Low Multiplicity of Infection of Helicobacter pylori Suppresses Apoptosis of B Lymphocytes

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Abstract

Helicobacter pylori infection of the human stomach causes chronic gastritis that can lead to gastric cancer. Because activated lymphocytes persist in the gastric mucosa, and because a high multiplicity of infection (MOI) of H. pylori is needed to induce apoptosis in vitro, we speculated that resistance of lymphocytes to apoptosis is an important feature of the immune response to H. pylori. Freshly isolated mouse splenocytes underwent substantial spontaneous apoptosis and displayed a biphasic response to H. pylori, in which low MOI (1-10) markedly inhibited apoptosis, whereas high MOI (>75) potentiated apoptosis. Low MOI reduced mitochondrial membrane depolarization, caspase-3 and caspase-9 activation, and cytochrome c release and increased Bcl-2 levels. Low MOI also induced cellular proliferation. When cells were subjected to fluorescence-activated cell sorting after coculture with H. pylori, CD19+ B cells were found to be protected from apoptosis and undergoing proliferation at low MOI, whereas CD3+ T cells did not exhibit this pattern. The protective effect of low MOI on apoptosis persisted even when B cells were isolated before activation. Immunophenotyping showed that all B-cell subsets examined were protected from apoptosis at low MOI. Additionally, gastric infection with H. pylori resulted in protection of splenic B cells from spontaneous apoptosis. Our results suggest that the low levels of H. pylori infection that occur in vivo are associated with B-cell survival and proliferation, consistent with their potential to evolve into mucosa-associated lymphoid tissue lymphoma. (Cancer Res 2006; 66(13): 6834-42)

Introduction

Helicobacter pylori is a Gram-negative microaerophilic bacterium that selectively colonizes the human stomach and causes chronic gastritis, peptic ulcers, and gastric cancer. The latter includes gastric mucosa–associated lymphoid tissue (MALT) lymphoma (1), which is a low-grade B-cell lymphoma that frequently responds well to H. pylori eradication (2). There is a vigorous innate and adaptive immune response to H. pylori, but the infection generally persists for the life of the host due in part to its ability to evade the antimicrobial effects of the immune response (3). We have reported that one potential cause of the ineffective immune response is the induction of apoptosis in macrophages (4, 5). T-lymphocyte apoptosis in response to H. pylori has also been put forth as mechanism for immune evasion (6), but a high multiplicity of infection (MOI) of 300 has been shown to be required to induce apoptosis (6).

Because we reported that macrophages readily undergo apoptosis at an MOI of 10 (4), we questioned whether lymphocytes may be resistant to H. pylori–induced apoptosis at a low MOI. Therefore, using mouse splenocytes, we conducted studies in freshly isolated lymphocytes with a range of MOIs, beginning with what is likely to occur in the stomach (MOI of 1-10; refs. 7, 8), up to the high levels used in most studies (MOI of 100-400; ref. 6). We now report that these cells were protected from spontaneous apoptosis by low MOI of H. pylori, whereas high MOI enhanced apoptosis. When splenocytes were sorted after activation, B cells were found to be protected from apoptosis and undergoing proliferation. These findings probed us to investigate the B-cell responses in more detail.

The ontogeny of B-cell subsets involves positive and negative selection based on the anatomic site of exposure to antigens, including bacterial stimuli, and costimulation by T cells. Splenic B cells have been divided into four major populations based on their stage of development (9): immature transitional type 1 (T1) and type 2 (T2) and mature follicular B (FoB) and marginal zone B cells. T1 cells are progenitor cells derived from the bone marrow that move to the spleen and differentiate into T2 cells in the follicular zone; T2 cells can differentiate into mature FoB or marginal zone B cells (10). Marginal zone B cells exhibit a survival response with bacterial stimulation (11). In the case of MALT lymphoma, lymphoid follicles in the stomach derive from clonally expanded marginal zone B cells (12, 13). Intriguingly, we found that all B-cell subsets were protected from apoptosis by low-MOI H. pylori infection, and that splenic B cells from mice with gastric H. pylori infection were also protected from apoptosis. Our results indicate that levels of H. pylori infection that occur in vivo are associated with B-cell survival and growth in nontransformed lymphocytes.

Materials and Methods

Mice, Cells, and Culture Conditions

Experiments were conducted with C57BL/6 mice (8-12 weeks; The Jackson Laboratory, Bar Harbor, ME). In some experiments, mice were infected with H. pylori SS1 (14), as described (4), and gastric and splenic tissues were harvested 4 or 12 months later. Splenocytes were isolated aseptically (15) in Krebs-Ringer/HEPES buffer (pH 7.4) with 0.5% bovine serum albumin and cultured in complete RPMI 1640. MOI was determined as the ratio of bacteria to eukaryotic cells (4, 8).

Bacteria

H. pylori SS1 was maintained under microaerobic conditions on brucella blood agar plates, with concentrations of bacteria determined by absorbance (8). Lysates of H. pylori were obtained by passage through a
French pressure cell at 20,000 p.s.i. (8). In some studies *H. pylori* was fixed in 2% formaldehyde before use. *Citrobacter rodentium* (DBS100) was grown as described (16) and lysed as above.

**Detection of Apoptosis**

Splenocytes (0.5 × 10^7 cells per well in 96-well plates) were stimulated with *H. pylori* at different MOI (1–400) for 0 to 24 hours. Apoptosis was analyzed by three different methods.

**DNA histogram analysis.** Cells were permeabilized with PBS/0.1% Triton X-100 and stained with propidium iodide (PI) using a cell cycle analysis kit (Roche Molecular Biochemicals, Indianapolis, IN). Cells were analyzed by flow cytometry using ModFit LT software (BD Biosciences, San Jose, CA), with the sub-G0-G1 peak representing the apoptotic population (17) as described (5).

**ELISA.** The Cell Death Detection ELISA Plus kit (Roche Molecular Biochemicals) was used, which is based on the determination of cytoplasmic histone-associated DNA fragments (4, 18). The relative amount of apoptosis was determined as a ratio to control level.

**Annexin V staining.** Cells were stained with Annexin V-FTTC or Annexin V-allophycocyanin and either PI or 7-aminooxycyanin D (7-AAD; Oncogene Research Products, San Diego, CA). Cells were analyzed with a flow cytometer (FACSCalibur, BD Biosciences) using CellQuest software (BD Biosciences) for Annexin V-FTTC/PI–stained cells, and FlowJo software (Tree Star, Inc., Ashland, OR) for the Annexin V-allophycocyanin/7-AAD–stained cells. The upper right (Annexin V-/PI+ or 7-AAD+) quadrant represents late apoptotic cells, and the lower right (Annexin V-/PI or 7-AAD-) quadrant represents early apoptotic cells, whereas the upper left (Annexin V+/PI+ or 7-AAD+) and lower left (Annexin V+/PI- or 7-AAD-) quadrants represent necrotic and viable cells, respectively (5, 18, 19). It has been reported that in lymphocytes, the upper right quadrant defines late apoptosis, and this correlates well with other assays of apoptosis (20, 21).

**Proliferation Analysis**

Bromodeoxyuridine (Brdurd) incorporation was used as an estimate for cell growth and DNA synthesis. After 24 hours of cell culture, BrdUrd was added for 16 hours, and incorporation was determined by ELISA (Roche Molecular Biochemicals). In each experiment, relative proliferation was determined as a ratio to control level.

**Cell Viability**

Viability of splenocytes was determined by a colorimetric assay with the cell proliferation kit II (Roche Molecular Biochemicals), in which the conversion of the tetrazolium salt 2,3-bis(2-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide to formazan is measured spectrophotometrically (5).

**Mitochondrial Membrane Potential**

The electron gradient across the mitochondrial membrane space during normal respiration is the mitochondrial membrane potential (ΔΨm). Loss of ΔΨm (depolarization) was measured by flow cytometry after staining with a Mitotracker kit (Calbiochem, La Jolla, CA; ref. 5). The cationic dye fluoresces red as it aggregates inside healthy mitochondria. In apoptotic cells, if the ΔΨm collapses the dye stays as monomer in the cytoplasm and emits green fluorescence (FI-2), which is detected by flow cytometry.

**Measurement of Caspase-3 Activity**

Caspase-3 activity was measured by the cleavage of the chromogenic tetrapeptide (Ac-DEVD-p-nitroanilide). In brief, 2 × 10^7 cells were lysed, combined with substrate in the reaction mixture, and incubated at 37°C in the incubation chamber of a microplate reader, and kinetic analysis of absorbance was done (5).

**Western Blot Analysis**

After coculture with *H. pylori*, splenocytes were lysed, and 50 μg of protein per lane were separated by SDS-PAGE using 10% gels and transferred onto Immobilon-P membranes (Millipore, Bedford, MA) by electroblotting. Membranes were blocked with 5% nonfat dry milk for 2 hours at room temperature. The following antibodies and dilutions were used for immunoblots: rabbit monoclonal anti-caspase-3 (1:1,000; Cell Signaling, Beverly, MA), rabbit polyclonal anti-caspase-9 (1:1,000; Cell Signaling), rabbit polyclonal anti-caspase-8 (1:2,000; Abcam, Cambridge, MA), rabbit polyclonal anti-Bcl-2 (1:1,000; Calbiochem), and mouse monoclonal anti-β-actin (1:5,000; Sigma, St. Louis, MO). For cytochrome c, cytoplasmic fractions were prepared using a cytosolic/mitochondrial fractionation kit (Oncogene Research Products); 20 μg protein per lane were separated on 16% SDS Tris-HCl gels and transferred (3); and a rabbit polyclonal anti-cytochrome c antibody was used (1:1,000; Cell Signaling). Detection was done by chemiluminescence for all experiments (5).

**Fluorescence-Activated Cell Sorting of Splenocytes**

In the first series of experiments, total splenocytes were cocultured with *H. pylori* followed by sorting of B and T lymphocytes. B cells were stained with a phycoerythrin-conjugated rat monoclonal anti-CD19 antibody (clone 1D3; BD PharMingen, San Diego, CA), and T cells were stained with a phycoerythrin-conjugated hamster monoclonal anti-CD3ε chain antibody (clone 145-2C11; BD PharMingen). Stained lymphocytes were separated by fluorescence-activated cell sorting (FACS; BD FACSVantage). Cell purities were >93% on reanalysis. Immediately after sorting, apoptosis and proliferation were assessed by ELISA and BrdUrd incorporation, respectively. In the second series of experiments, splenic B lymphocytes were isolated before stimulation. B cells were stained with anti-CD19 antibody and subjected to FACS (BD FACSAria). Cell purity was >99% on reanalysis. Isolated B cells were placed in complete culture medium and activated with *H. pylori*. After 24 hours of incubation, apoptosis and proliferation were assessed by ELISA and BrdUrd incorporation, respectively.

**Immunophenotyping of Splenic Lymphocytes by Flow Cytometry**

Total splenocytes. Splenocytes were cultured in the presence or absence of *H. pylori* and then stained for phenotyping and apoptosis analysis. For phenotyping, splenocytes were incubated with phycoerythrin-conjugated anti-CD19 antibody to identify B cells within the total population, and apoptosis was detected with Annexin V-allophycocyanin/7-AAD as described above. Data were collected on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software.

B cells. B lymphocytes were isolated from splenocytes by negative selection using an auto-MACS–automated cell sorter (Miltenyi Biotechnology, Auburn, CA). Pooled splenocytes were depleted of RBC and then incubated with anti-CD43 antibodies coupled to magnetic beads to deplete CD43-bearing (non-B cell) leukocytes (9). The purity of B cells isolated was >95% by flow cytometry using anti-CD19 antibody. Isolated B cells were placed in complete culture medium and activated with *H. pylori*. Cells were harvested and labeled with FITC-conjugated anti-IgM (Jackson Immunoresearch, West Grove, PA), and biotin-conjugated anti-IgD was detected with streptavidin–phycoerythrin (BD Pharmingen). The IgM<sup>high</sup>IgD<sup>low</sup> population represents T1 + marginal zone; IgM<sup>low</sup>IgD<sup>high</sup> represents T2; and a rabbit polyclonal anti-caspase-8 (1:1,000; Cell Signaling) was used (1:1,000; Cell Signaling). Detection was done by chemiluminescence for all experiments (5).

**Statistical Analysis**

Values shown represent the mean ± SE of separate experiments. For comparisons between multiple groups, the Fisher's protected least significant difference test was used, and for comparisons between two groups, the Student's t test was used.

**Results**

**Protection from apoptosis and stimulation of proliferation with low MOI of *H. pylori*.” To determine whether lymphocytes may be resistant to *H. pylori*–induced apoptosis, we used freshly isolated mouse splenocytes as a source of lymphocytes to avoid issues related to the resistance of apoptosis of tumor cell lines and
to allow us to assess both T and B lymphocytes. In splenocytes from naive C57BL/6 mice, there was substantial induction of spontaneous apoptosis at 24 hours (Fig. 1A), which developed in a time-dependent manner (Fig. 1B). This level of apoptosis is consistent with previous reports in freshly isolated splenocytes (22–24). Exposure of splenocytes to low MOI of H. pylori (1, 5, and 10)–protected cells from apoptosis (Fig. 1A and C), with a maximal decrease of 48.1 ± 5.6%. There was also a concentration-dependent increase in apoptosis that became apparent at MOI above 50 and peaked at MOI of 100 to 400. The differential effect of low and high MOI on apoptosis was confirmed by DNA fragmentation ELISA (Fig. 1A) and cell staining with Annexin/PI (Fig. 1D). The protection at low MOI and potentiation at high MOI began at 12 hours after isolation and continued to 24 hours (Fig. 1B).

Additionally, cellular proliferation was activated by low MOI, and this effect was lost at MOI of 25 (Fig. 1E).

When we assessed the requirement for live bacteria in the modulation of apoptosis, we found that protection from apoptosis at low MOI was completely maintained when H. pylori was subjected to fixation or lysis (Fig. 1F). Potentiation of apoptosis at high MOI still occurred, although it was somewhat attenuated compared with live bacteria. When we used Citrobacter rodentium, which causes colitis in mice (16), we found that there was no protection from apoptosis at low MOI (Fig. 1F). To further assess the overall survival effect on splenocytes, we measured cell viability and found that there was a significant increase with low MOI and a marked decrease at high MOI. These findings occurred with fixed or lysed H. pylori in addition to live bacteria (Fig. 1G).

![Figure 1. Low MOI of H. pylori protects splenocytes from spontaneous apoptosis and stimulates cellular proliferation. A, effect of different concentrations of H. pylori SS1, expressed as multiplicity of infection, on apoptosis. Obtained by both PI staining of fixed cells followed by flow cytometry and quantification of apoptosis by ModFit LT software and by DNA fragmentation ELISA. B, time course of apoptosis in response to low and high MOI of H. pylori. C, representative histogram plots of PI-stained cells with % cells in the sub-G0-G1 fraction, indicative of apoptosis, marked. D, representative plots of Annexin V versus PI for the conditions as marked. Percentage of cells in the Annexin V+/PI+ quadrant, indicative of late apoptosis. E, effect of MOI on cellular proliferation, as determined by BrdUrd incorporation. F, effect of different bacterial preparations on splenocyte apoptosis. HP, live intact H. pylori; HP Fixed, formalin fixed H. pylori; HP Lys, H. pylori French press lysate. C. rod Lys, Citrobacter rodentium French press lysate. G, 2,3-bis-(2-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyaniline assay of cell viability in splenocytes stimulated with different preparation of H. pylori. *, P < 0.05; **, P < 0.01 increased versus unstimulated cells; x, P < 0.05; xx, P < 0.01 decreased versus unstimulated cells (A, B, and E-G). In (A) to (G), a minimum of four mice were used in separate experiments, each done in duplicate or triplicate.
Low MOI of \textit{H. pylori} reduces mitochondrial membrane depolarization, caspase-3 and caspase-9 activation, and cytochrome c release and increases Bcl-2 levels. Because we have found that macrophage apoptosis caused by \textit{H. pylori} was associated with mitochondrial membrane depolarization and caspase-3 activation (5), we assessed these variables in splenocytes. Control splenocytes exhibited substantial mitochondrial membrane depolarization, whereas low MOI \textit{H. pylori} reversed this pattern, and high MOI exacerbated it (Fig. 2A). Consistent with these findings, we found that caspase-3 activity was reduced by 49.8 ± 3.9% and increased by 205.6 ± 5.5% in the splenocytes exposed to MOI of 5 and 100, respectively (Fig. 2B). Western blotting (Fig. 2C) revealed that compared with control cells, MOI 5 treatment resulted in increased uncleaved caspase-3 and decreased cleaved caspase-3, indicating less active enzyme. In parallel with the return of apoptosis in the MOI 100–treated cells, there was a decrease in uncleaved and an increase in cleaved caspase-3 compared with the MOI 5–treated cells.

Because of these changes in caspase-3, we assessed upstream caspases. Similar to caspase-3, there was increased uncleaved caspase-9 at MOI of 5 compared with control or MOI 100–treated cells, indicating protection from caspase-9 activation (Fig. 2C). In contrast, levels of uncleaved caspase-8 were not significantly affected by \textit{H. pylori} (Fig. 2C). These data suggest that the preventive effect of low MOI of \textit{H. pylori} on apoptosis is caspase-8–independent and may be a direct effect on the mitochondria. Therefore, we assessed cytochrome c and found that at low MOI, there was a significant reduction in cytosolic cytochrome c levels, indicating prevention of mitochondrial cytochrome c release to the cytosol (Fig. 2C). The antiapoptotic protein Bcl-2 is associated with protection from mitochondrial membrane depolarization (25); consistent with this, we found that Bcl-2 protein levels were increased in cells exposed to low MOI \textit{H. pylori} and returned to low levels in MOI 100–treated cells (Fig. 2D).

We also tested whether low MOI of \textit{H. pylori} could protect cells from chemically induced apoptosis. With induction by either the protein kinase inhibitor, staurosporine, or the transcriptional inhibitor actinomycin D, there was no protection with addition of low MOI of \textit{H. pylori}. Representative data for percent apoptosis determined by Annexin V staining and flow cytometry were as follows: control, 35.1 ± 0.6%; staurosporine (0.5 μmol/L), 76.7 ± 3.2%; staurosporine + \textit{H. pylori}, 74.6 ± 2.4%; actinomycin D (0.5 μg/mL), 54.5 ± 0.7; actinomycin D + \textit{H. pylori}, 65.8 ± 0.2. These data suggest that the spontaneous apoptosis in the splenocytes that can be reduced by low MOI of \textit{H. pylori} is likely occurring through different pathways than those activated by these chemical inducers.

**Activation of total splenocytes results in protection from apoptosis of B cells at low MOI of \textit{H. pylori} and induction of T-cell apoptosis at high MOI.** We sought to determine which cells are affected by the low and high MOI \textit{H. pylori}. In initial experiments, we removed monocytes/macrophages by adherence and found that the remaining cells still retained protection from apoptosis when stimulated with low MOI \textit{H. pylori} (data not shown). After 24 hours of coculture of total splenocytes with \textit{H. pylori}, splenocytes were subjected to FACS using antibodies to T cells (anti-CD3) and B cells (anti-CD19). In the control cells, 23.5 ± 2.1% were CD3⁺ and 49.4 ± 3.5% were CD19⁺, similar to levels reported in previous studies (22). CD3⁺ cells exhibited significant induction of apoptosis above control levels with MOI of 100 but were not protected at MOI 5 (Fig. 3A). The CD19⁻ cells representing the non-B cells had a similar pattern. In contrast, the CD19⁻ cells had clear protection from apoptosis at MOI 5, with the same result seen with the CD3⁻ cells, representing mainly B cells (Fig. 3A).
To confirm the protection from apoptosis in the B cells, we studied CD19+-gated cells during the time course of development of early and late apoptosis. As shown in Fig. 4A (top), in unstimulated total splenocytes, from time 0 to 8 hours, there was an increase in both early (Annexin V+/7-AAD-) and late (Annexin V+/7-AAD+) apoptosis and progression of early apoptotic cells into late apoptosis at 24 hours. In the MOI 5-treated cells, this progression to late apoptosis at 24 hours was prevented (Fig. 4A, middle). In contrast, with MOI 100 stimulation, there was an increase in early apoptosis at 8 hours compared with control cells and a marked progression of apoptosis from early to late at 24 hours (Fig. 4A, bottom). When the CD19+-gated cells were assessed (Fig. 4B), the results mirrored those in the total splenocytes, with the progression of early apoptotic cells to late apoptosis (Fig. 4B).
Isolated B cells maintain protection from apoptosis. Because it has been reported that H. pylori–associated MALT lymphoma involves B-cell stimulation by T cells, we determined whether T cells were required for the protection from apoptosis in the B cells. We isolated CD19+ cells from splenocytes by FACS before stimulation and found that after 24 hours of coculture with H. pylori, the protective effect of low MOI of H. pylori was maintained, and as in the total splenocytes, the CD19+ cells failed to be protected at low MOI and exhibited potentiation of apoptosis at high MOI (Fig. 3C).

Because we had found that FACS-isolated B cells were protected from apoptosis at low MOI, we sought to confirm these findings by a second technique for B-cell isolation (MACS) and for apoptosis determination (Annexin V/7-AAD staining). In initial experiments, we found that the MACS-purified B cells had higher rates of spontaneous apoptosis at 24 hours of coculture with H. pylori, the protective effect of low MOI of H. pylori was maintained, and as in the total splenocytes, the CD19+ cells failed to be protected at low MOI and exhibited potentiation of apoptosis at high MOI (Fig. 3C).

Figure 5. Apoptosis in purified B-cell subsets is inhibited by low MOI of H. pylori. B cells were isolated by negative selection using anti-CD43–labeled magnetic beads (MACS) and stained using anti-IgD and anti-IgM. The first column (A) represents contour plots for B-cell subpopulations defined as T1 + marginal zone (MZ; IgMhighIgDlow), T2 (IgMhighIgDhigh), and mature FoB (IgMlowIgDhigh) cells. The boxes are percentage of cells in each of the B-cell subsets. The total B cells and gated subsets were then further analyzed for early and late apoptosis using staining for Annexin V-APC/7-AAD and flow cytometry in the second through fifth columns (B). Representative data from one experiment. Similar results were observed in five experiments, each using pooled cells from three mice.

Low MOI of H. pylori prevents progression of apoptosis in B-cell subsets. To analyze the subpopulations, the purified B cells were immunophenotyped by staining with IgM and IgD (Fig. 5A). As shown in Fig. 5B, the T1 + marginal zone cells exhibited substantial increases in both late apoptosis (Annexin V+/7-AAD+) and necrosis (Annexin V−/7-AAD−) from 0 to 18 hours, which were reduced by MOI of 5 of H. pylori, with a doubling of live cells (Annexin V−/7-AAD−). When live B cells were gated, we also found that CD9+ marginal zone B cells had a significant increase in number with MOI of 5 (1.5±0.15-fold increase versus unstimulated control, P < 0.05, n = 5) compared with the other cell subsets (data not shown). The MOI 100 treatment exacerbated the progression to death compared with the unstimulated cells, as highlighted by the very low percentage of live cells (9.2%). In the mature FoB cells, there was a similar pattern of events with progression to late apoptosis and necrosis in the unstimulated cells that was attenuated with MOI of 5 and exacerbated with MOI of 100. The T2 cells also exhibited spontaneous apoptosis at 18 hours, but there were substantially higher levels of surviving cells when compared with the T1/marginal zone and mature FoB cells. H. pylori at MOI of 5 reduced the progression to late apoptosis in the T2 cells, and MOI of 5 markedly increased the late apoptosis and reduced the live cells compared with the unstimulated cells.

top) again prevented by exposure to H. pylori MOI of 5 (Fig. 4B, middle) and increased by MOI 100 (Fig. 4B, bottom) compared with unstimulated cells.
In summary, all three of the B-cell subsets undergo spontaneous apoptosis with the levels of apoptosis in the order of T1/marginal zone > FoB > T2, and there was protection from late apoptosis in all three populations with low MOI of *H. pylori*, with the increase in live cells most pronounced in the T1/marginal zone and FoB cells. Taken together, these data indicate that the low MOI of *H. pylori* has a global effect on B-cell subsets, and that the net effect is increased cells available for response to antigenic stimulation and subsequent differentiation.

Low MOI of *H. pylori* stimulates proliferation of B cells within total splenocytes but not when these cells are isolated before activation. Because we had observed an increase in proliferation at low MOI in Fig. 1, we determined which populations of lymphocytes were responsible for this response. When total splenocytes were activated, most of the increase in proliferation with MOI of 5 derived from the B cells (Fig. 3B), with CD19+ and CD3− cells showing 4- and 3-fold increases, respectively, compared with only a small increase in the CD3+ cells that did not occur in the CD19− cells. However, when the FACS-separated B cells were activated, they lost the proliferative response to low MOI *H. pylori* (Fig. 3D), indicating that the proliferation of B cells is likely to be dependent on exposure to T cells activated by *H. pylori*.

Infection of the gastric mucosa with *H. pylori* imparts protection from apoptosis in splenic B cells. Because isolation of large numbers of lymphocytes from the lamina propria of the mouse stomach is not feasible and induction of immune responses in splenocytes of infected mice has been reported (26), we studied effects of gastric infection on splenocyte apoptosis. In initial experiments with splenocytes from mice infected for 4 months, at which time gastritis is consistently apparent (4, 14), we found a significant reduction in spontaneous apoptosis 24 hours after isolation compared with splenocytes from naive mice (Fig. 6A). We then conducted studies in mice infected with *H. pylori* for 12 months. These mice did not possess more extensive gastritis than the 4-month mice, but we found even more survival in the splenocytes from infected mice with a 38.0 ± 8.9% reduction in apoptosis compared with naive mice (Fig. 6B). Sorting experiments showed that the protection from apoptosis in the splenocytes from infected mice derived from the CD19+ B cells, which was confirmed by similar results in the CD3− population (Fig. 6B).

**Discussion**

In our model system, splenocytes underwent a time-dependent increase in apoptosis without activation; similar results have been reported (24), as has protection from apoptosis by mitogenic stimuli, such as phorbol 12-myristate 13-acetate and concanavalin A (22). Although it is accepted that immature T and B cells are predisposed toward apoptosis, similar findings have been reported in mature splenic T (23) and B cells (24). Spontaneous apoptosis has been reported to be inhibited by protein kinase C (PKC) activation (22–24) and exacerbated by PKC inhibition in both T and B cells (23, 24). However, B lymphocyte survival has also been shown to be mediated by PKC-independent pathways (10). In our studies, we found that low MOI of *H. pylori* could not inhibit staurosporine-induced apoptosis, suggesting that the protective effect of *H. pylori* is not likely to be mediated by an effect on protein kinases. Additionally, we found that apoptosis induced by the transcriptional inhibitor actinomycin D was not inhibitable with low MOI of *H. pylori*. This indicates that low MOI *H. pylori* is not a general inhibitor of apoptosis, but rather, its effect is most relevant to regulation of B-lymphocyte homeostasis. The protective effect of *H. pylori* is not likely to be mediated by effects on Fas because there was no inhibition of Fas ligand or receptor expression with low MOI of *H. pylori* (data not shown). B cells have been shown to be protected from apoptosis by interleukin-4 (24). However, it has been shown that splenocytes do not produce interleukin-4 with *H. pylori* (data not shown). B cells have been shown to be protected from apoptosis by interleukin-4 (24). However, it has been shown that splenocytes do not produce interleukin-4 with *H. pylori* treatment (26). Additionally, it is unlikely that the protective effects of low MOI require T cell–derived cytokines because our data show that isolated B cells were still protected from apoptosis in the absence of T cells.

Our sorting experiments indicated that T cells were not protected from apoptosis at low MOI and exhibited apoptosis at high MOI. Similar findings have been reported in the Jurkat T cell line, although it should be noted that these cells have low basal apoptosis because they are a tumor cell line (6). In contrast, we found that CD19+ B cells were protected from apoptosis and underwent proliferation with exposure to low MOI of *H. pylori*. Transfection of the *H. pylori* product CagA in an interleukin-3-dependent B-cell line has been shown to protect cells from hydroxyurea-induced apoptosis (27). This cell line did not exhibit spontaneous apoptosis, and CagA inhibited proliferation (27), whereas we found that low MOI of *H. pylori* induced proliferation of B cells. This leads to the question as to what bacterial factors may be responsible for the survival of B cells. A recent study of strains isolated from patients found no association of major *H. pylori* virulence genes or adhesins with MALT lymphoma when compared with gastritis alone, leading to the conclusion that proinflammatory strains are not linked to MALT pathogenesis (28). Similarly, we have found that strains deficient in the *H. pylori*......
proteins cagA, ure1, and picB did not lose the ability to protect cells from apoptosis (data not shown). Additionally, our data may be Helicobacter pylori specific because when we tested the Gram-negative colitis-inducing pathogen C. rodentium, there was no protection from apoptosis at low MOI.

Our most striking result is that it was the splenic B cells that were protected from spontaneous apoptosis by low MOI of H. pylori. It is believed that upon antigenic stimulation, T cells die, whereas T2 cells differentiate into mature FoB or marginal zone B cells (29, 30). The differentiation of T2 cells into mature FoB or marginal zone B cells may depend on the nature of the antigen encountered (31). Our data indicate that low MOI of H. pylori results in protection from apoptosis in each of the subsets we assessed, suggesting that H. pylori may exert its survival effects at both early and late stages of B-cell development. It has been shown that the MALT lymphoma of the H. pylori–infected stomach derives from marginal zone B cells (13, 32). Our experiments in MACS-purified B cells suggest that splenic marginal zone B cells are protected from apoptosis and are significantly increased in number following infection with low MOI of H. pylori. This would be consistent with the established role of the marginal zone B cells as sensors of bacterial stimuli and their importance in innate immunity (11, 13). However, because the marginal zone B cells represent a small proportion of the total splenic B cells (33), it is likely that the other B-cell subsets also contribute to the reduction in apoptosis that we have observed.

Isolated B cells retained protection from spontaneous apoptosis when exposed to low MOI of H. pylori. This was observed whether cells were separated by FACS using antibody to CD19, or negative selection using antibody to CD43 to remove non-B cells by MACS. These data seem to be in conflict with the previous view that MALT lymphoma involves T-cell help in that invading tumor B cells in the stomach require the presence of T cells (10, 34, 35). However, it is important to realize that our studies are conducted in non-tumorigenic naive splenocytes. Moreover, because we found that all of the subsets were protected from apoptosis at low MOI H. pylori, this suggests that we are observing an innate survival phenomenon rather than a T helper cell–mediated process. The loss of B-cell proliferation when B cells were exposed to low MOI after isolation indicates that for proliferation, H. pylori–specific T-cell help is needed. Taken together, these data suggest that low levels of H. pylori infection can have a direct effect on B cells to active survival pathways, but that expansion of these cells as is seen in MALT lymphoma may require further stimulation by T cell–derived factors. It remains to be determined what mechanisms may be mediating the interaction of the low MOI of H. pylori with the induction of the B-cell survival. Certainly, the implication that B-cell receptor activation and the effects of B cell–activating factor (BAFF) are critical to the survival of splenic B-cell populations (10, 36) would suggest that these elements could be involved in the survival effect induced by low MOI H. pylori that we have observed.

In our in vivo data show that when mice have gastric infection with H. pylori, their splenic B cells exhibit protection from spontaneous apoptosis. This suggests that there is communication between the stomach and the spleen. This might occur either by H. pylori–derived antigens or other bacterial components reaching the spleen through the bloodstream, or by gastric lymphocytes traveling to the spleen after activation by H. pylori directly or by antigen-presenting cells. The possibility that there is a systemic effect on lymphocyte survival by the gastric infection is highly provocative. Our finding that the splenocytes of the H. pylori–infected mice exhibit a phenotype of increased B-cell survival is consistent with the finding that the human host has been shown to make antibodies to numerous H. pylori antigens shortly after infection (37), indicative of systemic B-cell activation. The C57BL/6 mice that we used in our studies did not develop lymphoid follicles, germinal centers, or lymphoproliferative lesions that are observed in human MALT lymphoma and can occur in late-stage infection of BALB/c mice infected for ≥18 months with Helicobacter felis (35, 38) or Helicobacter heilmannii (38). The propensity of BALB/c mice to develop MALT lymphoma has been linked to their predisposition to exhibit Th2 responses (35, 38, 39). Because C57BL/6 mice are known to develop Th1-skewed responses to H. pylori (26), which mimics the case in human infection (40), we considered it appropriate to use this mouse model for our studies of lymphocyte responses. It will be intriguing to compare findings in C57BL/6 mice with those in BALB/c mice in future investigations. In summary, our report provides new insights into the persistence of H. pylori infection and may have relevance to the pathogenesis of MALT lymphoma because our major observation is that exposure of lymphocytes to biologically relevant concentrations of H. pylori (7, 8) leads to enhanced B-cell survival.

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