Heat Shock Protein 60, via MyD88 Innate Signaling, Protects B Cells from Apoptosis, Spontaneous and Induced[1]

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We recently reported that heat shock protein 60 (HSP60) via TLR4 signaling activates B cells and induces them to proliferate and secrete IL-10. We now report that HSP60 inhibits mouse B cell apoptosis, spontaneous or induced by dexamethasone or anti-IgM activation. Unlike HSP60 enhancement of B cell proliferation and IL-10 secretion, TLR4 signaling was not required for the inhibition of apoptosis by HSP60; nevertheless, MyD88 was essential. Inhibition of apoptosis by HSP60 was associated with up-regulation of the antiapoptotic molecules Bcl-2, Bcl-x_L, and survivin, maintenance of the mitochondrial transmembrane potential, and inhibition of caspase-3 activation. Moreover, B cells incubated with HSP60 manifested prolonged survival following transfer into recipient mice. These results extend the varied role of HSP60 in the innate regulation of the adaptive immune response. *The Journal of Immunology,* **2009, 183: 890–896.**

The adaptive immune response is regulated, among other factors, by a balance between the proliferation of activated immune cells and their death. Apoptosis is required both to terminate the adaptive immune response and to regulate lymphocyte maturation and selection (1, 2). The balance between activation signals, apoptosis-inducing signals, and survival signals is notable in B cells. Mature B cells undergo spontaneous apoptosis in vitro, and their survival requires cytokines or antigenic stimulation (3, 4). B cell activation by anti-IgM Ab induces B cell proliferation; however, BCR multimerization of surface Ig also can induce apoptosis (5, 6). Activation of mature B cells by Ags requires a second signal that inhibits surface Ig-mediated apoptosis; this signal can originate from interacting T cells. By prolonging or shortening the life of Ag-activated B cells, factors that regulate B cell apoptosis play an important role in regulating the humoral immune response. Here, we report that heat shock protein 60 (HSP60)[3] is such a factor.

HSP60 has long been known as an important intracellular chaperon that functions to help nascent or denatured proteins fold into proper shape (7). However, HSP60 also has been discovered to function outside the cell as a signal to the immune system, both as an Ag for T cells (8–12) and B cells (13) and as a ligand for the innate immune system via TLR signaling (14–17). The local expression of HSP60 appears to serve as an immune biomarker (18); expression of HSP60 was shown to be up-regulated on the surface of activated T cells (19, 20) and at sites of inflammation (21). We recently reported that human HSP60 activates naive mouse B cells to up-regulate their expression of MHC class II and costimulatory molecules and to proliferate and secrete IL-10 (22). These effects of HSP60 were found to be largely dependent on innate TLR4 and MyD88 signaling.

In view of the importance of apoptosis in regulating immune repertoires and responses, we undertook to learn whether extracellular HSP60 might affect B cell survival and apoptosis. The results provide additional information on the complex influences of HSP60 on the immune system.

Materials and Methods

Mice

Female C57BL/6J mice were purchased from Harlan Olac. TLR4 knockout mice, GFP transgenic mice, and C3HeB/FeJ and C3H/HeJ mice were obtained from The Jackson Laboratory. MyD88 knockout mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan) (23). The mice were maintained in a specific pathogen-free facility and were used at the age of 5–8 wk. The experiments were conducted under the supervision of the Institute Animal Welfare Committee according to established practice.

Reagents

Dexamethasone (DEX) was purchased from Sigma-Aldrich. CpG, a phosphorothioate oligonucleotide was synthesized at the Oligonucleotide Synthesis Unit of the Weizmann Institute of Science (Rehovot, Israel). The oligonucleotide CpG contains two 9-mer segments, ^{59}TCCATAAC GTTGCAAACGTTCTG^{39}. Anti-IgM was purchased from Jackson ImmunoReaserch Laboratories. Ab against B220 was purchased from eBioscience.

Purification of B cells

Spleen cell suspensions were depleted of RBC by treatment with red blood lysis buffer (Sigma-Aldrich). B cells were then purified by negative selection with a B cell isolation kit containing biotin-conjugated mAbs to CD43, CD4, and Ter-119 (Miltenyi Biotec). This procedure routinely yielded B cell preparations that were >95% positive for the B220 marker, determined by FACS analysis. In the experiments, purified B cells were cultured for 12 or 48 h in medium alone (untreated) or in the presence of different concentrations of stimulators, as indicated.

HSP60 preparation and LPS reagents

Human HSP60 was prepared as described (24). Endotoxin contamination of the preparations was determined using the kinetic turbidmetric *Limulus* amebocyte lysate test, performed by an independent laboratory (Biological Industries); endotoxin content was <0.0001 endotoxin unit/μg protein, corresponding to <0.01 pg LPS equivalents/μg recombinant HSP60. We used LPS of *Escherichia coli* strain 055:B5 (chromatographically purified and phenol extracted; Sigma-Aldrich) or LPS of *Salmonella minnesota* Re595 (Sigma-Aldrich).

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[3] Abbreviations used in this paper: HSP60, heat shock protein 60; DEX, dexamethasone; $\Delta\Psi_m$, mitochondrial transmembrane potential.

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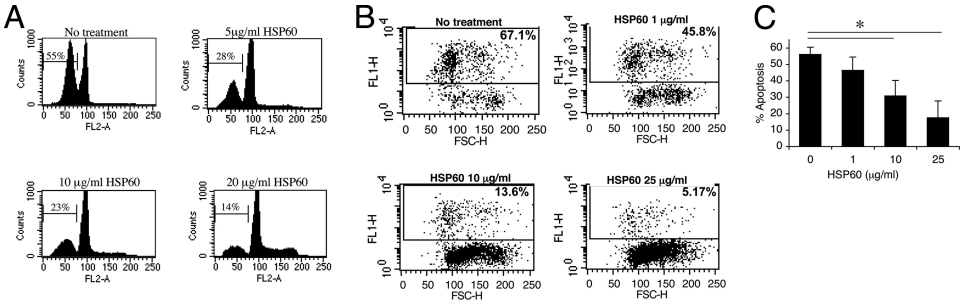

FIGURE 1. HSP60 protects B cells from spontaneous apoptosis. *A*, B cells were treated with HSP60 and were analyzed for apoptosis by propidium iodide staining. The histograms show the DNA content. The percentage of apoptotic cells, as determined by the sub-G_1 DNA content, is shown at the left of each histogram. These data are representative of three experiments; statistical analysis of the means and SDs of the three experiments showed significant differences from the untreated group at 10 and 20 μg/ml HSP60. *B*, Purified B cells were cultured for 48 h in medium alone (untreated) or in the presence of different concentrations of HSP60, as indicated. The resulting populations were analyzed for apoptosis by the APO-Direct TUNEL kit staining. The percentage of apoptotic cells, as determined by flourescein-dUTP$^+$ cells, is shown at the right corner of each dot plot. Similar data were obtained in each of three experiments; statistical analysis of the means and SDs of the three experiments is shown in *C*. *, $p < 0.05$ vs no treatment by the two-tailed Welch *t* test.

Western blot analysis

Whole-cell lysates were made using lysis buffer containing 137 mM NaCl, 20 mM Tris HCl (pH 7.5) 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, and 2 mM EDTA. Proteins were quantified using the BCA assay kit (Pierce), and 30–60 μg of protein was separated in 15% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Proteins were detected after 1 h at room temperature with monoclonal anti Bcl-2, anti Bcl-x_L, or anti-survivin (eBioscience). Polyclonal Ab anti-total ERK1/2 was obtained from Sigma-Aldrich. The blots were incubated with HRP-conjugated anti-rabbit IgG or anti-mouse IgG Abs (Jackson ImmunoReaserch Laboratories) and then developed by ECL.

Flow cytometric analysis

B cell apoptosis was detected by flow cytometry. Briefly, purified B cells were seeded in 24-well plates, 5×10^5 per well, and incubated for 48 h at 37°C and 5% CO_2 in the presence of HSP60 at the indicated concentration. For cell cycle analysis, the cells were washed once in cold PBS, fixed in 2 ml cold methanol (−20°C) for 30 min, centrifuged, and resuspended in 0.5 ml of PBS containing RNase A (100 μg/ml) and propidium iodide (50 μg/ml). The cells were then subjected to flow cytometric analysis; for each sample 10,000 cells were collected and cell cycle distribution was analyzed according to relative DNA content (propidium iodide staining). Cell debris was electronically gated out at low forward light scatter, and the percentage of cells in different cell cycle phases was computed using CellQuest software (BD Biosciences). The cells were then collected, stained by an APO-Direct TUNEL kit (Phoenix Flow Systems), and mitochondrial potential was measured by DePsipher (R&D Systems) according to the manufacturer's procedures. Intracellular caspase activity was measured by an ApoStat kit (R&D Systems) as indicated in the manufacturer's protocol. The cells were analyzed by flow cytometry using a FACSort (BD Biosciences) and CellQuest software (BD Biosciences). Forward light scatter is labeled by FSC-H, intensity (area) in the FL2 channel is termed FL2-A.

Apoptosis induction and determination

Spontaneous apoptosis was measured after culturing B cells with full medium without activation and was determined after 48 h. The amount of apoptosis was quantified by propidium iodide staining and APO-Direct TUNEL kit staining. Apoptosis was induced by incubating the cells with 5 nM DEX or with 1 μg/ml goat anti-mouse IgM for 48 h.

Statistical analysis

The InStat 2.01 program (GraphPad Software) was used for statistical analysis, by using the Welch *t* test, two-sided. Differences were considered statistically significant at $p < 0.05$. The statistical significance of repeated flow cytometric analyses was done by combining data from the various repeats and analyzing the means and SDs of the different groups (see Figures).

Results

HSP60 protects B cells from spontaneous apoptosis

To learn whether HSP60, a B cell mitogen (22), can also protect B cells from spontaneous apoptosis in vitro, we treated freshly isolated B cells with different concentrations of HSP60. Fig. 1*A* shows that spontaneous apoptosis of the B cells was inhibited in a dose-dependent manner by incubation with HSP60. We confirmed this conclusion using the TUNEL assay. Fig. 1*B* shows that B cells cultured with medium alone manifested 67% TUNEL-positive apoptotic cells; coculturing the B cells with increasing concentrations of HSP60 decreased B cell apoptosis to 5.17% at 25 μg/ml. Fig. 1*C* shows an analysis of the results pooled from three similar experiments.

HSP60 rescues B cells from apoptosis induced by anti-IgM

Although anti-IgM stimulates B cell proliferation in vitro, a substantial proportion of mature B cells also undergo apoptosis

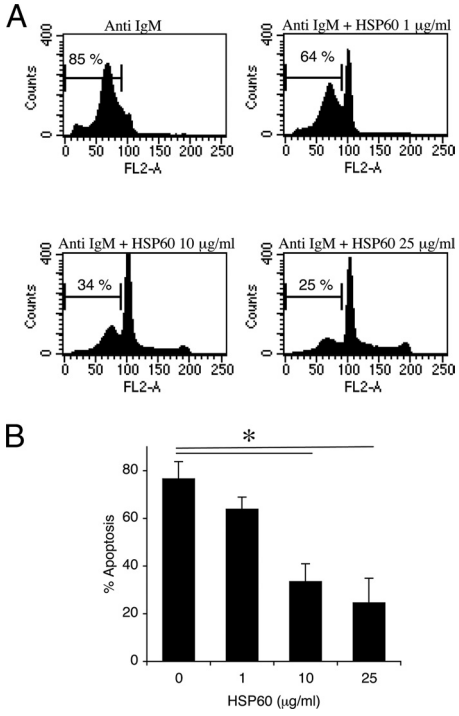

FIGURE 2. HSP60 rescues B cells from apoptosis induced by anti-IgM. *A*, B cells were cultured with goat anti-mouse IgM (1 μg/ml; control) or with anti-IgM in the presence of different concentrations (1, 10, or 25 μg/ml) of HSP60. The resulting populations were analyzed for apoptosis by propidium iodide staining. Similar data were obtained in each of three experiments; statistical analysis of the means and SDs of the three experiments is shown in *B*. *, $p < 0.05$ vs untreated with HSP60 by the two-tailed Welch *t* test.

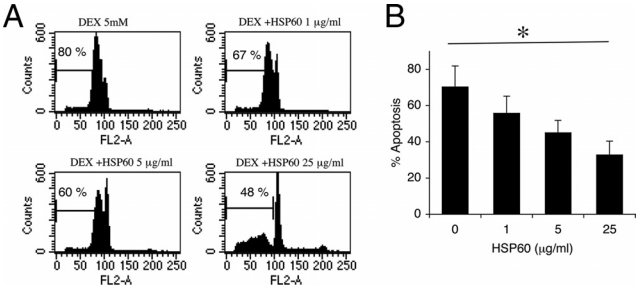

FIGURE 3. HSP60 rescues B cells from apoptosis induced by DEX. *A*, B cells were cultured with DEX (5 nM) or with DEX in the presence of different concentrations of HSP60 (1, 5, or 25 μg/ml). The resulting populations were analyzed for apoptosis. Similar data were obtained in each of three experiments; statistical analysis of the means and SDs of the three experiments is shown in *B*. *, $p < 0.05$ vs untreated with HSP60 by the two-tailed Welch *t* test.

in response to BCR cross-linking (5), a phenomenon called activation-induced cell death. We tested whether HSP60 could signal B cells to inhibit activation-induced cell death. Fig. 2*A* shows that B cells cultured with anti-IgM alone underwent 85% apoptosis, but coculturing the B cells with increasing concentrations of HSP60 decreased B cell apoptosis to 25% at 25 μg/ml. We confirmed these results using the TUNEL assay (not shown). Fig. 2*B* shows an analysis of the results pooled from three similar experiments.

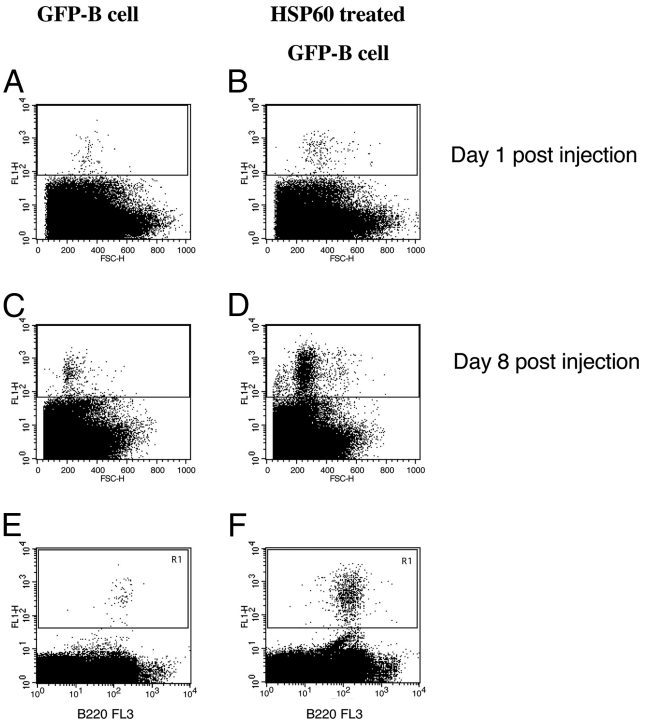

FIGURE 4. HSP60-treated B cells manifest prolonged survival in vivo. B cells from GFP-C57BL6 mice were cultured for 12 h with HSP60 (30 μg/ml). The cells were then washed extensively and 20×10^6 cells were injected i.p. into wild-type C57BL/6J mice. After 1 and 8 days, the mice were sacrificed and their spleens were analyzed for GFP^+ cells by flow cytometry. *A*, *C*, and *E*, Untreated B cells; *B*, *D*, and *F*, HSP60-treated B cells; *E* and *F* designate B cells stained at 8 days with anti-B220 Ab. In the experiment shown here, there were 2.8- and 5.3-fold increases of GFP B cells following HSP60 activation after 1 or 8 days, respectively. An additional experiment showed an increase of 12.9-fold of GFP B cells following HSP60 activation.

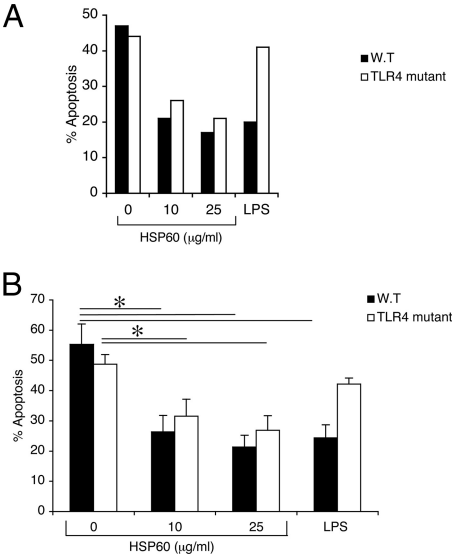

FIGURE 5. HSP60 can protect TLR4 mutant B cells from apoptosis. *A*, B cells from C3H/HeB or C3H/HeJ (TLR4 mutant) mice were cultured with concentrations of HSP60 of 10 or 25 μg/ml or with 1 μg/ml of LPS as indicated. The resulting populations were analyzed for apoptosis by propidium iodide staining. Similar data were obtained in each of three experiments; statistical analysis of the means and SE of the three experiments is shown in *B*. *, $p < 0.05$ vs untreated with HSP60 by the two-tailed Welch *t* test. W.T, wild type.

HSP60 rescues B cells from apoptosis induced by DEX

Glucocorticoids have important antiinflammatory and immunosuppressive properties (25), induce apoptosis of lymphocytes, and are widely used as therapeutic agents in many disorders (26). Treatment of mice with DEX leads to depletion of B cells in the bone marrow (27) and spleen (28). Fig. 3*A* shows that incubation of B cells with DEX (5 nM) induced massive apoptosis in culture; that is, ~80% apoptosis was detected after 48 h. However, exposure of B cells to increasing concentrations of HSP60 effectively inhibited the DEX-induced apoptosis; treatment with 25 μg/ml HSP60 reduced the apoptosis from 80% to 48%. These results were confirmed by TUNEL assay (not shown). Fig. 3*B* shows an analysis of the results pooled from three similar experiments.

HSP60-treated B cells manifest prolonged survival in vivo

We used B cells expressing GFP as probes to learn whether HSP60 treatment could enhance the survival of B cells in the in vivo environment. B cells were isolated from GFP mice and incubated for 12 h with 30 μg/ml HSP60 or with medium alone. The B cells were then washed extensively, and 20×10^6 GFP-labeled B cells were injected i.p. into wild-type recipient C57BL/6J mice. By counting the numbers of GFP-labeled cells in the recipients, we could detect the effect of HSP60 exposure on B cell survival. The spleen, bone marrow, lymph nodes, and peripheral blood of the recipient mice were collected on days 1 and 8 postinjection, and the green fluorescent cells were counted by FACS analysis. After 1 day, we could detect GFP-labeled B cells mainly in the spleens; the HSP60-treated B cells were three times more prevalent than the untreated B cells (Fig. 4, *A* and *B*). HSP60-treated GFP-labeled B cells also could be detected in small numbers in the bone marrow, lymph nodes, and peripheral blood (not shown). Eight days after injection, the HSP60-treated B cells had increased in number and were 5.3-fold more prevalent than were the untreated B cells (Fig. 4, *C* and *D*). Staining the spleen cells for both

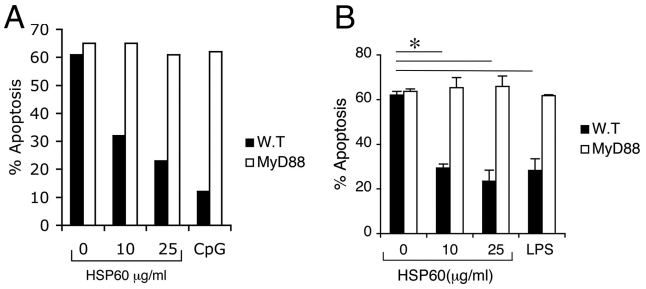

FIGURE 6. MyD88 is required for protection from apoptosis induced by HSP60. *A*, B cells from wild-type (W.T) or MyD88$^{-/-}$ (MyD88) mice were cultured in the presence of concentrations of HSP60 (1, 10, or 25 μg/ml) or CpG (1 μg/ml) as indicated. The resulting populations were analyzed for apoptosis by propidium iodide staining. The histograms show the DNA content of the B cells. The percentage of apoptotic cells, as determined by the sub-G_1 DNA content, is shown. Similar data were obtained in each of three experiments; statistical analysis of the means and SDs of the three experiments including LPS is shown in *B*. *, $p < 0.05$ vs untreated with HSP60 by the two-tailed Welch *t* test.

GFP and B220 at 8 days confirmed the increased numbers of HSP60-treated B cells (see Fig. 4, *E* and *F*). These results indicate that exposure to HSP60 in vitro can enhance the survival of B cells in vivo.

HSP60 can protect TLR4 mutant B cells from apoptosis

We previously reported that the ability of HSP60 to induce proliferation and cytokine secretion by B cells was dependent on TLR4 signaling (22). To learn whether TLR4 signaling also mediates the protection from apoptosis induced by HSP60, we analyzed the effect of HSP60 on the survival of B cells isolated from wild-type C3H/HeB mice and from TLR4 mutant C3H/HeJ mice. Fig. 5*A* shows that treatment with LPS, a known ligand for TLR4 (29), could significantly inhibit apoptosis in wild-type mice, but was much less effective in TLR4 mutant mice ($p < 0.05$). HSP60 in concentrations of 10 or 25 μg/ml, however, significantly protected TLR4 mutant B cells and wild-type B cells to the same degree (Fig. 5*B*). Similar results were obtained when we compared wild-type TLR4$^{+/+}$ B cells to TLR4$^{-/-}$ knockout B cells (not shown). These results clearly indicate that HSP60-induced protection from apoptosis is not dependent on TLR4 signaling.

MyD88 is required for HSP60-induced protection from apoptosis

To learn if the effect of HSP60 required MyD88 innate signaling, we tested the B cells of MyD88 knockout mice; CpG, known to act in a MyD88-dependent manner, served as a control. We found that HSP60 could not protect MyD88$^{-/-}$ B cells from anti-IgM-induced apoptosis (Fig. 6*A*) or from spontaneous apoptosis (not shown). These results indicate that HSP60-induced B cell protection from apoptosis is dependent on MyD88, although it can proceed in the absence of functional TLR4. Similar data were obtained in each of three experiments including an LPS control for TLR4 signaling (Fig. 6*B*).

HSP60 induces increased expression of antiapoptotic molecules: Bcl-2, Bcl-x_L, and survivin

Members of the Bcl-2 family are well-known down-regulators of apoptosis (30). We therefore tested whether HSP60 might influence the levels of Bcl-2 and Bcl-x_L in B cells of C57BL/6

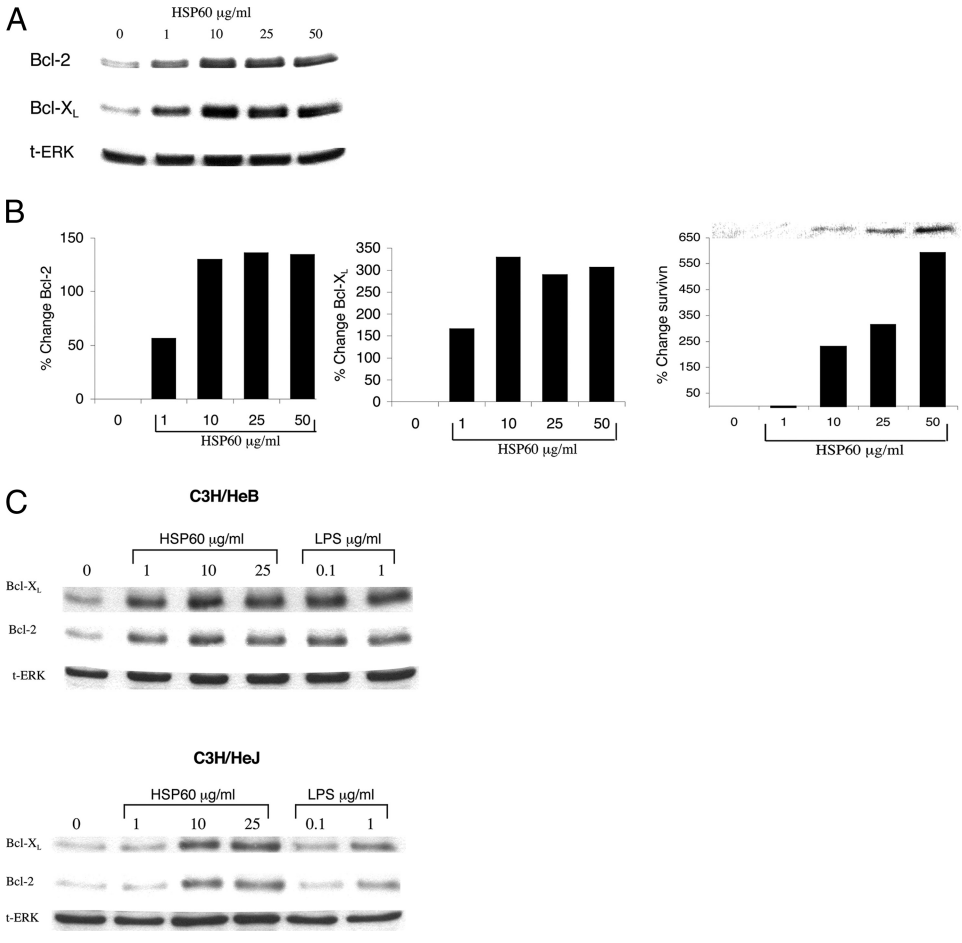

FIGURE 7. HSP60 induces increased expression of antiapoptotic molecules: Bcl-2, Bcl-x_L, and survivin. B cells were cultured in the presence of the indicated concentrations of HSP60. Total protein extracts (25 μg) were prepared from the cultures and analyzed for Bcl-x_L and Bcl-2 (*A*) or survivin protein expression (*B*) by Western blotting. Relative protein levels were determined by densitometry and reported as percentage enhancement compared with control B cells cultured with medium alone. Total Erk (t-ERK) was used as a loading control. One representative experiment of three with similar results is shown. *C*, Purified B cells from C3H/HeB or C3H/HeJ mice were cultured for 48 h in the presence of different concentrations of HSP60 (1, 10, or 25 μg/ml) or 0.1 or 1 μg/ml LPS as indicated. Total protein extracts (30 μg) were prepared from the cultures and analyzed for Bcl-x_L and Bcl-2 protein expression by Western blotting. Total Erk (t-ERK) was used as a loading control. One representative experiment of two with similar results is shown.

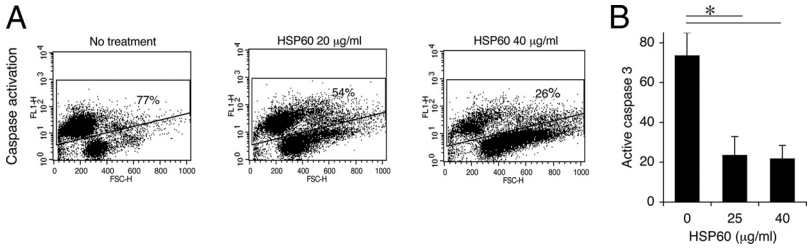

FIGURE 8. HSP60 down-regulates activation of caspase. *A*, B cells were cultured in the presence of concentrations of HSP60 of 20 or 40 μg/ml as indicated. The resulting populations were analyzed for apoptosis by intracellular caspase staining using the ApoStat kit (R&D Systems). Apoptosis was determined by flow cytometry. The dot plot FL-1 axis represents the cells that express active caspase. Similar data were obtained in each of three experiments; statistical analysis of the means and SDs of the three experiments is shown in *B*. *, $p < 0.05$ vs no treatment by the two-tailed Welch *t* test.

mice. Western blot analysis of whole-cell lysates showed that 1–50 μg/ml HSP60 induced a significant increase in the percentage of Bcl-2 and Bcl-x_L quantified using computerized densitometry (Fig. 7, *A* and *B*). Fig. 7*C* shows that C3H/HeB mice, which express functionally active TLR4, up-regulated Bcl-x_L and Bcl-2 in response to either HSP60 or LPS; in contrast, C3H/HeJ mice, which express a mutated TLR4 molecule, responded relatively more strongly to HSP60 than they did to LPS, which depends on TLR4 signaling. These results support the conclusion that the antiapoptotic effects of HSP60 on B cells are not dependent on TLR4.

Survivin, a member of the inhibitor of apoptosis family of proteins, plays a pivotal role in the regulation of cell death (31). Fig. 7*B* show that survivin was not expressed in resting B cells, but incubation with increasing concentrations of HSP60 for 48 h markedly increased the expression of survivin. Thus, HSP60 induces the up-regulation of the antiapoptosis molecules survivin, Bcl-2, and Bcl-x_L in B cells.

HSP60 down-regulates caspase activation

Members of the caspase gene family (cysteine proteases with aspartate specificity) play significant roles in both inflammation and apoptosis (32). Caspases are essential for some of the characteristic changes in cell morphology and certain biochemical events associated with the execution and completion of apoptosis. To detect the effect of HSP60 on caspase activity, we used a cell-permeable, FITC-conjugated pan-caspase inhibitor (ApoStat); in this assay, caspase activity was measured by increased fluorescence in the tested cells. Fig. 8*A* shows that B cells treated with HSP60 manifested significantly decreased the levels of active caspases, that is, 26% at 40 μg/ml compared with 77% in untreated B cells. Similar data were obtained in each of three experiments (Fig. 8*B*).

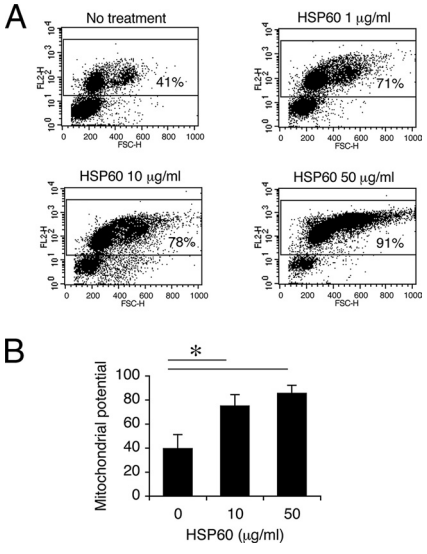

FIGURE 9. HSP60 inhibits the loss of $\Delta\Psi_m$ in B cell apoptosis. *A*, B cells were cultured in the presence of different concentrations of HSP60 (1, 10, or 50 μg/ml) as indicated. The resulting populations were analyzed for mitochondrial membrane potential staining using the DePsipher kit (R&D Systems). Apoptosis was determined by flow cytometry. Bright red fluorescence (FL-2) indicates high potential. Similar data were obtained in each of two experiments; statistical analysis of the means and SDs of the two experiments is shown in *B*. *, $p < 0.05$ vs untreated by the two-tailed Welch *t* test.

HSP60 blocks the loss of mitochondrial membrane potential occurring in B cell apoptosis

Cellular energy produced during mitochondrial respiration is stored as an electrochemical gradient across the mitochondrial membrane (mitochondrial transmembrane potential, $\Delta\Psi_m$); disruption of $\Delta\Psi_m$ has been shown to be one of the first intracellular changes following the onset of apoptosis (33). Therefore, we examined the effect of HSP60 on the $\Delta\Psi_m$ of B cells using DePsipher, a lipophilic cation that acts as a mitochondrial activity marker. DePsipher aggregates upon membrane polarization forming an orange-red fluorescent compound. If the mitochondrial potential is disturbed, the dye cannot access the membrane and the red fluorescence decreases. As shown in Fig. 9*A*, DePsipher accumulated in HSP60-treated B cells, reaching 91% at 50 μg/ml compared with only 41% in untreated B cells. Similar data were obtained in each of two experiments (Fig. 9*B*). Thus, HSP60 effectively blocked the loss of mitochondrial membrane potential occurring in the spontaneous apoptosis of B cells.

Discussion

In this study we report that HSP60 inhibits B cell apoptosis, spontaneous or induced, by DEX or anti-IgM. TLR4 was not involved in HSP60-enhanced survival; nevertheless, MyD88 was essential. Moreover, exposure of B cells to HSP60 in vitro prolonged their survival and enhanced their numbers in vivo. The effect of HSP60 on cell viability was associated with maintenance of the $\Delta\Psi_m$, up-regulation of the antiapoptotic Bcl-2, Bcl-x_L, and survivin molecules, and inhibition of caspase-3 activation.

Earlier, we reported that HSP60 induced B cells to proliferate and secrete IL-10 through a signaling pathway that required TLR4 and MyD88, but not TLR2 (22). However, we found here that the antiapoptotic effects of HSP60 were not mediated through TLR4, but that MyD88 was still required. It is possible that HSP60 may inhibit apoptosis through RP105 or through another TLR family member that uses MyD88 (34). It has been shown that an agonist Ab to RP105 protects B cells from apoptosis induced by irradiation or DEX and drives them to proliferate (35). However, MyD88 also acts downstream to the IL-1 and IL-18 receptors (23). It remains to

be discovered which, if any, of these mediators might participate in the down-regulation of apoptosis by HSP60.

Note that LPS could protect B cells from apoptosis only if functional TLR4 was present (Fig. 5); thus, the antiapoptosis effects of HSP60 on B cell survival cannot be attributed to LPS contamination. Moreover, the preparations of HSP60 we used were checked by the *Limulus* amebocyte lysate assay and found to contain only 0.01 pg of LPS per gram of HSP60; hence, a concentration of 10 μg/ml HSP60 should contain a concentration of 0.1 pg/ml LPS, a concentration below the sensitivity of B cells to LPS.

HSP60 has been discovered as a ligand of innate TLR receptors expressed on both innate immune cells (macrophages and dendritic cells (17, 36, 37)) and adaptive immune cells (T cells and B cells (14, 22)). These innate effects of HSP60 are complex: TLR signaling by HSP60 can induce responses that are either proinflammatory or antiinflammatory depending on the cell type and the concentration of HSP60. T cells, for example, respond via TLR2 to nanogram per milliliter concentrations of HSP60 (14, 15, 38); B cells, macrophages, and dendritic cells respond via TLR4 to microgram concentrations of HSP60 (39, 40). Macrophages and dendritic cells respond to HSP60 by activation and secretion of proinflammatory cytokines such as IL-12 and TNF-α (17, 39). The innate effect of HSP60 on B cells and T cells shifts their phenotype toward an antiinflammatory response: HSP60 down-regulates T cell chemotaxis (41) and secretion of IFN-γ and TNF-α upon activation by mitogenic anti-CD3 (38). HSP60 via TLR2 enhances T cell secretion of IL-10 and augments the function of $CD4^+CD25^+$ regulatory T cells (15). The effects of HSP60 on B cells, reported herein, are protection from apoptosis and enhanced survival. Additionally, HSP60 induces B cells to proliferate and to secrete IL-10 and IL-6, and it up-regulates the ability of B cells to act as APCs (22); Ag presentation by HSP60-treated B cells skews the T cell response toward a Th2 phenotype (22).

The innate effects of HSP60 on B cells appear to be specific for mammalian HSP60: *Mycobacterium bovis* HSP65 (MB-HSP65) or *E. coli* GroEL, bacterial homologs of HSP60, failed to induce B cells to proliferate or to secrete IL-10 (22). Nevertheless, these bacterial HSP60 molecules were reported to induce secretion of proinflammatory cytokines by human monocytes or mouse macrophages (40, 42). The different bacterial HSP60 molecules affect T cells differently: *E. coli* GroEL, but not *M. tuberculosis* HSP65, induces T cells to adhere to fibronectin (41). In short, the effects of HSP60, self and foreign, are varied, and it is an oversimplification to think that HSP60 is a "danger signal" only (17).

We do not know the concentrations of soluble HSP60 available to interact with innate receptors in vivo, but HSP60 is reported to be overexpressed at sites of inflammation (43). Elevated levels of circulating HSP60 were reported to be associated with early atherosclerosis (44) and with acute lung injury (45); average serum levels of several micrograms per milliliter were reported in these patients (44, 45). The local concentration of HSP60 at sites of inflammation, however, is not known.

HSP60 can modulate the immune response in various ways. In bacterial infection where foreign HSP60 is present, macrophages and dendritic cells, the first cells to migrate to the inflamed site, will be activated via their TLR4 receptors to secrete proinflammatory cytokines and present the bacterial Ags to Ag-specific T cells. In this way, foreign HSP60 can help activate host adaptive immunity against the pathogen. Indeed, pathogen HSP60 is itself a dominant bacterial Ag (46).

Antigenic dominance could be explained by the fact that the HSP60 molecule might serve as its own adjuvant (47) by interacting simultaneously with innate receptors on APCs and Ag receptors on specific T cells and B cells. As the foreign invaders and their foreign HSP60 variants are destroyed, the self-HSP60 that is also up-regulated at the inflammatory site might then act on T cells and B cells in various ways: low concentrations of self-HSP60 can activate regulatory T cells to down-regulate the inflammatory response (15). In contrast, higher levels of self-HSP60 can enhance the survival of Ag-specific B cells at the site, as shown here, to prolong Ab production and presentation of Ag to T cells (22). Moreover, the innate activation of B cells by HSP60 to secrete IL-10 would serve to down-regulate the inflammatory response (22).

There are additional mechanisms by which HSP60 can regulate the adaptive immune response. Activated T cells express HSP60 on their surface (48), and HSP60 expressed on activated effector T cells was found to serve as a target for anti-ergotypic T cells that down-regulate proinflammatory T cells (20). Self-HSP60 might then down-regulate effector T cell function both as an Ag for anti-ergotypic T cells (49–51) and as an innate TLR2 signal for $CD4^+CD25^+$ regulatory T cells (15). Thus, self-HSP60 might help to down-regulate the inflammatory process as the healing process progresses. The varied roles of HSP60 on the different innate and adaptive arms of the immune system are still not fully defined. It is clear even at this early stage, however, that HSP60 is exploited by the immune system as a biomarker for the state of the tissues in infection and in body maintenance (18). The administration of HSP60 or its epitope fragments can down-regulate autoimmune disease models in experimental animals (52, 53), and HSP60 peptide treatment is in advanced clinical trials in type 1 diabetes (54).

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Disclosures

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