

# *Helicobacter pylori* Membrane Vesicles Stimulate Innate Pro- and Anti-Inflammatory Responses and Induce Apoptosis in Jurkat T Cells

Jody Winter,\* Darren Letley, Joanne Rhead, John Atherton, Karen Robinson

Nottingham Digestive Diseases Centre Biomedical Research Unit, School of Clinical Sciences, Centre for Biomolecular Sciences, The University of Nottingham, Nottingham, United Kingdom

**Persistent *Helicobacter pylori* infection induces chronic inflammation in the human gastric mucosa, which is associated with development of peptic ulceration, gastric atrophy, and gastric adenocarcinoma. It has been postulated that secretion of immunomodulatory molecules by *H. pylori* facilitates bacterial persistence, and membrane vesicles (MV), which have the potential to cross the gastric epithelial barrier, may mediate delivery of these molecules to host immune cells. However, bacterial MV effects on human immune cells remain largely uncharacterized to date. In the present study, we investigated the immunomodulatory effects of *H. pylori* MV with and without the vacuolating cytotoxin, VacA, which inhibits human T cell activity. We show a high degree of variability in the toxin content of vesicles between two *H. pylori* strains (SS1 and 60190). Vesicles from the more toxigenic 60190 strain contain more VacA (s1i1 type) than vesicles from the SS1 strain (s2i2 VacA), but engineering the SS1 strain to produce s1i1 VacA did not increase the toxin content of its vesicles. Vesicles from all strains tested, including a 60190 isogenic mutant null for VacA, strongly induced interleukin-10 (IL-10) and IL-6 production by human peripheral blood mononuclear cells independently of the infection status of the donor. Finally, we show that *H. pylori* MV induce T cell apoptosis and that this is enhanced by, but not completely dependent on, the carriage of VacA. Together, these findings suggest a role for *H. pylori* MV in the stimulation of innate pro- and anti-inflammatory responses and in the suppression of T cell immunity.**

*Helicobacter pylori* is a Gram-negative microaerophilic bacterium that colonizes the stomachs of around half the world's population. Infection persists lifelong if untreated, stimulating chronic inflammation of the gastric mucosa (1, 2). Host, environmental, and bacterial factors affect disease risk, with carriage of more virulent bacterial strains associated with a higher incidence of ulceration and cancer (1, 2).

*H. pylori* produces multiple virulence factors, including the vacuolating cytotoxin VacA, which is polymorphic in its signal (s), intermediate (i), and middle (m) regions. The s1, i1, and m1 alleles confer higher toxin activity and broader cell specificity, while the s2, i2, and m2 alleles are less toxigenic (3–5). Carriage of strains producing more toxigenic forms of VacA is associated with a higher disease incidence (5, 6).

*H. pylori* is known to have profound effects on gastric epithelial cells, including stimulation of interleukin-8 (IL-8) production (7, 8), and toxic effects of VacA on epithelial cells are now well characterized (9). *In vitro*, the toxin induces vacuolation (10) and apoptosis (11–14) in epithelial cell lines, and oral administration of purified VacA causes epithelial damage in experimental animals (15–17).

During persistent colonization, *H. pylori* exerts both pro- and anti-inflammatory effects on the human immune system (18–21). However, since the majority of bacteria remain in the mucus overlying the gastric epithelial cell layer in the stomach lumen (22), it is unclear how bacteria or bacterial products cross this barrier to access cells of the host immune system and stimulate these responses. Secreted proteins might be readily broken down in the harsh gastric environment and might access host cells in relatively small quantities. VacA is now known to inhibit T cell proliferation and IL-2 production (23–27), but while these effects are striking *in vitro*, the secreted toxin would likely be present in the stomach lumen at low concentrations, and whole bacteria (carrying VacA

on their surfaces) are not generally thought to penetrate the epithelial barrier.

In common with other Gram-negative bacteria, *H. pylori* constitutively produces membrane vesicles (MV) (28, 29). These 20- to 200-nm diameter “blebs” from the outer surface of the bacterium contain mainly outer membrane and periplasmic components, including lipopolysaccharide (LPS), peptidoglycan, and proteins (30, 31). *H. pylori* MV are known to carry VacA and can deliver active toxin to epithelial cells, although the toxin is also secreted conventionally (29). Independently of VacA status, and in common with MV from other bacteria, *H. pylori* MV also stimulate proliferation, IL-8 secretion, and apoptosis in epithelial cells (32, 33). There is growing interest in the role of membrane vesicles in bacterial pathogenesis, both in *H. pylori* and more widely. However, MV-mediated bacterial effects on cells of the host immune system are poorly understood to date. *H. pylori* is known to secrete multiple immune-modulatory proteins, and membrane vesicle-mediated delivery of these might represent a possible route by which *H. pylori* can exert long-range effects on host immune cells,

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Address correspondence to Jody Winter, jody.winter@ntu.ac.uk.

\* Present address: Jody Winter, School of Science and Technology, Clifton Campus, Nottingham Trent University, Nottingham, United Kingdom.

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delivering discrete concentrated packages of a “cocktail” of *H. pylori* molecules, including VacA and other virulence factors.

In the present study, we set out to characterize the effects of *H. pylori* MV on human immune cells and to determine which, if any, are due to the carriage of VacA. We show that there are substantial differences in the quantity of VacA associated with MV between the more toxigenic 60190 strain and less toxigenic SS1. *H. pylori* MV from both strains strongly stimulate release of pro- and anti-inflammatory cytokines from human peripheral blood mononuclear cells (PBMCs), independently of VacA, and induce apoptosis in T cells. Toxic effects on T cells are enhanced by, but not dependent on, the presence of VacA.

## MATERIALS AND METHODS

***H. pylori* strains.** The strains used in this study were 60190 (ATCC 49503, VacA s1i1 m1), SS1<sup>WT</sup> (VacA s2i2 m2) (34), 60190  $\Delta$ vacA (35), and SS1<sup>s1i1</sup>. The SS1<sup>s1i1</sup> mutant was generated by replacing the s2i2-carrying region of SS1 wild-type vacA with the s1i1-carrying region from strain 60190 by natural transformation with plasmid pJR100 and allelic exchange by homologous recombination and chloramphenicol resistance marker rescue following established methods (5). The SS1 vacA open reading frame was replaced with the 60190 sequence (GenBank accession no. U05676) up to nucleotide 1684.

**MV purification.** *H. pylori* strains from frozen stocks were grown for 24 h on blood agar base 2 plates containing 7% (vol/vol) defibrinated horse blood (Oxoid Ltd., Basingstoke, United Kingdom) at 37°C in a microaerobic cabinet and then passaged once onto fresh blood plates and grown for a further 24 h. Bacteria were then inoculated into 500 ml brain heart infusion broth (Oxoid Ltd., Basingstoke, United Kingdom) containing 0.2%  $\beta$ -cyclodextrin and shaken at 150 rpm under microaerophilic conditions for 48 h. Bacterial cells were removed by centrifugation at 6,000  $\times$  g for 10 min and confirmed helical by Gram staining and microscopy. The culture supernatants were passaged sequentially through 0.45- $\mu$ m and then 0.20- $\mu$ m syringe filters. MV were precipitated at room temperature using 40% (wt/vol) ammonium sulfate for 1 h and then harvested by centrifugation at 10,000  $\times$  g for 15 min. The pellet containing MV was resuspended in 20 ml low-endotoxin Dulbecco’s phosphate-buffered saline (PBS) (Sigma-Aldrich Co. Ltd., Gillingham, Dorset, United Kingdom) and MV harvested by ultracentrifugation at 100,000  $\times$  g for 2 h at 4°C. The pellet containing purified MV was resuspended in 500  $\mu$ l PBS, adjusted to a 500- $\mu$ g/ml total protein concentration following quantification using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA), and stored at -20°C until use.

**TEM.** All MV preparations were confirmed to be free of bacterial cells and flagella by transmission electron microscopy (TEM). MV samples on 200-mesh Formvar-copper grids (Agar Scientific) were negatively stained using 0.5% uranyl acetate and visualized using a Fei Tecnai Biotwin electron microscope.

**Preparation of bacterial water extracts.** *H. pylori* strains were grown for 24 h on blood agar base 2 plates as described above, and then bacteria were harvested into 2 ml PBS, pelleted by centrifugation, and resuspended in 200  $\mu$ l sterile distilled water (dH<sub>2</sub>O). After vigorous vortexing, bacterial suspensions were incubated at room temperature for 30 min and then centrifuged at 10,000  $\times$  g for 5 min. Supernatants were stored at -20°C until use.

**SDS-PAGE and Western blotting.** MV proteins (5  $\mu$ g/lane) were reduced by boiling in buffer containing SDS and dithiothreitol (DTT) and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membrane, and probed with rabbit polyclonal anti-VacA p33 and anti-VacA p55 sera (each at 1:10,000) (generated in-house at The University of Nottingham Biomedical Services Unit) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:10,000). Bands were

visualized using Amersham ECL Prime Western blotting reagent (GE Healthcare).

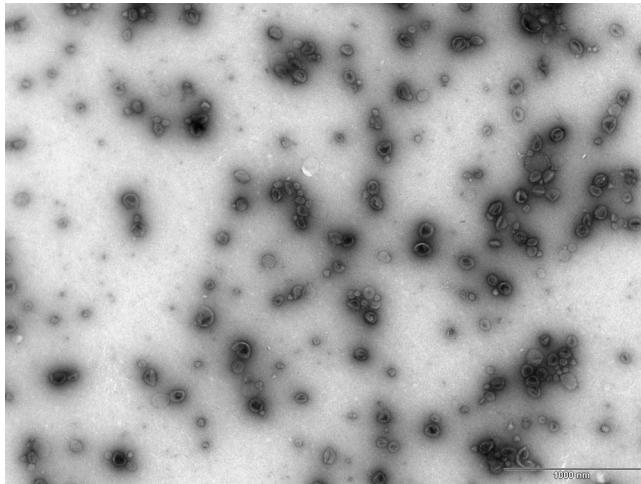
**Recombinant VacA purification.** Sequences encoding the p33 and p55 subunits of VacA were cloned from 60190 genomic DNA, recombinantly expressed in *Escherichia coli*, and purified essentially following the methods of González-Rivera et al. (2010) (36) except that both p33 and p55 domains were purified under denaturing conditions and refolded by dialysis prior to reconstitution of the toxin. The reconstituted toxin was highly active, inducing profound vacuolation of AGS cells and inhibition of Jurkat proliferation and IL-2 secretion, consistent with previously published reports (36).

**Vacuolation assay.** RK13 cells (ATCC CCL-37, a rabbit kidney cell line) were maintained in Ham’s F-12 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Sigma-Aldrich Co. Ltd., Gillingham, Dorset, United Kingdom). Cells were seeded into 96-well tissue culture-treated flat-bottom plates (Nalge Nunc International, Thermo Fisher Scientific Inc.) at  $1 \times 10^4$ /well and treated with 0 to 4  $\mu$ g/ml purified recombinant VacA, 50  $\mu$ g/ml MV, or appropriate buffer controls in the presence of 10 mM NH<sub>4</sub>Cl for 4 h. Vacuolation extent was assessed by light microscopy, with the mean proportion of vacuolated cells from 4 or 5 randomly selected microscopy fields across duplicate wells calculated for a minimum of 100 cells per condition. Representative data from two independent replicate experiments are shown.

**Stimulation of PBMCs by MV.** Blood was collected from patients into EDTA Vacutainers (Greiner Bio-One, Stonehouse, United Kingdom). Patients were attending the Queen’s Medical Centre, Nottingham, for routine upper gastrointestinal endoscopy, usually for investigation of dyspepsia, but were otherwise healthy. Written informed consent was obtained from all patients under the approval of the Nottingham Research Ethics Committee 2. Male and female patients with an age range of 17 to 72 (mean age, 45) were included. *H. pylori* infection status was determined by rapid urease test and bacterial culture from gastric biopsy specimens taken during endoscopy. For healthy donor studies, blood was taken from healthy volunteers of unknown *H. pylori* infection status, with written informed consent, also under approval from the University of Nottingham Medical School Ethics Committee. Blood cells were separated from plasma by centrifugation (725  $\times$  g, 10 min) and resuspended in washing medium (15 ml RPMI 1640 supplemented with 2% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine [all from Sigma]). The cell suspension was carefully layered on 5 ml Histopaque 1077 (Sigma) and centrifuged at 725  $\times$  g for 20 min. PBMCs were collected from the interface between the Histopaque and RPMI layers and washed twice by suspension in washing medium and centrifugation at 400  $\times$  g for 5 min. Purified PBMCs were suspended in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine, and cell viability was confirmed to be >99% by trypan blue staining and microscopy. PBMCs at  $1 \times 10^6$ /ml were coincubated in sterile 96-well plates with 0 to 25  $\mu$ g/ml MV or medium/buffer negative controls at 37°C in 5% CO<sub>2</sub> for 24 or 48 h as indicated. The T cell mitogen concanavalin A was used at 5  $\mu$ g/ml as a positive control. Cytokine concentrations in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (eBioscience) following the manufacturer’s instructions. ELISA sensitivity limits were calculated as means from triplicate wells containing no cytokine plus 3 times the standard deviation (SD) and were <3.2 pg/ml (IL-10), <5 pg/ml (IL-6 and IL-2), <1 pg/ml (IL-4 and IL-12p70), and <4 pg/ml (gamma interferon [IFN- $\gamma$ ]).

**Purification of CD4<sup>+</sup> cells from human blood.** CD4<sup>+</sup> cells were purified from healthy volunteer PBMCs using an EasySep Negative Selection human CD4<sup>+</sup> T cell enrichment kit (Stemcell Technologies) following the manufacturer’s instructions.

**T cell inhibition and apoptosis assays.** Jurkat T cells (a generous gift from R. McIntosh, Academic Clinical Oncology Department, University of Nottingham) were maintained in RPMI 1640 supplemented with 2 mM L-glutamine and 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub>.



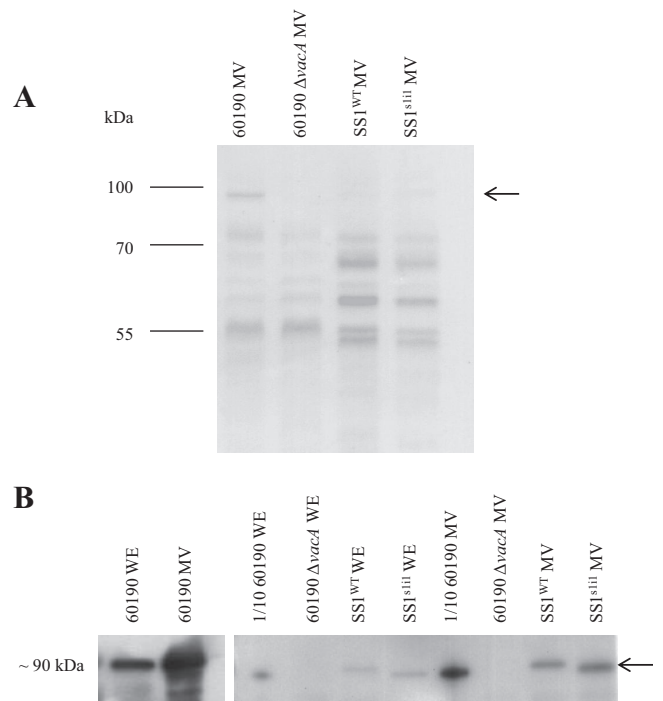
**FIG 1** Electron micrograph showing the size distribution, morphology, and purity of 60190 MV. Purified MV were diluted negatively stained using 0.5% uranyl acetate and visualized using a Tecnai FEI electron microscope to confirm vesicle purity. All MV were free from bacteria, flagella, and other bacterial debris. A representative image of 60190 MV at a magnification of  $\times 20,500$  is shown. Scale bar, 1,000 nm.

For inhibition assays, Jurkat or CD4<sup>+</sup> T cells enriched from PBMCs at  $1 \times 10^6$ /ml and 200  $\mu$ l per well in a sterile 96-well plate were pretreated for 1 h with MV, recombinant VacA, or appropriate buffer controls at the indicated concentrations and then stimulated with 50 ng/ml phorbol myristate acetate (PMA) and 1  $\mu$ M ionomycin for 48 h at 37°C in 5% CO<sub>2</sub>. To determine the extent of proliferation, CellTiter 96 Aqueous One reagent (Promega) was used following the manufacturer's instructions. This reagent, based on the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, causes a color change directly proportional to the number of metabolically active cells in the solution. The IL-2 concentration in each culture supernatant was quantified by ELISA (eBioScience). For apoptosis, viability, and cytotoxicity assays, Jurkat or CD4<sup>+</sup> T cells enriched from PBMCs at  $1 \times 10^5$ /ml and 100  $\mu$ l per well in a sterile 96-well plate were incubated for 24 h at 37°C in 5% CO<sub>2</sub> in the presence of MV, recombinant VacA, or appropriate buffer controls at the indicated concentrations. Proteinase K-treated MV (and an equivalent buffer-only control) were preincubated with 100  $\mu$ g/ml proteinase K for 60 min at 37°C following the methods of Bomberger et al. (37), and then proteinase K was inactivated using P1860 protease inhibitor cocktail (Sigma) before adding the MV to the T cells. An ApoTox-Glo triplex assay (Promega) was used following the manufacturer's instructions to simultaneously determine viability (live-cell protease activity, resulting in a fluorescent signal proportional to the number of living cells per well), cytotoxicity (dead-cell protease activity, resulting in a fluorescent signal proportional to the number of cells that have lost membrane integrity per well), and apoptosis (a luminescent signal proportional to caspase 3/7 activity per well). Data for the first two parameters were combined to give a dead/live cell, or cytotoxicity/viability, ratio for each well.

**Statistical analysis.** Data were analyzed by one-way analysis of variance and Tukey's posttest unless otherwise stated. Graphs show representatives of two to four independently repeated experiments, carried out in triplicate unless otherwise stated, and error bars show standard deviations for replicates.

## RESULTS

*H. pylori* MV were harvested from filtered culture supernatants by ultracentrifugation. Before determining their effects on immune cells, we characterized them by TEM, SDS-PAGE, and Western blotting to quantify VacA.

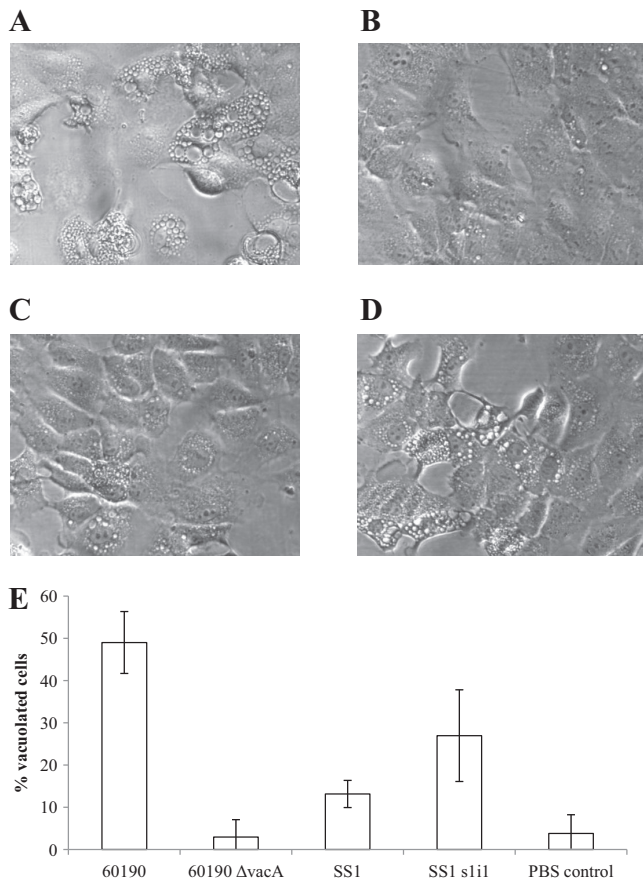


**FIG 2** Comparison of protein profiles and VacA contents in membrane vesicles (MV) from 60190 and SS1 strains. Reduced proteins (5  $\mu$ g/lane) were separated on 12% SDS-polyacrylamide gels and Coomassie blue stained (A) or transferred onto nitrocellulose and immunoblotted for VacA (B). Isogenic mutant MV contained proteins similar to those in MV purified from their respective parental strains, but MV from the 60190 and SS1 strain backgrounds had different protein profiles. VacA (arrow) was barely detectable in MV from the SS1 strains but present in much larger quantities in 60190 MV. For comparison, water extracts (WE) from whole bacteria are shown. High VacA quantities in 60190 samples caused overexposure of blots, so 0.5- $\mu$ g/lane (1/10) 60190 samples were analyzed directly alongside SS1 samples, and 5- $\mu$ g/lane 60190 samples developed in parallel on separate film are also shown (left).

**MV with or without VacA have similar morphologies and are produced in similar quantities.** Strains 60190 (expressing sl11m1-type VacA) and an isogenic mutant null for VacA (69010  $\Delta$ vacA) produced similar quantities of MV in liquid culture. The yield was approximately 3.8 mg MV protein per liter culture after 48 h of culture (final optical density at 600 nm [OD<sub>600</sub>], 0.32 to 0.39). Strains SS1<sup>WT</sup> (expressing s2i2m2 VacA) and SS1<sup>sl11</sup> (an isogenic mutant engineered to express sl11m2 VacA) produced 3.6 to 4.4 mg MV protein per liter culture after 48 h (final OD<sub>600</sub>, 0.14 to 0.20). All four strains were confirmed to be >99% helical after 48 h of liquid culture. The purified MV from all four strains had similar dimensions and morphologies, and all preparations were free of bacteria, bacterial cell wall debris, and flagella when visualized by transmission electron microscopy (a representative image is shown in Fig. 1).

**There is substantial strain variation in the quantity of VacA associated with MV.** Equal concentrations of 60190 MV with and without VacA were analyzed by SDS-PAGE and Western blotting for VacA (Fig. 2). Although secreted p88 VacA sometimes separates into p33 and p55 domains, all MV-associated VacA migrated at a position consistent with intact p88 on SDS-PAGE. VacA protein was completely absent from 60190  $\Delta$ vacA MV, as expected. Protein profiles of MV from the two strains were otherwise indis-





**FIG 3** MV containing active VacA cause vacuolation in RK13 cells. (A to D) RK13 cells were incubated for 4 h in the presence of 50  $\mu\text{g/ml}$  MV purified from strain 60190 (carrying active s1i1m1 VacA) (A), 60190  $\Delta vacA$  (B), SS1<sup>WT</sup> (carrying s2i2m2 VacA with impaired vacuolating activity) (C), or SS1<sup>s1i1</sup> (carrying relatively small quantities of active s1i1m2 VacA) (D). (E) Mean proportion of heavily vacuolated cells ( $\pm$ SD) across a minimum of four randomly selected microscopy fields ( $>100$  cells) per condition.

tinguishable by SDS-PAGE, consistent with the isogenic nature of the  $\Delta vacA$  mutant.

MV from SS1<sup>WT</sup> and SS1<sup>s1i1</sup> had protein profiles markedly different from that of the 60190 MV, reflecting the high level of strain-to-strain variability in *H. pylori*. When crude water extracts from whole bacteria were compared by Western blotting, the 60190 extracts contained substantially more VacA than the SS1 extracts. MV from strain 60190 contained very high concentrations of VacA, but in MV from strains SS1<sup>WT</sup> and SS1<sup>s1i1</sup> VacA was barely detectable on the same blot. In order to compare VacA contents in 60190 and SS1 preparations on the same blot without overexposure, 60190 samples had to be diluted 10-fold, indicating marked strain-to-strain variation in the quantities of VacA associated with MV (Fig. 2).

**VacA remains active when vesicle associated.** As expected, RK13 cells were heavily vacuolated (49% of cells were vacuolated) following 4 h of incubation in the presence of 50  $\mu\text{g/ml}$  60190 MV (Fig. 3A), but application of 60190  $\Delta vacA$  MV did not induce significant vacuolation above background levels (3% vacuolated cells with 60190  $\Delta vacA$  MV versus 4% with PBS only) (Fig. 3B and E). SS1<sup>WT</sup> MV induced minimal vacuolation (13%), and SS1<sup>s1i1</sup>

MV induced vacuolation at an intermediate level (27%) (Fig. 3C to E), consistent with the delivery of small quantities of s2i2 and s1i1 VacA, respectively.

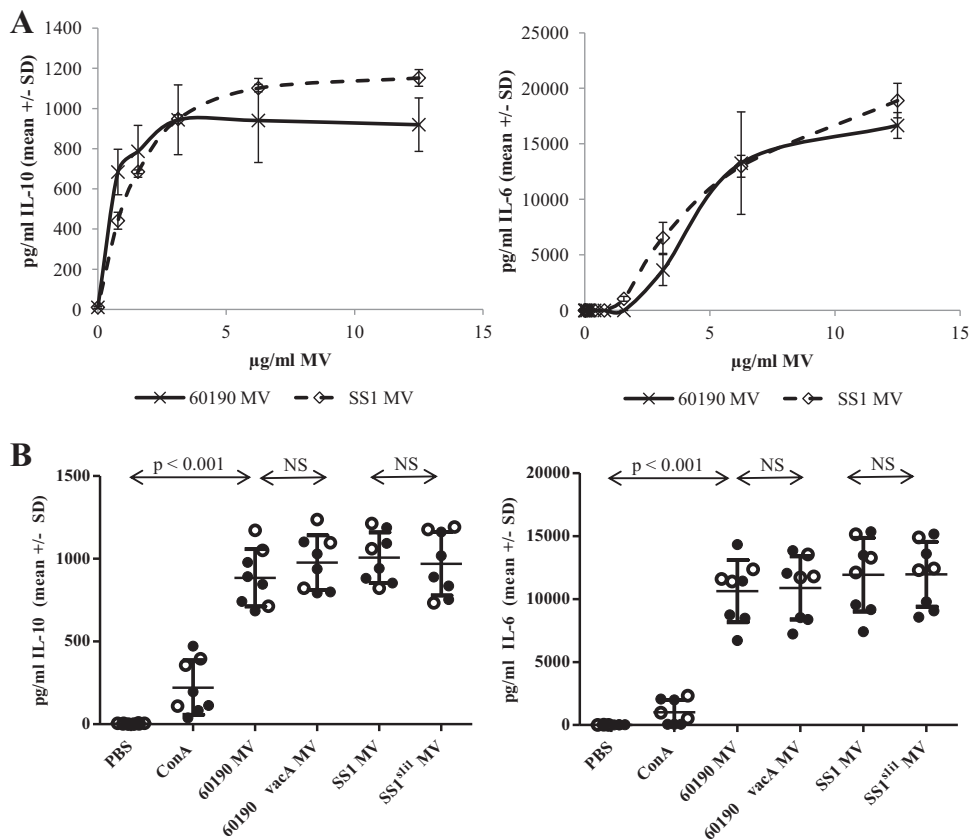
**MV stimulate release of IL-6 and IL-10 (but not IL-2, IL-4, IL-12p70, or IFN- $\gamma$ ) from PBMCs in a dose-dependent manner.** Having characterized the VacA protein contents and vacuolating activities of 60190, 60190  $\Delta vacA$ , SS1<sup>WT</sup>, and SS1<sup>s1i1</sup> MV, we aimed to investigate effects of MV on human immune cells. First, peripheral blood mononuclear cells (PBMCs) were purified from healthy donors of unknown *H. pylori* infection status and cultured in the presence of 0 to 12.5  $\mu\text{g/ml}$  SS1 or 60190 MV for 48 h. Cytokines secreted into the culture medium were quantified by ELISA. *H. pylori* MV strongly stimulated the release of IL-10 and IL-6 from PBMCs, in a dose-dependent manner (Fig. 4A). Incubation with up to 25  $\mu\text{g/ml}$  MV did not stimulate IL-2, IL-4, IL-12p70, or IFN- $\gamma$  secretion at concentrations above the limit of sensitivity of the assays (data not shown), indicating that the cytokine response in PBMCs from healthy donors was largely innate rather than T cell derived. *H. pylori* MV also stimulated a dose-dependent proliferative response in human PBMCs, which was maximal at approximately 3  $\mu\text{g/ml}$  (see Fig. S1 in the supplemental material).

Next, we compared the effects of MV at 10  $\mu\text{g/ml}$  from the two wild-type *H. pylori* strains, 60190 (VacA s1i1m1) and SS1 (VacA s2i2m2), on PBMCs from a panel of patients presenting at the Queens Medical Centre, Nottingham, with gastric symptoms for routine endoscopy. PBMCs were incubated for 24 h in the presence of 5  $\mu\text{g/ml}$  concanavalin A (a T cell mitogen), 10  $\mu\text{g/ml}$  MV, or a buffer-only control, and then IL-10 and IL-6 levels in culture medium were determined by ELISA. MV from both strains stimulated high levels of IL-6 and IL-10 production by PBMCs from all patients tested ( $P < 0.001$  compared with PBS-treated control cells) (Fig. 4B).

We hypothesized that MV might activate a memory T cell response in PBMCs from *H. pylori*-infected patients. In order to determine whether the strong cytokine response to *H. pylori* MV was innate or adaptive, we stratified the data according to the infection status of the patients (Fig. 4B). There was no significant difference in the levels of cytokines produced by PBMCs from infected and uninfected patients in response to *H. pylori* MV ( $P = 0.26$  for IL-10 and  $P = 0.34$  for IL-6 by unpaired Student *t* tests comparing infected-group against uninfected-group responses to 60190 MV). This indicates that human PBMCs mount a strong, innate cytokine response to *H. pylori* MV, likely due to the delivery of concentrated pathogen-associated molecular patterns (PAMPs) such as LPS and peptidoglycan.

Since VacA can directly activate mast cells to produce IL-6 and IL-10 (38), we hypothesized that MV containing active VacA might provoke more pronounced cytokine responses. In order to examine the contribution of VacA to MV-induced IL-10 and IL-6 secretion from PBMCs, the effects of MV from 60190  $\Delta vacA$  and from the isogenic SS1 mutant engineered to produce more toxic s1i1 VacA (SS1<sup>s1i1</sup>) were also measured (Fig. 4B). All four MV types stimulated the production of similar levels of IL-10 and IL-6 ( $P > 0.05$ ), indicating that VacA is not required at the concentration of MV tested.

***H. pylori* MV are toxic to Jurkat T cells.** We expected MV to stimulate IL-2 production by T cells present in the PBMC cultures, but this was not the case. PBMCs stimulated with MV produced IL-10 and IL-6, as described above, but the anticipated T cell-



**FIG 4** MV stimulate IL-10 and IL-6 production from PBMCs. (A) PBMCs from healthy donors of unknown *H. pylori* infection status were incubated for 48 h in the presence of 0 to 12.5 µg/ml SS1<sup>WT</sup> or 60190 MV, and then IL-10 (left) and IL-6 (right) concentrations in culture supernatants were measured by ELISA. Similar data were obtained from independent experiments using PBMCs from three different donors; representative data from one donor are shown. (B) PBMCs from *H. pylori*-infected (closed circles) and uninfected (open circles) patients were incubated for 24 h in the presence of 10 µg/ml MV, 5 µg/ml concanavalin A, or an equivalent volume of PBS, then IL-10 (left) and IL-6 (right) concentrations in culture supernatants were measured by ELISA. NS, not significant.

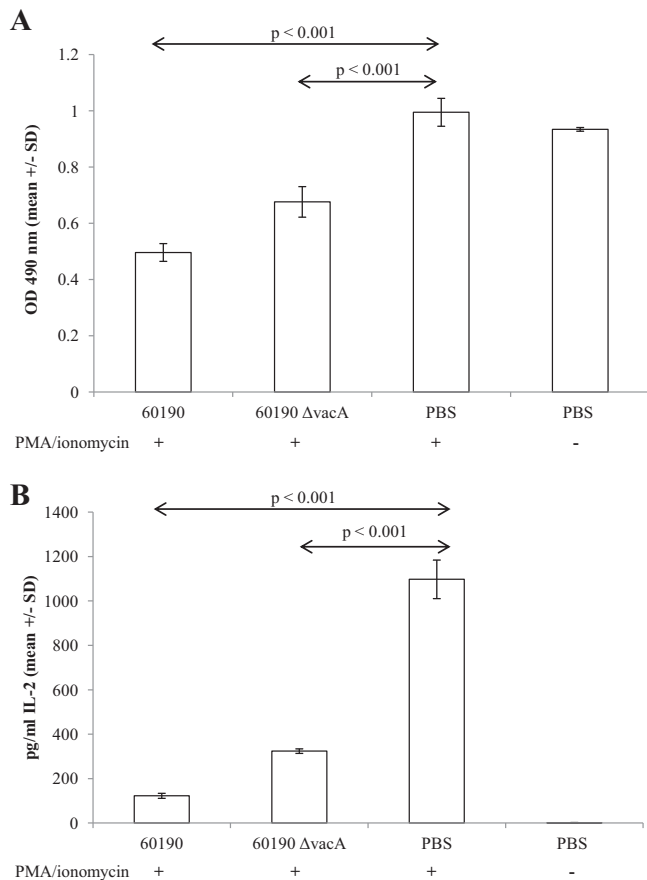
derived IL-2 response was absent. Since free soluble VacA inhibits T cell proliferation and IL-2 secretion (23–25, 27, 39), we set out to determine whether or not MV-associated VacA exerts inhibitory effects on T cells. Jurkat cells were pretreated for 1 h with 25 µg/ml MV and then stimulated for 48 h using PMA and ionomycin, following methods described by Gebert et al. (23). PMA-ionomycin-stimulated cells proliferated (i.e., the number of metabolically active cells per well increased) compared with unstimulated control cells (Fig. 5A, PBS controls with or without PMA-ionomycin). Pretreatment with MV caused a loss of metabolically active cells rather than proliferation, indicating a toxic effect on MV on Jurkat cells ( $P < 0.001$ ) (Fig. 5A). Consistent with this, PMA-ionomycin-driven IL-2 production was markedly inhibited by pretreatment with MV ( $P < 0.001$ ) (Fig. 5B). These cytotoxic effects were dose dependent, with MV-mediated cytotoxicity detectable at 60190 MV doses of 6.25 µg/ml and above (data not shown).

**MV induce apoptosis in Jurkat T cells, which is enhanced by, but not dependent on, the presence of VacA.** Since Jurkat cells pretreated with MV died in proliferation assays, we decided to characterize the toxic effect of MV on T cells further by measuring apoptosis. Free soluble VacA is known to induce apoptosis in gastric epithelial cells (11–14) but not in Jurkat cells (23, 27, 39). *H. pylori* MV effects on gastric epithelial cells are complex, with induction of proliferation at low doses and of apoptosis at higher doses (33), but their effects on immune cells are unknown.

We cultured Jurkat cells in the presence of 25 µg/ml MV for 24 h and then measured intracellular and extracellular protease and cleaved caspase 3/7 activities using an ApoTox-Glo triplex assay (Promega) to determine levels of cell viability, cytotoxicity, and apoptosis, respectively.

*H. pylori* MV markedly decreased Jurkat cell viability and increased cytotoxicity compared with those for cells treated with an equivalent volume of PBS without MV ( $P < 0.001$  for 60190 and SS1<sup>s111</sup> MV,  $P < 0.01$  for 60190 Δ*vacA* MV, and  $P < 0.05$  for SS1 MV) (Fig. 6A). MV carrying more active forms of VacA had a more profound cytotoxic effect than MV with s2i2m2-type VacA or no VacA, although this difference did not reach statistical significance in the SS1 background ( $P < 0.01$  for 60190 versus 60190 Δ*vacA*), but 60190 Δ*vacA* MV remained cytotoxic in the absence of VacA.

*H. pylori* MV induced apoptosis in Jurkat cells (Fig. 6B). This was enhanced by, but not completely dependent on, the presence of VacA: Jurkat cells treated with 60190 MV had significantly more caspase 3/7 activity than those treated with 60190 Δ*vacA* MV ( $P < 0.001$ ). 60190 MV at 25 µg/ml were more potent than the apoptosis-inducing chemical staurosporine at 1 µM ( $P < 0.001$ ). SS1 and SS1<sup>s111</sup> MV also induced apoptosis ( $P < 0.001$ ) but to a lesser extent than MV from either of the 60190 background strains ( $P < 0.001$ ). An MV dose of 25 µg/ml was the minimum required to induce apoptosis in Jurkat cells.

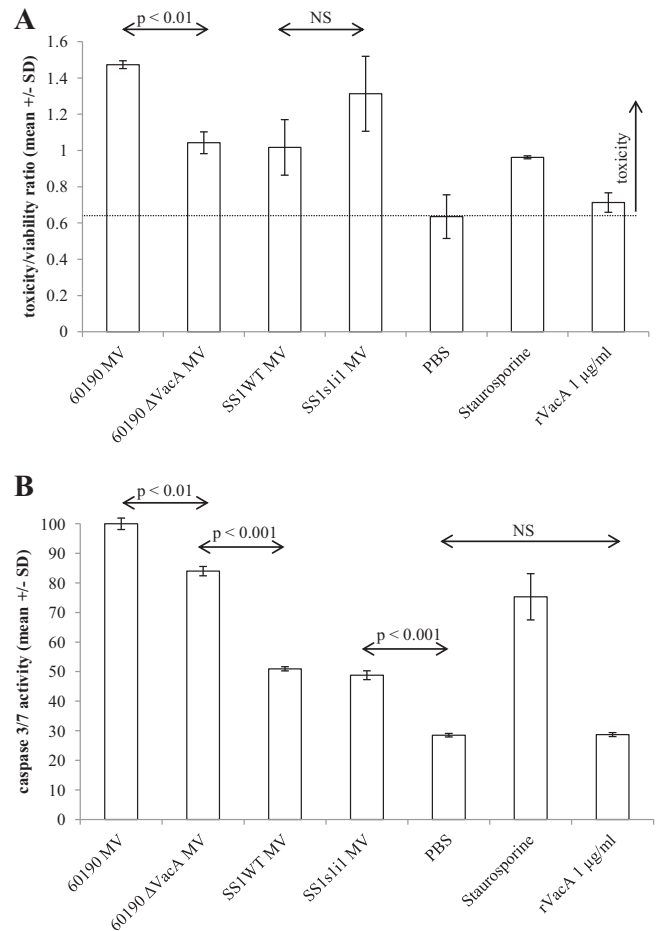


**FIG 5** Pretreatment with MV reduces Jurkat cell viability and IL-2 production in response to PMA and ionomycin stimulation. Jurkat cells were pretreated with 25  $\mu$ g/ml MV for 1 h and then stimulated with PMA and ionomycin. After 48 h, the concentration of metabolically active cells per well was measured using a CellTiter colorimetric assay (A), and IL-2 concentrations in culture supernatants were quantified by ELISA (B).

In order to confirm that VacA enhanced MV-induced Jurkat apoptosis but did not itself induce apoptosis when free and soluble, we treated Jurkat cells with purified recombinant s111m1 VacA expressed in *E. coli* using the 60190 *vacA* p33 and p55 genes and prepared following the method of González-Rivera et al. (36) (Fig. 7A). The activity of the recombinant toxin was confirmed by vacuolation assay on RK13 cells (Fig. 7B), and, as expected, recombinant VacA inhibited IL-2 secretion from PMA-ionomycin-stimulated Jurkat cells (Fig. 7C) but did not induce apoptosis (Fig. 6B), consistent with the published reports of other researchers (23, 27, 39).

Since 60190  $\Delta$ *vacA* MV were capable of inducing Jurkat cell apoptosis but the effect was enhanced by the presence of VacA in the vesicles, we hypothesized that VacA on the vesicle surface promoted binding to and/or uptake into Jurkat cells. To test this hypothesis, we used proteinase K to remove surface-bound proteins, including VacA from 60190 MV, following the methods of Bomberger et al. (37). As predicted, proteinase K-treated 60190 MV still induced Jurkat cell apoptosis, but only 60190  $\Delta$ *vacA* MV levels (Fig. 8).

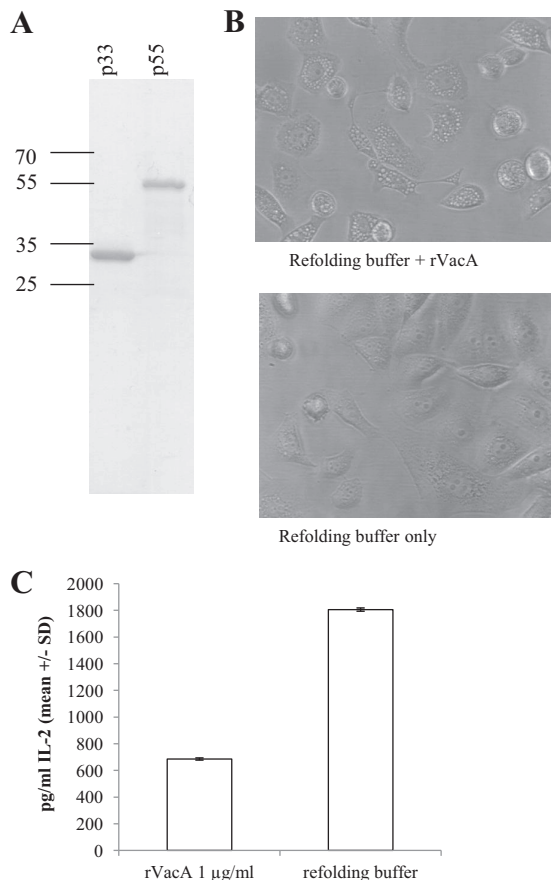
**MV effects on CD4<sup>+</sup> T cells purified from human blood.** The Jurkat cell line is a widely used IL-2-producing leukemic T cell



**FIG 6** *H. pylori* MV induce apoptosis in Jurkat cells. Jurkat cells were incubated in the presence of 25  $\mu$ g/ml MV for 24 h, and then cytotoxicity, viability, and caspase 3/7 activity were quantified using an ApoTox-Glo triplex assay. Mean values from triplicate wells are shown ( $\pm$ SD). (A) Overall cytotoxicity expressed as a toxicity/viability ratio with reference to healthy cells treated with a PBS control. (B) Caspase 3/7 activity normalized to the maximum level seen across all conditions tested.

line, but experimental results using such cell lines might not always be representative of host-pathogen interactions *in vivo*. To confirm *H. pylori* MV effects on native human T cells, we purified CD4<sup>+</sup> T cells from human blood and repeated the proliferation and apoptosis assays described above.

The Jurkat cell line proliferates rapidly even in the absence of stimulation, so addition of PMA and ionomycin did not markedly increase the number of metabolically active cells per well in Jurkat cell assays (Fig. 5). Native CD4<sup>+</sup> cells grow more slowly *in vitro*, and proliferation was markedly increased in response to PMA and ionomycin. Pretreatment with 25  $\mu$ g/ml *H. pylori* 60190 MV (with or without VacA) significantly inhibited this proliferative response ( $P < 0.001$ ) (Fig. 9A). Similar to the results obtained with Jurkat cells (Fig. 6), incubation with 25  $\mu$ g/ml 60190 MV induced apoptosis in native CD4<sup>+</sup> cells at levels comparable to those with the chemical apoptosis inducer staurosporine at 1  $\mu$ M ( $P < 0.05$ ) (Fig. 9B). However, MV from the other *H. pylori* strains tested did not induce significant levels of apoptosis, indicating that MV-associated *H. pylori* components, including VacA and other, as-



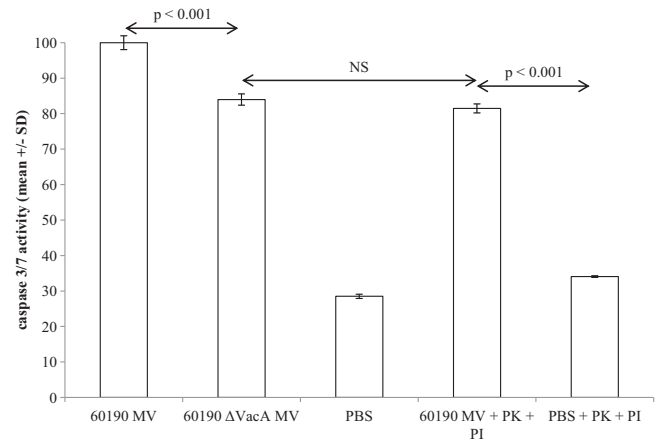
**FIG 7** Recombinant VacA is active on epithelial and Jurkat cells. (A) Recombinant s111m1-type VacA (rVacA) was reconstituted from purified, refolded p33 and p55 subunits. Purity was confirmed by SDS-PAGE. (B) RK13 cells were incubated overnight in the presence of 1 µg/ml rVacA and vacuolation confirmed by light microscopy. (C) Jurkat cells were pretreated with rVacA or buffer control for 1 h and then stimulated with PMA and ionomycin. After 48 h, IL-2 secretion was quantified by ELISA.

yet-unidentified factors may contribute to T cell inhibition during infection.

## DISCUSSION

*H. pylori* infects humans lifelong, inducing profound immunological changes, including chronic inflammation of the stomach lining (gastritis) and elevated levels of IL-10-producing regulatory T cells (19). Coevolution of bacterium and host has likely resulted in a balance between induction of an inflammatory response in the host gastric mucosa, perhaps to promote release of host nutrients into the stomach lumen, and an anti-inflammatory response to prevent clearance of the bacteria by the host immune system (18).

Gram-negative bacteria constitutively produce MV, and multiple functions have been attributed to them, including envelope stress response (40) and toxin delivery (reviewed by Deatherage and Cookson [41]). There is growing interest in MV because they are now believed to play a role in bacterial pathogenesis for many species. Since MV resemble the bacterial surface, they can act as decoys for host factors such as antibodies and antimicrobial peptides (42–44) to promote bacterial survival and persistence. MV from a number of bacterial species have been proven to deliver



**FIG 8** Induction of Jurkat cell apoptosis by MV is enhanced by, but not dependent on, carriage of VacA. 60190 MV were pretreated with proteinase K (PK) (inactivated using a protease inhibitor [PI] prior to cell culture). Treated and untreated MV were added to Jurkat cells at 25 µg/ml and caspase 3/7 activity measured after 24 h of incubation using an ApoTox-Glo triplex assay. Data are expressed as the percentage of maximal apoptosis induction and are means from triplicate wells.

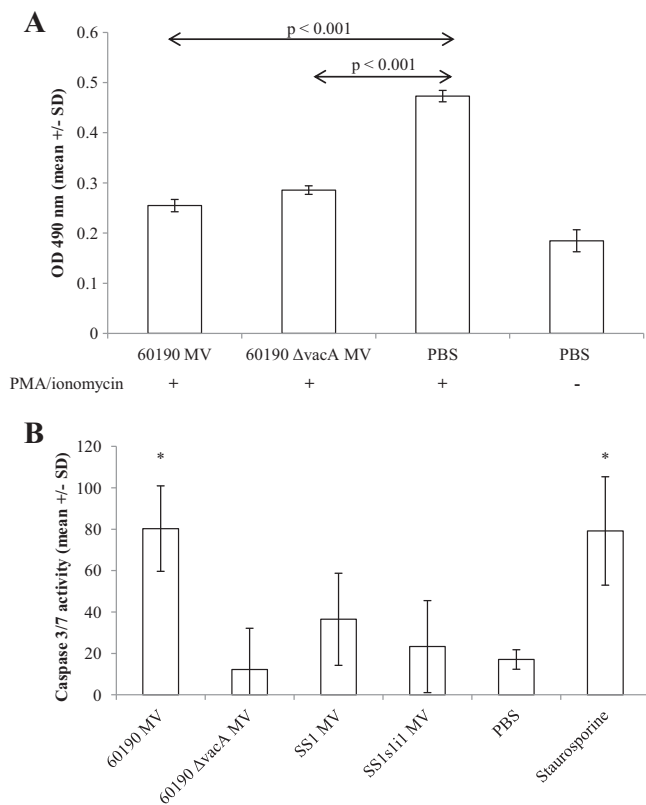
virulence factors and PAMPs to host epithelial cells, inducing production of the proinflammatory cytokine IL-8, but direct effects of MV on host immune cells remain largely uncharacterized to date. Consequently, although we have focused on *H. pylori*, the findings described here may also be of interest to researchers studying mechanisms of immune modulation and pathogenesis in other bacterial infections.

*H. pylori* clearance is T cell dependent in a mouse infection model (45), and hence there has been great interest in the discovery of two proteins secreted by *H. pylori*, VacA and  $\gamma$ -glutamyl transferase (GGT), which inhibit human T cells via distinct mechanisms (23, 46). However, it is not yet clear how, and in what quantities, these factors access T cells in the lamina propria underlying the gastric epithelial barrier when the infecting bacteria remain on or close to the apical surface of the epithelium.

In addition to free soluble secretion, both VacA and GGT are packaged into MV (31, 47) by *H. pylori*. MV are more likely to cross the epithelial barrier than whole bacteria and so might exert long-range effects on host immune cells. They could potentially protect VacA and GGT from degradation/inactivation during transit, and they would facilitate simultaneous delivery of multiple *H. pylori* molecules to a single host cell in a concentrated “dose,” preventing rapid dilution of bacterial secreted factors in the stomach lumen and potentially contributing to *H. pylori*-mediated carcinogenesis. With this in mind, we set out to characterize immunomodulatory effects of *H. pylori* MV on human immune cells, first using PBMCs to broadly assess cytokine responses to the MV and then focusing on T cell-specific effects.

We show that *H. pylori* MV are strong innate stimulators of human immune cells, inducing proliferation and release of high concentrations of both proinflammatory (IL-6) and anti-inflammatory (IL-10) cytokines. Since PBMCs from people without *H. pylori* infection produced quantities of IL-6 and IL-10 similar to those produced by PBMCs from patients with a current infection, these cytokines are likely to be produced innately (e.g., by monocytes and NK cells rather than by *H. pylori*-specific T cells) in





**FIG 9** *H. pylori* MV effects on CD4<sup>+</sup> T cells purified from human blood. (A) CD4<sup>+</sup> T cells purified from human blood were pretreated with 25 µg/ml MV for 1 h and then stimulated with PMA and ionomycin (results for an unstimulated control are also shown). After 48 h, the concentration of metabolically active cells per well was measured using a CellTiter colorimetric assay. (B) Alternatively, CD4<sup>+</sup> T cells were incubated in the presence of 25 µg/ml MV, PBS, or the chemical apoptosis-inducer staurosporine for 24 h, and then levels of apoptosis were determined by measuring caspase 3/7 activity using an Apo-Tox-Glo triplex assay. Data are expressed as the percentage of maximal apoptosis induction and are means from triplicate wells.

response to MV-associated PAMPs such as LPS and peptidoglycan. Despite high levels of strain-to-strain variability in their contents, MV from strains with 60190 or SS1 backgrounds stimulated indistinguishable cytokine responses independent of the presence, absence, or type of VacA. MV, like whole bacteria, are also known to induce IL-8 release from gastric epithelial cells (7, 8), so it is possible that MV are involved in stimulating and maintaining pro- and anti-inflammatory host responses during persistent infection, via direct effects on both epithelial and innate immune cells.

Given that *H. pylori* MV are enriched for LPS (28, 48), strong innate stimulation of IL-6 and IL-10 from human PBMCs was not unexpected. However, the complete absence of T cell activation, characterized by IL-2 secretion, led us to examine MV effects on T cells more closely. Since MV-associated VacA is active on gastric epithelial cells, we expected to find that MV carrying VacA were capable of delivering the toxin to T cells, resulting in inhibition of proliferation and IL-2 secretion as previously established (23–27) for free toxin. In fact, any such VacA-mediated T cell inhibition was masked by the strong toxic effect of the vesicles themselves. *H. pylori* culture supernatant has been previously reported to induce apoptosis in T cells (49), and here we show that MV alone are able

to induce apoptosis to similar, or higher, levels than the chemical inducer staurosporine used as a positive control in this study. Although active recombinant VacA in free soluble form did not induce T cell apoptosis, MV-mediated apoptosis was enhanced by, but not completely dependent on, carriage of the toxin. MV from the less toxic SS1 strain did not induce T cell apoptosis to the same extent as MV from 60190, even when the SS1 strain was engineered to produce a more active sli1 VacA toxin form. Comparison of MV contents by SDS-PAGE and Western blotting revealed striking differences in relative VacA concentrations between the 60190 and SS1 strain backgrounds.

Further investigation of strain-strain variability in MV contents and activities is now under way in our laboratory, and we also aim to define the specific components of *H. pylori* MV that induce T cell apoptosis and the mechanisms by which they do so. We propose that membrane vesicle-mediated delivery of *H. pylori* immune-modulatory factors to host immune cells may contribute to bacterial pathogenesis.

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