors of NFAT activation, stimulated NFAT-GFP T cells did not express GFP (Fig. 2*C* and data not shown). Stimulated cells treated with VacA expressed GFP similar to the PBS-treated cells, regardless of whether the primary cells were TCR/CD28-stimulated or stimulated with PMA/ionomycin (Fig. 2*C* and data not shown). These results, taken together with our findings that VacA causes only modest effects on IL-2 secretion in primary T_h cells, suggest that VacA can inhibit proliferation of primary human T_h cells via an NFAT-independent mechanism.

VacA Inhibits IL-2-Driven Proliferation of Primary Human T_h Cells but Not IL-2-Dependent Survival. To gain further insight into the mechanism used by VacA to suppress activation-induced proliferation of primary T cells, we investigated the kinetics of VacA-mediated effects. CFSE-labeled primary human T_h cells were TCR/CD28-stimulated as described and treated with PBS, wild-type VacA, or VacA-Δ(6–27) at different time points after stimulation. T cell proliferation was assessed 5 days after stimulation by flow cytometric analysis. VacA inhibited proliferation of T_h cells even when added 48 h after stimulation, suggesting that VacA effects are largely independent of early TCR/CD28 signals (Fig. 3*A*). In contrast, cyclosporin A and FK506 completely inhibited T_h cell proliferation when added within the first 24 h but had little effect when added 48 h after TCR/CD28 stimulation (data not shown).

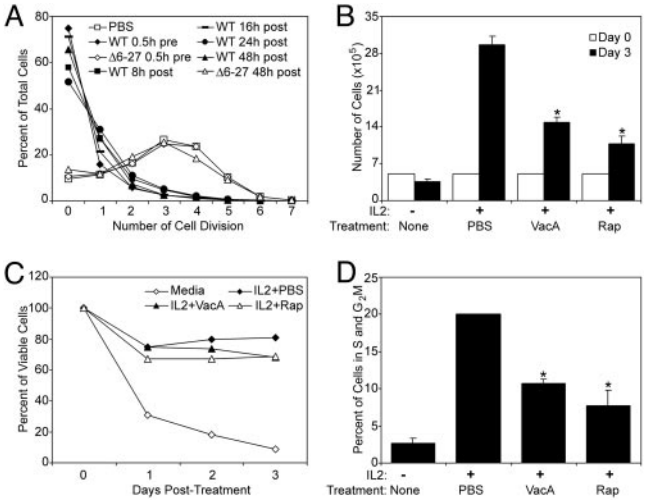

Fig. 3. VacA inhibits IL-2-driven proliferation of primary human T_h cells. (*A*) Wild-type (WT) VacA (10 μg/ml) or VacA-Δ(6–27) (10 μg/ml) were added to CFSE-labeled purified primary human $CD4^+$ T cells at the indicated time points either preceding or after (pre- or post-) TCR/CD28 stimulation. Activated cells were expanded in IL-2-containing media, and cell proliferation was analyzed by flow cytometry at day 5 after stimulation. (*B*) Primary human T_h cells were TCR/CD28 stimulated for 48 h and expanded in the presence of IL-2 for 2 additional days. At day 4 after stimulation, T cells were removed from IL-2 and treated with PBS, wild-type VacA (10 μg/ml), or rapamycin (Rap; 200 ng/ml) for 24 h. After 24 h, IL-2 was added back to the media as indicated, and cells were treated again with the different additives and expanded in fresh media containing supplemental IL-2 for 3 days. Cell proliferation was assessed by cell counting with a hemacytometer. Results represent the mean ± SD from triplicate samples. *, $P < 0.001$ when compared with the PBS-treated cells. (*C*) Viability of T_h cells was determined by flow cytometric gating for viable cells based on forward and side scatter properties at days 1, 2, and 3 after TCR/CD28 stimulation. (*D*) For cell-cycle analysis, primary human T_h cells were treated as described in *B*. Cell-cycle distribution was analyzed at 36 h after IL-2 stimulation by using PI staining and flow cytometry analysis, as described in *Materials and Methods*. The percentages of total cells in S phase and G_2M phase are shown. Results represent the mean ± SD from triplicate samples. *, $P < 0.01$ when compared with the PBS-treated cells. Results are representative of at least two experiments using cells from different donors and different toxin preparations.

We next tested whether VacA blocked IL-2-dependent proliferation of primary T cells at later time points (96 h) after stimulation, a stage in which T cell proliferation and survival depend solely on IL-2 signals (29). For these experiments, T_h cells were TCR/CD28-stimulated for 48 h and expanded in the presence of IL-2 for an additional 2 days. At day 4 postactivation, T cells were removed from IL-2 and treated with VacA or other additives for 24 h. IL-2 was then added back to the medium, and cells were expanded for an additional 3 days. Cell counts were performed to assess cellular proliferation at day 3 after IL-2 stimulation. As expected, activated T cells treated with PBS and stimulated with IL-2 proliferated ≈6-fold from day 0 to day 3, and no proliferation was observed in the absence of IL-2 (Fig. 3*B*). In the presence of VacA, however, T cell numbers increased only ≈3-fold ($P < 0.001$), similar to cells treated with rapamycin (Fig. 3*B*), an immunosuppressive drug that blocks IL-2-driven proliferation of T cells (30, 31). Similar results were obtained when proliferation was assessed via CFSE fluorescence (data not shown). These data indicate that VacA inhibits IL-2-driven proliferation of activated primary human T_h cells.

IL-2 signals are required not only for activation-induced T cell proliferation but also for survival of these cells (29). Therefore, we also monitored the viability of the activated T_h cells cultured in the presence or absence of VacA. As expected, the majority of activated T cells (>90%) incubated without IL-2 for 3 days underwent apoptosis (Fig. 3*C*). In contrast, VacA treatment did not result in a significant increase in cell death (Fig. 3*C*), suggesting that VacA inhibits IL-2-driven proliferation without altering IL-2-dependent survival.

VacA Attenuates IL-2-Dependent Cell Cycle Progression in Primary Human T_h Cells. To determine whether VacA-mediated inhibition of IL-2-driven proliferation is due to a perturbation of cell cycle progression, we assessed cell cycle progression of activated T cells in the presence of VacA. Primary human T_h cells were TCR/CD28-stimulated and expanded in IL-2-containing medium as described above. Day 4-activated T_h cells were removed from IL-2 for 24 h to induce G_1-phase cell-cycle arrest (32) and were then treated with VacA or other additives. IL-2 was then added back to the medium, and cell cycle distribution was analyzed at different time points through PI staining. The activated T_h cells arrested in G_1-phase reentered the cell cycle ≈24–36 h after IL-2 stimulation, as seen by the increased number of T_h cells in S and G_2M phases (Fig. 3*D*). In contrast, cells treated with either rapamycin (which inhibits IL-2-induced cell cycle progression by arresting the cells at the G_1 phase) (30, 31) or VacA displayed impaired IL-2-induced cell cycle progression (Fig. 3*D*). We also determined the rate of DNA synthesis in VacA-treated T_h cells by assessing incorporation of BrdUrd, a thymidine analog that is incorporated into newly synthesized DNA during S phase. VacA-treated activated T cells were ≈2-fold less efficient in the rate of BrdUrd uptake when compared with PBS-treated control cells (data not shown), thus corroborating the PI results (Fig. 3*D*). Taken together, these data provide strong evidence that VacA inhibits IL-2-driven proliferation in activated primary human T_h cells by suppressing cell cycle progression.

The N-Terminal Hydrophobic Domain of VacA Is Required for Inhibition of Primary Human T_h Cell Proliferation. Structure–function analyses have revealed that an intact structure of a hydrophobic domain within the VacA N-terminal region is required for the formation of anion-selective membrane channels (Table 1) (10–14). Because VacA-Δ(6–27), a VacA mutant that lacks the entire hydrophobic domain, did not suppress either proliferation of primary T_h cells (Fig. 3*A*) or IL-2 secretion in Jurkat T cells (Fig. 2*A*), we hypothesized that the formation of VacA anion-selective channels may play an important role in the process by which VacA inhibits proliferation of activated T cells. To further test this hypothesis, we examined the effects of two VacA mutant toxins that contain single amino acid substitutions in the hydrophobic domain of VacA (VacA-P9A and VacA-G14A) (12) on proliferation of primary human T_h cells. These VacA mutants proteins, including VacA-Δ(6–27), are defective in channel-forming activity but retain other structural and functional characteristics of the wild-type VacA protein, including the ability to form oligomeric structures and the capacity to bind and enter cells (Table 1) (10–12). In contrast to wild-type VacA, these mutant proteins did not cause any detectable inhibition of T cell proliferation (Fig. 4). These data indicate that an intact VacA N-terminal hydrophobic domain is required for VacA-mediated inhibition of T cell proliferation and suggest that the formation of VacA anion-selective membrane channels is important for the suppression of activation-induced proliferation of primary T_h cells.

VacA-Δ(6–27) Inhibits the Immunosuppressive Effects of Wild-Type VacA on T Cells. In studies of VacA-induced effects on HeLa cells and AGS cells (a human gastric epithelial cell line), the VacA mutant toxin [VacA-Δ(6–27)] has been reported to exhibit a dominant-negative phenotype (11). When mixed in an equimolar ratio with wild-type VacA, VacA-Δ(6–27) blocks the capacity of wild-type VacA to cause cell vacuolation (11), form anion-

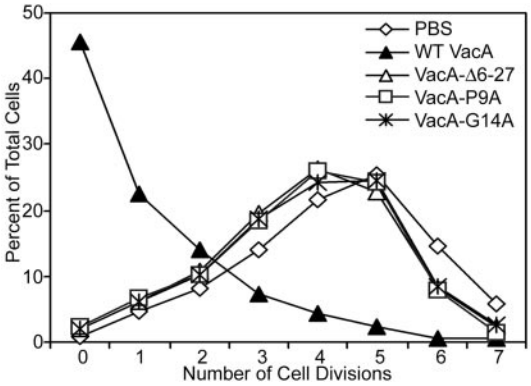

Fig. 4. Analysis of VacA mutant proteins demonstrates that an intact N-terminal hydrophobic domain is required for VacA-mediated effects on T cell proliferation. Purified primary human T_h cells were CFSE-labeled and treated with wild-type VacA (WT VacA, 10 μg/ml), one of three different mutant toxins (each 10 μg/ml), or PBS for 1 h. Cells were then TCR/CD28 stimulated for 48 h, expanded in IL-2-containing media, and analyzed by flow cytometry at day 5 after stimulation, as described in *Materials and Methods*. Results are representative of three experiments using cells from different donors and different toxin preparations.

selective membrane channels (11), induce cytochrome *c* release (10), and induce apoptosis (33). The inhibitory actions of VacA-Δ(6–27) are thought to be due to the formation of inactive mixed-oligomeric complexes, comprised of both wild-type and mutant toxin (11, 34, 35). To investigate whether VacA-Δ(6–27) could block the actions of wild-type VacA on T_h cells, the two toxins were mixed and added at various stoichiometric ratios to CFSE-labeled resting T_h cells. CFSE-labeled primary human T_h cells were subsequently TCR/CD28-stimulated, and proliferation was evaluated by flow cytometric analysis 5 days poststimulation. VacA-Δ(6–27) potently blocked wild-type VacA-mediated inhibition of T cell proliferation in a dominant-negative fashion, because it was partially effective even in the presence of 20-fold molar excess of wild-type VacA (Fig. 5*A*). VacA-Δ(6–27) also blocked the inhibitory effects of wild-type VacA on IL-2 secretion in Jurkat T cells, regardless of whether the cells were TCR/CD28-stimulated or stimulated with PMA/ionomycin (Fig. 5*B* and data not shown). Importantly, the dominant-negative phenotype exhibited by the VacA-Δ(6–27) mutant toxin was specific for the Δ6–27 mutation, because two

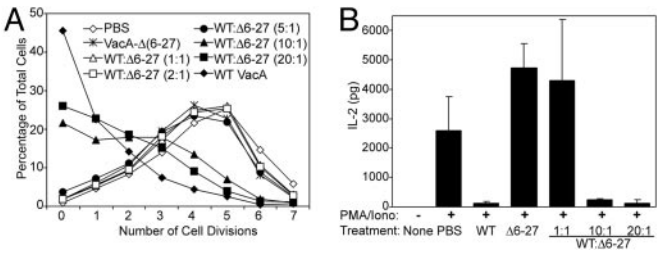

Fig. 5. Effects of a dominant-negative mutant VacA toxin. (*A*) Primary human T_h cells were CFSE-labeled and then treated for 1 h with wild-type VacA (10 μg/ml) and VacA-Δ(6–27) in different ratios (WT:Δ6–27) as indicated. Cells were then TCR/CD28 stimulated and expanded in IL-2-supplemented media, as described in *Materials and Methods*. Cell proliferation was analyzed by flow cytometric analysis on day 5 after stimulation. (*B*) Jurkat T cells were pretreated with wild-type VacA and VacA-Δ(6–27) as in *A* for 1 h as indicated, activated with PMA (50 ng/ml) and ionomycin (500 ng/ml) and then incubated for 24 h. Culture supernatants were assayed for IL-2 secretion (pg/ml) by using a CBA assay as described in *Materials and Methods*. Results are representative of three experiments using cells from different donors, different preparations of Jurkat T cell lines, and different toxin preparations.

other mutant toxins containing point mutations within the VacA N-terminal hydrophobic domain (P9A and G14A) did not exhibit a dominant-negative phenotype (data not shown).

Discussion

Here we show that VacA inhibits the proliferation of TCR/CD28-stimulated primary human $CD4^+$ T_h cells (Fig. 1). In addition, our data demonstrate a qualitative difference between VacA-mediated effects on IL-2 secretion by Jurkat T cells and primary human T_h cells (Fig. 2). In contrast to the potent inhibitory effects of VacA on IL-2 secretion in Jurkat T cells (Fig. 2*A*) (17, 22), VacA-treated primary T cells retain the capacity to secrete high levels of IL-2 (Fig. 2*A*). The suppressive effect of VacA on Jurkat T cell proliferation has previously been attributed to suppression of IL-2 expression, occurring at the level of transcriptional regulation due to NFAT inhibition (17, 22). In contrast, our results provide evidence that VacA-mediated inhibition of primary human T_h cell proliferation occurs at a later stage of T cell activation, wherein the immunosuppressive drugs cyclosporine A and FK506 (NFAT-activation inhibitors) are no longer inhibitory (Fig. 3 *A* and *B* and data not shown). Specifically, we show that VacA impairs IL-2-driven cell-cycle progression in activated primary human T_h cells (Fig. 3*B*), resulting in an inhibition of IL-2-driven proliferation. This effect of VacA resembles the actions of the immunosuppressive drugs rapamycin and sanglifehrin A (31, 36), two known inhibitors of IL-2-driven proliferation.

We also show, based on analysis of single-point mutant toxins, that the inhibitory effects of VacA on primary human T_h cell proliferation and IL-2 secretion in Jurkat T cells depend on the integrity of the VacA N-terminal hydrophobic domain, which is required for formation of membrane channels (Fig. 4). This provides strong evidence that VacA-mediated effects on T cell proliferation depend on the formation of membrane channels. This conclusion is consistent with data reported by Boncristiano *et al*. (17), who showed that various VacA effects on Jurkat T cells could be blocked by NPPB, a nonspecific chloride channel inhibitor. One of the VacA mutant toxins in the current study, VacA-Δ(6–27) (11), potently blocked the effects of wild-type VacA on T cells (Fig. 5). This dominant-negative mutant VacA protein interacts with wild-type toxin, resulting in the formation of nonfunctional mixed-oligomeric structures (11, 34). Inhibition of wild-type VacA activity by a dominant-negative mutant protein is consistent with a model in which the formation of oligomeric VacA structures, such as membrane channels, is required for VacA-induced effects on T cells. We hypothesize that formation of anion-selective VacA membrane channels (13, 14) induces membrane depolarization of T cells, and that this phenomenon is mechanistically important in the inhibition of IL-2-dependent T cell proliferation. Consistent with this view, Boncristiano *et al*. (17) recently reported that VacA could block ionophore-stimulated influx of calcium into T cells. Furthermore, a previous study demonstrated that activation of glycine-gated chloride channels induces membrane depolarization of T cells, resulting in a decreased open probability of plasma membrane calcium channels and in the inhibition of IL-2-dependent proliferation without affecting IL-2 secretion or NFAT activation (37).

The current results, along with two recent studies (17, 22), provide evidence that VacA interferes with the proliferation of T cells via multiple mechanisms. VacA is capable of inhibiting T cell activation by blocking the activation of NFAT (17, 22), and we now show that VacA is also capable of inhibiting IL-2-driven proliferation of T cells. In addition to these effects, VacA has been reported to interfere with T cell activation through a channel-independent mechanism that involves activation of intracellular signaling through the mitogen-activated protein kinases MKK3/6 and p38 and the Rac-specific nucleotide ex-

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change factor, Vav (17). Together, these findings suggest that the effects of VacA on the immune system are likely to be multifactorial and more complex than initially thought.

Gastric biopsies from *H. pylori*-infected individuals consistently demonstrate infiltration of $CD4^+$ T_h cells (20, 21), and specific anti-*H. pylori* T cells have been detected in the gastric mucosa (21, 38). Nevertheless, *H. pylori* is able to evade the immune response and establish persistent infection. Experiments in mice indicate that the quality of the T_h cell response is critically important for eradication of *H. pylori* and prevention of *H. pylori*-induced pathology (18, 19). We propose that VacA might inhibit the clonal expansion and thus the acquisition of effector functions of infiltrating T_h cells that have already been activated by *H. pylori* antigens. This immunosuppressive activity of VacA is likely to play an important role in the process by which *H. pylori* evades the adaptive immune response.

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