Heat Shock Protein 60 Activates B Cells via the TLR4-MyD88 Pathway[1]

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We recently reported that soluble 60-kDa heat shock protein (HSP60) can directly activate T cells via TLR2 signaling to enhance their Th2 response. In this study we investigated whether HSP60 might also activate B cells by an innate signaling pathway. We found that human HSP60 (but not the *Escherichia coli* GroEL or the *Mycobacterial* HSP65 molecules) induced naive mouse B cells to proliferate and to secrete IL-10 and IL-6. In addition, the HSP60-treated B cells up-regulated their expression of MHC class II and accessory molecules CD69, CD40, and B7-2. We tested the functional ability of HSP60-treated B cells to activate an allogeneic T cell response and found enhanced secretion of both IL-10 and IFN-γ by the responding T cells. The effects of HSP60 were found to be largely dependent on TLR4 and MyD88 signaling; B cells from TLR4-mutant mice or from MyD88 knockout mice showed decreased responses to HSP60. Care was taken to rule out contamination of the HSP60 with LPS as a causative factor. These findings add B cells to the complex web of interactions by which HSP60 can regulate immune responses. *The Journal of Immunology,* 2005, 175: 3594–3602.

B cells are a principal component of the adaptive immune system, but also serve various innate immune functions. In addition to secreting Abs, B cells play an important role as APCs (1). Mature B cells express TLR4 and TLR2 and respond to innate stimuli such as LPS and peptidoglycan (PGL)[4] (2–5). Activation of B cells up-regulates their expression of MHC class II (MHC II) and costimulatory molecules. Recent studies have demonstrated that naive B cells can differentiate into polarized B cells with different cytokine profiles; Be1 cells activate Th1 responses, and Be2 cells activate Th2 responses (6).

The 60-kDa heat shock protein (HSP60) is a dominant immunogen in both foreign immunity and autoimmunity (7). HSP60 has also been identified as a ligand for the innate immune system. We reported recently that human T cells can directly respond to soluble HSP60 via TLR2; HSP60 activation increased T cell adhesion to fibronectin and inhibited T cell chemotaxis to stromal cell-derived factor-1α (8), and HSP60 treatment down-regulated IFN-γ and TNF-α and enhanced IL-10 secretion from activated T cells (9). Recently it was shown that T cells can respond innately to recombinant HSP60 produced in a eukaryotic system free of LPS and other bacterial contaminants (10).

The present study was undertaken to test whether soluble HSP60 might exert innate immune effects on B cell physiology. In this study we report the effects of HSP60 on B cell proliferation, activation, and cytokine secretion. We also tested the effects of HSP60 activation on the ability of B cells to stimulate allogeneic T cell proliferation and cytokine secretion. We found that signaling via TLR4 and MyD88 was critical in the response of B cells to HSP60. Contamination of HSP60 with LPS or other bacterial molecules did not appear to be involved in these innate immune effects.

Materials and Methods

Mice

Female C57BL/6J and BALB/c mice were purchased from Harlan Olac. C3HeB/FeJ, C3H/HeJ, and NOD mice were obtained from The Jackson Laboratory. MyD88-knockout mice and TLR2-knockout mice were provided by Prof. S. Akira (Osaka University, Osaka, Japan) (11, 12). The mice were maintained in a specific pathogen-free facility and were used at the age of 5–8 wk.

Purification of naive 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, as determined by FACS analysis.

HSP60 preparation and reagents

Human HSP60 was prepared as previously described (13). Endotoxin contamination of the preparations was determined using the kinetic-turbidmetric *Limulus* amebocyte lysate assay, performed by an independent laboratory; the endotoxin content was <0.0001 endotoxin units/μg protein, corresponding to <0.01 pg of LPS equivalents/μg recombinant HSP60. We used LPS of the *Escherichia coli* strain 055:B5 (chromatographically purified and phenol extracted; Sigma-Aldrich) or LPS of *Salmonella minnesota* Re595. PGL of *Staphylococcus aureus*, polymyxin B (PmB), PmB agarose beads, and maganin II amide were purchased from Sigma-Aldrich. *Mycobacterium bovis* HSP65 (MB-HSP65) and *E. coli* GroEL were purchased from StressGen Biotechnologies, and a preparation of recombinant histamine-tagged GroEL purified from *E. coli* was provided by Prof. A. Horovitz (Weizmann Institute of Science, Rehovot, Israel) (14).

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Received for publication November 9, 2004. Accepted for publication June 30, 2005.

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[1] This work was supported by the European Union and he Center for the Study of Emerging Diseases. I.R.C. is the incumbent of the Mauerberger Chair in Immunology at the Weizmann Institute, the Director of the Center for the Study of Emerging Diseases, Jerusalem, and the Director of the National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev.

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[4] Abbreviations used in this paper: PGL, peptidoglycan; HSP60, 60-kDa heat shock protein; MB, *Mycobacterium bovis*; MHC II, MHC class II; PmB, polymyxin B.

0022-1767/05/$02.00

Inhibition of HSP60 or LPS

HSP60 (5 μg/ml) or LPS (0.1 μg/ml) was preincubated for 1 h with anti-HSP60 mAb 8C6 or control IgG, both at 10 μg/ml, or with PmB (10 μg/ml) at 37°C and then used to activate B cells. Heat treatment of HSP60 or LPS (both at 1 mg/ml) was performed by boiling for 20 min. The treated preparations were then diluted (HSP60 to 5 μg/ml; LPS to 0.1 μg/ml) and tested for their ability to activate B cells. Inhibition of HSP60 or LPS by maganin II amide was performed as previously described (15). Briefly, B cells were preincubated with increasing concentrations of maganin II amide at 37°C. After 30 min, 0.01 μg/ml LPS or 0.5 μg/ml HSP60 was added. B cell proliferation was followed after 72 h, as described below. Removal of LPS and LPS-associated molecules from HSP60 using PmB agarose was performed as previously described (16). Briefly, aliquots of 1 ml of PmB agarose were poured into disposable columns (Pierce) and washed three times in 20 vol of PBS. HSP60 (500 μl) at 500 μg/ml was loaded onto each 1-ml column, the columns were shaken for 1 min, incubated at room temperature for 60 min, and eluted with PBS in 250-μl fractions. The concentration of HSP60 in the fractions collected from PmB agarose columns was determined using the bicinchoninic acid protein kit (Pierce).

B cell proliferation

Proliferation assays of B cells were performed using 96-well, round-bottom microtiter plates; 2.5×10^5 purified B cells were incubated in triplicate at 37°C in 5% CO_2 in 200 μl of RPMI 1640 mixed with DMEM supplemented with 5% FBS (HyClone), 5×10^{-5} M β-ME, 10 mM L-glutamate 12.5 mM HEPES, sodium pyruvate, nonessential amino acids, and 1% penicillin/streptomycin. HSP60 was added at concentrations of 0.1–50 μg/ml. After 48 or 72 h, the cells were pulsed with 1 μCi of [^{3}H]thymidine for 6 h, and [^{3}H]thymidine incorporation was measured using a 96-well plate beta counter. The mean cpm ± SD were calculated for each triplicate.

Cytokine and IgG3 determinations

Cytokines in culture medium were determined by ELISA for IL-10, IL-6, IFN-γ, TNF-α, and IL-4 (OptiEIA kits; BD Pharmingen) following the manufacturer's instructions. Standard curves were established using mouse recombinant cytokines; the assay detection limit was 16–32 pg/ml. The amount of IgG3 in culture supernatants was determined by capture ELISA using goat anti-mouse IgG3 at 2 μg/ml and goat anti-mouse IgG3-alkaline phosphatase at 0.5 μg/ml; both Abs were obtained from Southern Biotechnology Associates.

Flow cytometric analysis

B cell expression of surface activation and costimulatory molecules was determined by flow cytometry. Briefly, purified B cells were seeded in 96-well plates (2.5×10^5/well) and stimulated in triplicate for 18 h at 37°C in 5% CO_2. The cells were then collected, washed with PBS/0.5% BSA, and incubated for 30 min with Ab to the anti-FcγRIII/II 2.4G2 (BD Pharmingen) to block nonspecific binding. After washing, the cells were incubated for 45 min with FITC-conjugated anti-mouse CD86, CD80, or CD69 (Serotec) or with FITC-conjugated anti-mouse IA/IE, PE-conjugated CD40, or isotype control Ab (eBioscience). The cells were analyzed by flow cytometry using a FACSort (BD Biosciences) and CellQuest software (BD Biosciences).

Allogeneic T cell response

T cells were isolated from C57BL/6J mice by negative selection of non-T cells using the Pan T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. B cells were isolated from C3HeB/FeJ or C3H/HeJ mice, and treated for 14 h with HSP60 (25 μg/ml). The cells were washed three times with 50 ml of HBSS containing 2% FBS, counted, and gamma irradiated at 2500 rad before coculture with the allogeneic C57BL/6J T cells. Proliferation assays were performed in triplicates of 1.5×10^5 T cells/well seeded together with titrated numbers of irradiated B cells in a total volume of 200 μl in round-bottom, 96-well plates for 72 h. The proliferation of T cells was measured using [^{3}H]thymidine (Amersham Biosciences) at 1 μCi/well for the last 8 h of culture. Cytokines were measured in the culture medium as described above.

Statistical analysis

The InStat 2.01 program (GraphPad) was used for statistical analysis. Statistical analysis was performed using the two-sided Welch t test. Differences were considered statistically significant at $p < 0.05$.

Results

Soluble HSP60 induces B cell proliferation

Splenic B cells were isolated from naive C57BL/6J mice and purified by negative selection. The purified B cells were then incubated with HSP60 or *E. coli* LPS at different concentrations (0.1–50 μg/ml) for 48 h. B cell proliferation was measured by [^{3}H]thymidine incorporation for the last 7 h of culture. Fig. 1 shows that HSP60 and *E. coli* LPS, at similar concentrations (weight per volume), induced proliferation of the mouse B cells in a dose-dependent manner.

HSP60-induced B cell activation is not due to endotoxin contamination

In this study we used recombinant HSP60, so we tested whether the effects of HSP60 might be due to contamination with bacterial mitogens such as LPS. The endotoxin level detected in the recombinant protein was <0.0001 endotoxin units/μg, which corresponds to <0.01 pg of LPS in 1 μg of recombinant protein, a concentration probably less than that needed for significant B cell activation. In addition, several lines of experimental evidence support the conclusion that the effects we observed were due to HSP60 and not to LPS or other bacterial contaminants (Fig. 2).

First, the biological activity of the HSP60 preparation was blocked by specific Ab to HSP60; an IgG mAb raised against HSP60, 8C6 (10 μg/ml), significantly blocked the B cell proliferation induced by HSP60, but not that induced by LPS. A mouse isotype control IgG had no effect. Secondly, boiling the samples for 20 min blocked the ability of HSP60 to induce B cell activation, but had no effect on the activation induced by LPS. Thirdly, PmB, a known inhibitor of LPS, blocked the effect of LPS, but not that of HSP60. Fourthly, we used PmB agarose to clean possible contaminants from our HSP60 preparation. It was recently reported by Gao and Tsan (16) that applying HSP60 to PmB agarose columns could successfully remove LPS and LPS-associated factor(s); the same treatment completely eliminated the ability of their HSP60 preparation to induce macrophages to secrete TNF-α. Fig. 2*B* shows that HSP60 eluted from the PmB agarose column was fully active. Fifthly, maganin II amide totally inhibited the effect of *E. coli* LPS, but did not inhibit the effect of our HSP60 preparation on B cells (Fig. 2*C*). It was recently reported that HSP60 binds LPS (15); in that article the authors used maganin II amide, which inhibited the activity of LPS and/or structurally related molecules tightly bound to HSP60. Finally, MB-HSP65 or *E. coli* GroEL,

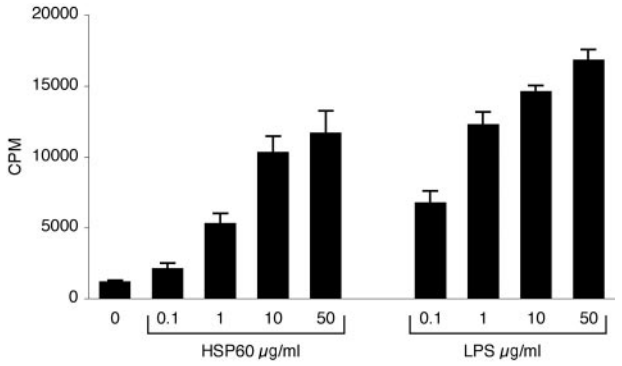

FIGURE 1. HSP60 or LPS induces B cell proliferation. B cells were incubated with various concentrations of HSP60 or *E. coli* LPS for 72 h and assayed for [^{3}H]thymidine incorporation. Results are presented as the mean cpm incorporated ± SD of triplicate cultures. The data are representative of five independent experiments using B cells pooled from two or three mice.

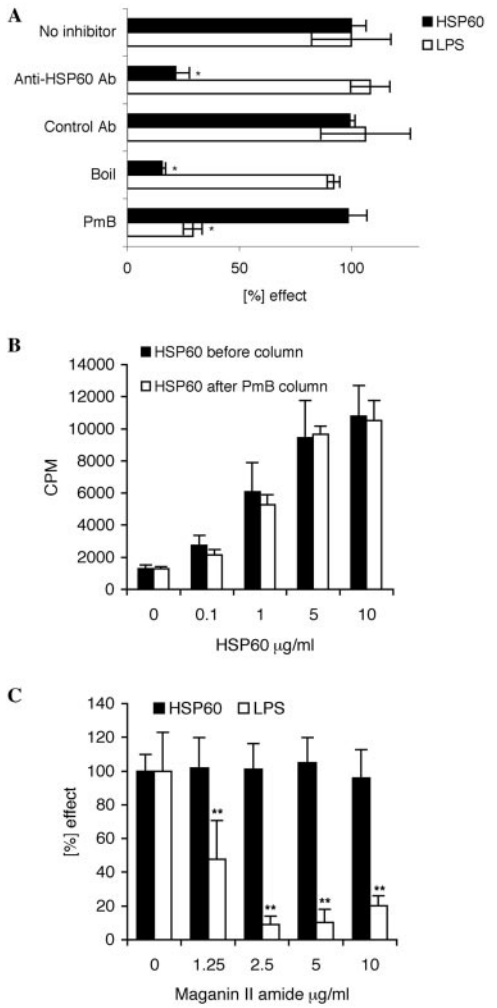

FIGURE 2. HSP60-induced proliferation is not due to LPS contamination. B cells were activated by incubation with HSP60 (5 μg/ml) or *E. coli* LPS (0.1 μg/ml). Test HSP60 or LPS was preincubated with anti-HSP60 mAb 8C6, control mouse IgG, or PmB (10 μg/ml) for 1 h and then used to activate B cells. In separate experiments, HSP60 or LPS was heat-treated (100°C for 20 min) for 72 h before testing. The results are expressed as the percentage of cpm ± SD incorporation induced by treated HSP60 or LPS compared with untreated HSP60 or LPS (indicated as 100%; *A*). One representative experiment of three is shown. *, $p < 0.05$ vs the HSP60 or LPS treated with no inhibition, by the two-tailed Welch *t* test. LPS and LPS-associated molecules were removed by passing through a PmB agarose column; HSP60 eluted from the column was quantified for protein concentration and used to activate B cells. B cell proliferation was measured after 72 h of incubation. The results are presented as the mean cpm incorporated ± SD of quadruplicate cultures (*B*). Maganin II amide, B cells were preincubated with increasing concentrations of maganin II amide at 37°C for 30 min, and then medium, 0.01 μg/ml LPS, or 0.5 μg/ml HSP60 was added for 72 h. The results are expressed as the percentage of cpm ± SD incorporation induced by treated HSP60 or LPS compared with untreated HSP60 or LPS (indicated as 100%; *C*). **, $p < 0.05$ vs HSP60 before passing through a PmB column, by the two-tailed Welch *t* test.

bacterial homologues of HSP60, failed to induce B cells to proliferate or to secrete IL-10 (Fig. 3). These bacterial HSP60 molecules were reported to induce the secretion of proinflammatory cytokines by human monocytes or mouse macrophages (17, 18). It is noteworthy, therefore, that the innate response of B cells, unlike that of macrophages or dendritic cells, was specific to the mammalian HSP60. Moreover, because MB-HSP65 and the *E. coli* GroEL were also isolated from bacteria, the failure of these recombinant preparations to activate B cells is compatible with the conclusion that the effects of HSP60 were not due to contamination with bacterial molecules.

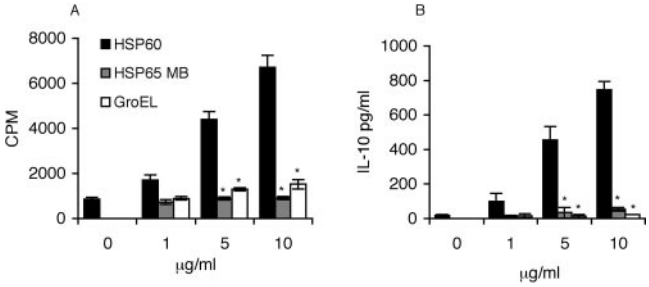

FIGURE 3. Bacterial MB-HSP65 and GroEL fail to activate B cell proliferation and cytokine secretion. B cells from C57BL/6J mice were treated or not with MB-HSP65, GroEL, or human HSP60 at various concentrations for 72 h and assayed for [^{3}H]thymidine incorporation (*A*) and IL-10 secretion (*B*). The results represent the mean ± SD of triplicate wells. One representative experiment of two is shown. *, $p < 0.05$ vs HSP60-treated cells, by the two-tailed Welch *t* test.

HSP60 induces expression of costimulatory molecules on B cells

One of the consequences of B cell activation is the enhanced expression of costimulatory molecules. To test whether HSP60 induces the expression of B cell activation markers, purified B cells from C57BL/6 mice were cultured in the presence of HSP60 (20 μg/ml). After 16 h, the cells were harvested, stained with anti-mouse FcR for blocking nonspecific binding sites, and incubated with Abs specific to MHC II, CD86 (B7-2), CD40, CD69, or CD80 (B7-1). Cell surface expression was analyzed by FACS. HSP60 induced up-regulation of MHC II, CD86 (B7-2), CD40, and the early activation marker CD69 (Fig. 4, *A–D*). No up-regulation of CD80 (B7-1) was observed (Fig. 4*E*).

HSP60 induces B2 cells to secrete cytokines

Cytokines are capable of regulating the development of T cell subsets; for example, IFN-γ and IL-2 induce Th1 responses, whereas IL-6 and IL-10 induce Th2 responses (19, 20). We investigated the effects of HSP60 on the induction of cytokine secretion by B cells. B cells were incubated with HSP60 (5–25 μg/ml) for 72 h, and the culture supernatants were tested for cytokine content by ELISA. Untreated B cells did not release any detectable cytokines. However, B cells stimulated with HSP60 produced significant quantities of IL-10 and IL-6 in a dose-dependent manner (Fig. 5, *B* and *D*). IL-10 became detectable after 2 days in culture and accumulated rapidly between days 2 and 3 (Fig. 5*A*); IL-6 was detected as early as after 10 h in culture (Fig. 5*C*). We could not detect IL-4, IL-5, IFN-γ, or TNF-α in the culture medium. B cells from BALB/c and NOD mice also produced IL-10 and IL-6 in response to HSP60 (data not shown).

CD5$^+$ (Ly1$^+$) B1 cells are reported to be a major source of IL-10 (21). The negative selection kit we used to purify B cells contained Ab to CD43, a marker present on most CD5$^+$ B cells, so most CD5$^+$ cells should have been depleted. To further exclude the participation of CD5$^+$ B cells, an additional step of depletion was performed using anti-CD5 magnetic beads. This specific depletion of CD5$^+$ B cells had no significant effect on IL-10 and IL-6 levels (data not shown). This indicates that the cytokines we measured in response to HSP60 were synthesized largely by CD5$^-$ B2 cells.

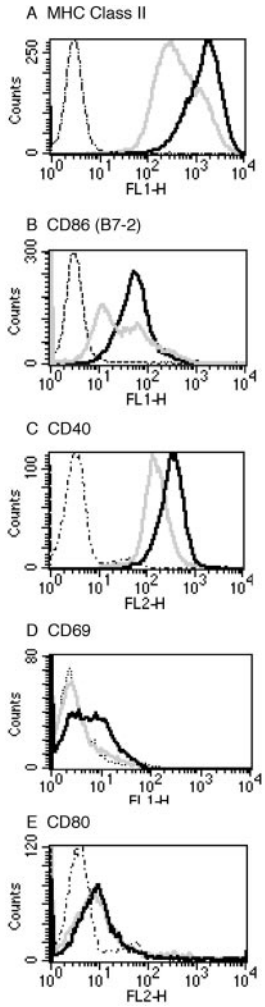

FIGURE 4. HSP60 induces the expression of costimulatory molecules. B cells from C57BL/6J mice were treated or not with HSP60 (20 μg/ml). After 16 h, the cells were stained with Ab against CD16/32 to block nonspecific binding; washed; stained with Ab to MHC II (*A*), CD86 (*B*), CD40 (*C*), CD69 (*D*), or CD80 (*E*); and analyzed by flow cytometry. The dotted line represents isotype control staining of HSP60-treated cells, the gray line shows unstimulated cells, and the black line shows HSP60-treated cells. The data are representative of four experiments.

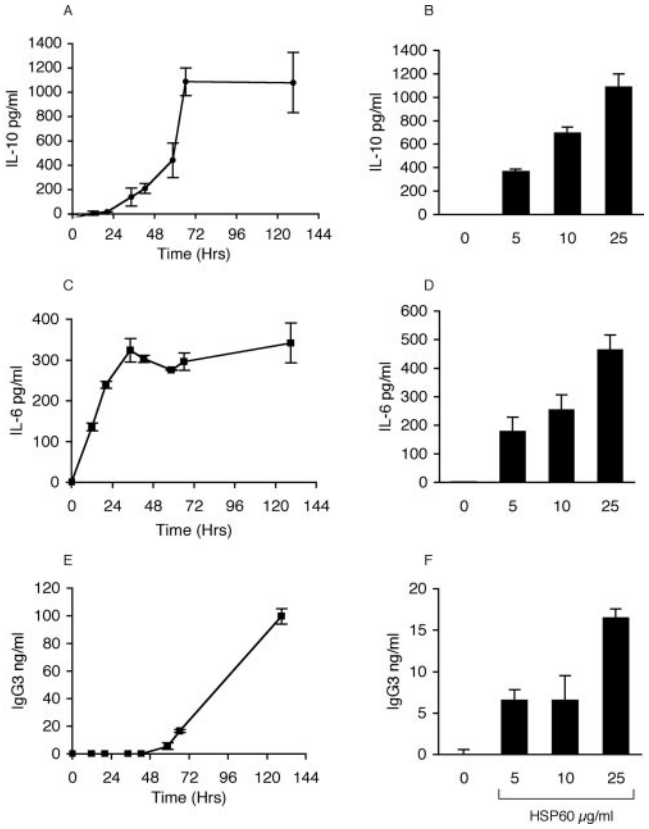

FIGURE 5. HSP60 induces B cells to secrete cytokines. B cells from C57BL/6J mice were treated or not with HSP60 (5–25 μg/ml). After 72 h, the supernatant media were collected, and the contents of IL-10 (*B*), IL-6 (*D*), and IgG3 (*F*) were determined by ELISA. For the kinetic experiments, B cells were treated with 25 μg/ml HSP60, supernatants were collected at the indicated time points, and the contents of IL-10 (*A*), IL-6 (*C*), and IgG3 (*E*) were determined by ELISA. The results represent the mean ± SD of triplicate wells. One representative experiment of three is shown.

HSP60 induces an isotype switch to IgG3

We tested whether HSP60 might also stimulate B cells to secrete Abs and found that HSP60 triggered Ig switching to IgG3 in a dose-dependent manner (Fig. 5*F*). IgG3 became detectable after 3 days in culture and accumulated rapidly between days 3 and 5 (Fig. 5*E*). These results show that, similar to other known B cell stimulants, HSP60 induces both proliferation and activation of B cells and is capable of activating the Ig switch machinery.

HSP60 activates B cells via TLR4

Soluble HSP60 was shown to activate T cells via the innate receptor TLR2 (8). To study the role of TLR2 in HSP60 signaling of B cells, we tested whether HSP60 could activate B cells from TLR2-knockout mice. TLR2-knockout mouse B cells responded well to HSP60; as expected, these B cells failed to respond to the TLR2 ligand PGL. In addition, B cells from TLR2$^{-/-}$ mice responded to HSP60 by IL-6 and IL-10 secretion and up-regulation of B7-2, CD40, CD69, and MHC II (not shown). Thus, TLR2 signaling does not appear to be involved in the activation of B cells by HSP60.

A TLR4 mutation in C3H/HeJ mice renders these mice resistant to the effects of bacterial LPS (22). To determine whether TLR4 signaling might be involved in B cell activation induced by HSP60, we compared the responses of B cells isolated from C3HeB/FeJ (wild-type TLR4) and C3H/HeJ (mutant TLR4) mice. We found that HSP60 induced B cells from the C3HeB/FeJ mice to proliferate; secrete IL-10, IL-6, and IgG3; and up-regulate their expression of costimulatory molecules (Fig. 6). In contrast, B cells from C3H/HeJ mice were low responders to stimulation with HSP60; the TLR4-mutant B cells were hyporesponsive to HSP60 in proliferation (Fig. 6*A*), and produced significantly less IL-10 (Fig. 6*B*), IL-6, and IgG3 (Fig. 6, *C* and *D*). The TLR4-mutant B cells also expressed significantly less of the accessory molecules CD86 and CD69 in response to HSP60 (Fig. 5*E*). Surprisingly, the up-regulation of MHC II induced by HSP60 was not dependent on the presence of functional TLR4 and was also observed in C3H/HeJ B cells (Fig. 6*E*, *lower panel*). Note, also, that B cells bearing the mutated TLR4 could still respond weakly to HSP60 by proliferation and cytokine secretion (Fig. 6).

MyD88 is required for the response to HSP60

MyD88 is a general adaptor protein that plays an important role in TLR family signaling; it was shown that LPS signals B cells via TLR4 and MyD88 (4, 23). However, a pathway for TLR4 activation by LPS independent of MyD88 has also been reported (24).

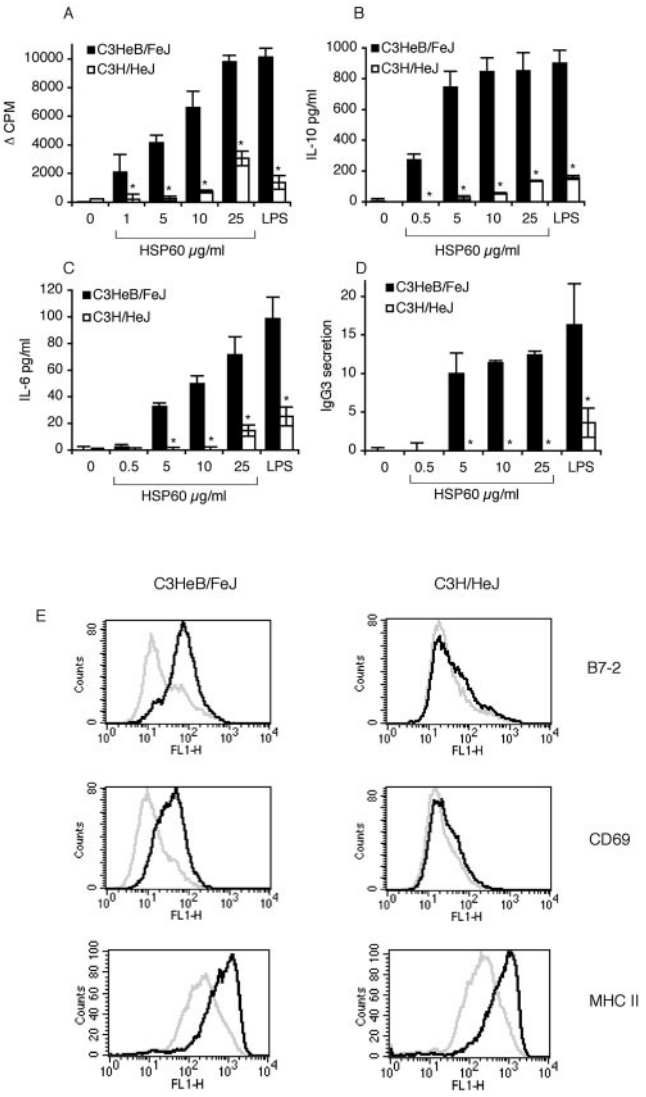

FIGURE 6. HSP60 activates B cells via TLR4. B cells from C57BL/6J, C3HeB/FeJ, or C3H-HeJ mice were treated or not with HSP60 (1–25 μg/ml) or *S. minnesota* LPS (0.1 μg/ml) for 72 h and assayed for [^{3}H]thymidine incorporation. The mean cpm tested minus the background cpm of the unstimulated cells $\pm$ SD are shown (*A*). The cytokine levels in the media were determined by ELISA for IL-10 (*B*), IL-6 (*C*), and IgG3 (*D*). The results represent the mean $\pm$ SD of triplicate wells. *, $p < 0.05$ vs C3HeB/FeJ-treated cells, by the two-tailed Welch *t* test. For cell surface markers, B cells from C3HeB/FeJ or C3H-HeJ were treated or not with HSP60 (20 μg/ml); after 18 h the cells were treated with Ab to CD16/32 to block nonspecific binding, washed, and stained with Ab against B7-2, CD69, or MHC II (*E*). One representative experiment of three is shown.

Therefore, we tested the ability of HSP60 to activate B cells from MyD88-deficient mice. Both wild-type MyD88$^{+/+}$ B cells and B cells from MyD88$^{+/-}$ mice (not shown) showed an increased proliferative response to HSP60 in a dose-dependent manner. However, MyD88-deficient B cells did not proliferate in response to HSP60, even when cultured with high concentrations of HSP60 (Fig. 7*A*). IL-10 production in response to HSP60 was also absent in B cells lacking MyD88 (Fig. 7*B*). Finally, up-regulation of the surface activation markers CD69, B7-2, and MHC II were also dependent on the presence of MyD88 (Fig. 7*C*). Thus, MyD88 signaling is required for the activation of B cells by HSP60.

HSP60 increases the capacity of B cells to stimulate allogeneic T cells

To learn whether B cell activation by HSP60 might have functional consequences, we studied the ability of B cells, treated or not with HSP60, to stimulate allogeneic T cells in vitro. Resting B cells possess little or no costimulatory activity and have been reported to be ineffective APCs for naive T cells (25). We found that B cells treated with HSP60 induced a greater degree of allogeneic T cell proliferation than did the untreated B cells (Fig. 8*A*). To learn whether TLR4 is involved in the enhanced allogeneic stimulation induced by HSP60-treated B cells, we compared the allogeneic T cell response to HSP60-activated B cells from C3HeB/FeJ (wild-type TLR4) with that of C3H/HeJ (mutated TLR4) mice. B cells from C3HeB/FeJ and C3H/HeJ mice were treated with HSP60 for 16 h, washed, irradiated with 2500 rad, and then cocultured with allogeneic C57BL/6J T cells. B cells from TLR4-mutant C3H/HeJ were much less able to induce T cell proliferation (Fig. 8, compare *A* and *B*).

We also analyzed the cytokines produced by the allogeneic T cells in response to HSP60-activated B cells. HSP60-activated B cells induced allogenic T cells to secrete high levels of IFN-γ compared with untreated B cells (Fig. 8*C*). The ability of HSP60-activated B cells to induce T cell secretion of IFN-γ was also dependent on the presence of functional TLR4; HSP60-activated B cells from C3H/HeJ mice induced lower levels of IFN-γ secretion than those from C3HeB/FeJ mice (Fig. 8, compare *C* and *D*). Analysis of the presence of the Th2 cytokine IL-10 in the allogeneic response showed that activated B cells from C3HeB/FeJ mice, but not B cells from TLR4-mutant C3H/HeJ mice, induced significant levels of IL-10 secretion (Fig. 8, compare *E* and *F*). Irradiated B cells, cultured alone, secreted only low levels of IL-10 and undetectable levels of IFN-γ (not shown). Thus, most cytokines detected in the supernatants of the allogenic T cell response probably did not come from the irradiated B cells, but, rather, from the responding T cells.

Discussion

The results of this study demonstrate that soluble HSP60 can activate B cells via TLR4-MyD88 signaling. Recently, two studies have suggested that the immunostimulatory properties of HSP60 on macrophages may be due to endotoxin contamination (16, 26). A new study by Osterloh et al. (10) showed that T cells could respond to low endotoxin, recombinant human HSP60 or to murine HSP60 expressed by eukaryotic cell lines free of LPS and other bacterial contaminants. The response of B cells we measured to HSP60 was not likely to be due to bacterial contamination. This conclusion rests on the following evidence. 1) The *Limulus* amebocyte lysate assay showed that the LPS content of our HSP60 preparation was <0.01 pg of LPS/μg 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 (see Fig. 1). 2) Specific anti-HSP60 Ab binding to HSP60 inhibited the B cell proliferation induced by HSP60; the same Ab had no effect on LPS-induced proliferation (see Fig. 2). 3) The specific inhibitor of LPS, PmB, inhibited the activity of LPS, but not that of HSP60. 4) Boiling abolished the effects of HSP60, but had no effect on LPS. 5) Maganin II amide, an LPS inhibitor that was recently shown to inhibit the activity of LPS and/or structurally related molecules tightly bound to HSP60 in macrophages (15), did not inhibit the effect of our HSP60 on B cells (Fig. 2*C*). 6) Passing HSP60 through a PmB agarose column that removes LPS and LPS-associated factor(s) had no effect on the HSP60 activity observed in this study (Fig. 2*B*). 7) Other proteins isolated from

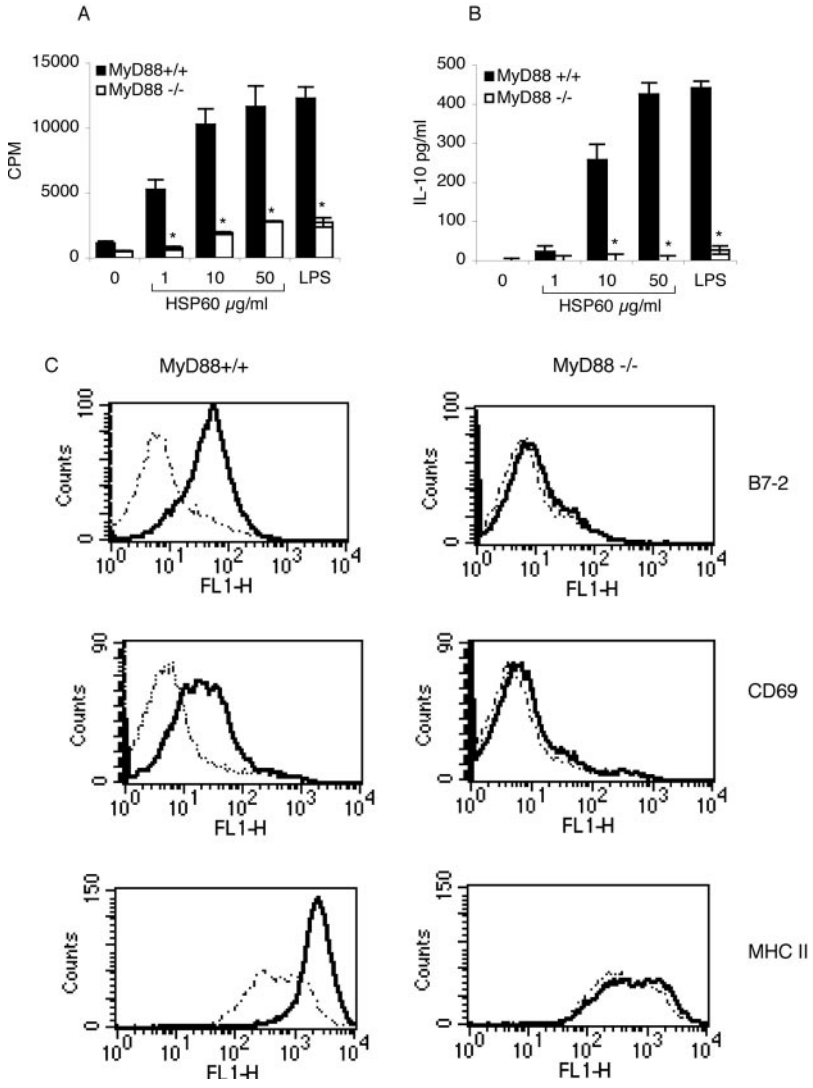

FIGURE 7. MyD88 is required for the response to HSP60. B cells from MyD88$^{+/+}$ or MyD88$^{-/-}$ mice were activated or not with HSP60 (1–50 μg/ml) and assayed for incorporation of [^{3}H]thymidine (*A*). The mean cpm incorporated ± SD of triplicate cultures are shown. *, $p < 0.05$ vs MyD88$^{+/+}$-treated cells, by the two-tailed Welch *t* test. IL-10 secretion in the culture media was determined; the mean concentration of IL-10 of triplicate cultures ± SD are shown (*B*). To detect surface markers, B cells were treated or not with HSP60 (20 μg/ml); after 16 h, the cells were treated with Ab to CD16/32 to block nonspecific binding, washed, and stained with Ab against B7-2, CD69, or MHCII (*E*). One representative experiment of three is shown. Cell surface markers B7-2, CD69, and MHC I are shown (*C*). One representative experiment of two is presented.

bacteria, such as MB-HSP65 and the bacterial HSP60 homologue GroEL isolated from *E. coli* failed to activate B cell proliferation or IL-10 secretion (Fig. 3). It is likely that macrophages, which respond to picogram per milliliter concentrations of LPS, are more sensitive to LPS contamination than are B cells, which require higher concentrations of LPS, making it even less likely that LPS contamination could explain our results.

Because our HSP60 was produced in *E. coli*, we were also concerned that the effects of HSP60 might be due to contamination with other bacterial products, such as lipoproteins, which were recently identified as the major factors responsible for the TLR2-mediated cell activation in commercial LPS (27). To exclude this possibility, we tested the ability of HSP60 to activate B cells from TLR2-deficient mice; such B cells responded to HSP60, whereas no response to the TLR2 ligand, PGL, was observed (not shown). Thus, the effects of HSP60 on B cells are not likely to be due to contamination by bacterial lipoproteins that act via TLR2. Taken together, these results argue against the likelihood that the B cell effects of HSP60 were really due to LPS contamination or to other TLR2 ligands, such as lipoproteins; the observed responses appear to be specific to HSP60.

HSP60 has been reported to act as an innate activator of T cells (8, 10), whereas HSP60 (or an HSP60/LPS complex) has been found to activate the innate immune systems of dendritic cells and macrophages (28–30). Table I summarizes the effects of HSP60 we report in this study on B cells compared with the effects of HSP60 or HSP60/LPS reported for other cell types. The present results show for the first time that self-HSP60 can act as a mitogen; we found that HSP60 can induce the proliferation of B cells, whereas macrophages, dendritic cells, and T cells were not reported to proliferate in response to HSP60/LPS.

With regard to cytokine secretion, previous studies have shown that human HSP60/LPS can induce proinflammatory responses in mouse or human macrophages and dendritic cells (28, 29, 31). In contrast to this proinflammatory cytokine pattern, B cells responding to HSP60 secreted high levels of the Th2 cytokine IL-10, low levels of IL-6, and no detectable levels of IL-4, IL-5, IFN-γ, or TNF-α. Note that IL-6 was recently found to control Th1/Th2 differentiation; IL-6 promotes Th2 differentiation and simultaneously inhibits Th1 polarization through two independent molecular mechanisms (20). We show in this study that HSP60-treated B cells shifted the allogeneic T cell response to a Th0 type, characterized by secretion of both IFN-γ and IL-10. In contrast to B cells, dendritic cells activated by HSP60/LPS promoted the secretion by allogeneic T cells of high levels of IFN-γ and IL-12 and low levels of IL-10, which is a Th1-promoting phenotype (28).

Regarding the regulation of surface markers, HSP60/LPS was shown to induce maturation of dendritic cells characterized by up-regulation of the costimulatory molecules CD40, MHC II, B7-2, and CD54 (28). B cells responding to HSP60 also up-regulate their

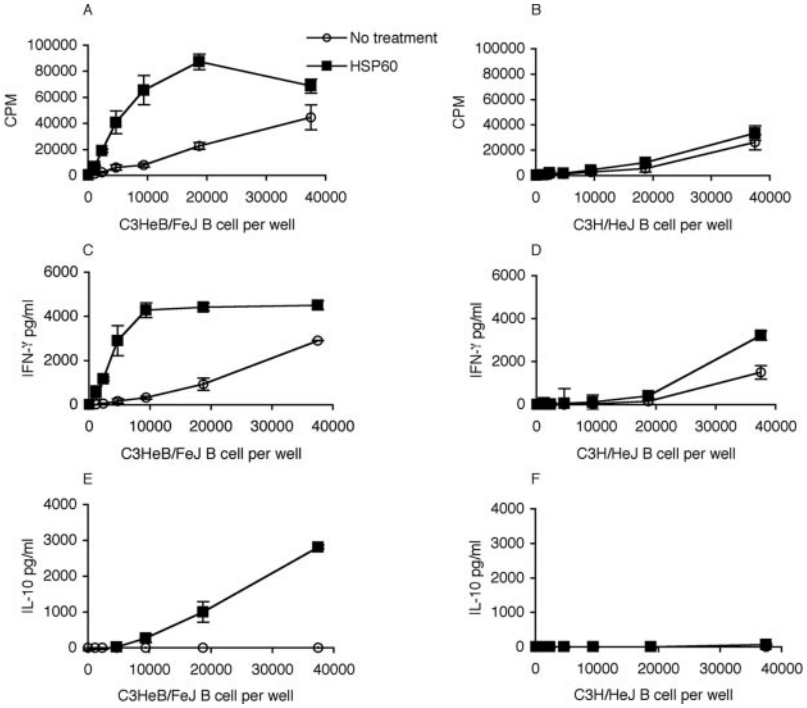

FIGURE 8. HSP60 increases the capacity of B cells to stimulate allogeneic T cells. B cells from C3HeB/FeJ or C3H-HeJ mice were treated or not with HSP60 (25 μg/ml). After 16 h, the cells were harvested and irradiated with 2500 rad, and graded numbers of B cells were cultured in the presence of 1×10^5 allogeneic T cells from C57BL/6J mice. After 3 days, the cells were pulsed with [^{3}H]thymidine, followed by harvesting and beta counting (*A* and *B*). For cytokine analysis, the supernatants were analyzed for the presence of IFN-γ (*C* and *D*) or IL-10 (*E* and *F*). Data are the mean $\pm$ SD of triplicate wells and are representative of two independent experiments.

expression of CD40, MHC II, B7-2, and CD69, although the B cells did not up-regulate the expression of CD54 (not shown). The effect of HSP60/LPS on dendritic cells and the effect of HSP60 on B cells therefore can enhance the ability of these cells to act as APCs. B7-2 is a costimulatory molecule important for Th2 responses; its binding to CD28 and CTLA-4 on T cells is involved in the generation of IL-4-producing cells that contribute to Th2-type responses (32). In contrast, B7-1 binding to CD28 on T cells has been shown to promote a Th1 phenotype (32). We found that B cells up-regulated the surface expression of B7-2 in response to HSP60; however, B7-1 expression was unchanged. The regulation of B7 molecule expression in response to HSP60 might be part of the mechanism by which B cells promote a Th0 allogeneic T cell response (see Fig. 8). The cytokine microenvironment in which the Ag is presented strongly influences the polarization of Th cells toward Th1, Th2, or Th0 in response to antigenic stimulation (33, 34). To date there is no information available about the effects of HSP60 on the expression of surface markers on macrophages. T cells, however, respond to HSP60 by down-regulation of the chemokine receptors CXCR4 and CCR7, and this inhibits their ability to migrate to stromal cell-derived factor-1α and Epstein-Barr virus-induced molecule 1 ligand chemokine (CCL19/ELC) (8).

TLR4 and TLR2 have been shown to be involved in signal transduction by HSP60 or HSP60/LPS complexes. Macrophages and dendritic cells use TLR4 signaling (28, 30); dendritic cells were also shown to respond via TLR2 (35). Human T cells respond

Table I. *Innate effects of human HSP60 on immune cells*[a]

Effect	Cell Type				References
	MO	DC	T cells	B cells	
Proliferation	No	No	No	Yes	
Cytokine secretion	TNF-α NO IL-12 IL-15 IL-6	TNF-α IL-12 IL-1β Low IL-10	ND	IL-10 Low IL-6	28, 29, 31
Surface markers	ND	CD40 $\uparrow$ MHC II $\uparrow$ B7-2 $\uparrow$ CD54 $\uparrow$	CXCR4 $\downarrow$ CCR7 $\downarrow$	CD40 $\uparrow$ MHC II $\uparrow$ B7-2 $\uparrow$ CD89 $\uparrow$	8, 28
TLR dependency	TLR4	TLR4 TLR2	TLR2	TLR4 Except MHC II $\uparrow$	8, 28, 30, 35
MyD88 dependency	Yes	ND	ND	Yes	35
Other functions	Activation	Maturation	Adhesion $\uparrow$ Migration $\downarrow$	IgG3 switch	8, 28, 29

[a] DC, dendritic cell; MO, macrophage.

to HSP60 via TLR2; no involvement of TLR4 was found (8). We show in this study that the B cell response to HSP60 is via TLR4. The responses of TLR4 mutants were significantly decreased, but not totally abolished; this might suggest the involvement of another receptor(s) or pathway(s) in B cells in response to HSP60. One interesting candidate might be RP105 (CD180). B cells, in addition to TLR4, express another member of the TLR family, the RP105 protein (36). Ogata et al. (36) reported an important role of RP105 in the regulation of LPS responsiveness. It is possible that the residual effect of HSP60 in TLR4-mutant cells might be due to activation of RP105. Up-regulation of MHC II was induced by HSP60 in the absence of functional TLR4; nevertheless, HSP60 could not induce the up-regulation of MHC II in MyD88-deficient B cells (Fig. 7*C*). The RP105 pathway was recently shown to act independently of MyD88 expression (37); therefore, we suspect that the MHC II up-regulation in the absence of TLR4 may not act through RP105. However, it is conceivable that RP105 might also use a MyD88-dependent pathway in B cell activation.

MyD88 is an adaptor molecule associated with TLR-mediated responses and was reported to be involved in the signaling pathway of HSP60 in dendritic cells (35). LPS was recently shown to induce maturation of dendritic cells via a pathway independent of MyD88 (24). In this study we show that B cell proliferation, up-regulation of surface molecules including MHC II, and cytokine secretion all depend on MyD88 activation by HSP60.

The *E. coli* HSP60 homologue GroEL and mycobacterial HSP65 were reported to induce the secretion of proinflammatory cytokines by macrophages via TLR4 (17, 18), but neither bacterial HSP60 molecule could interfere with the binding of human HSP60 to macrophages (17). Thus, different variants of HSP60 might bind to different receptors yet still act via TLR4 as a coreceptor for signaling. In this study we show that neither GroEL nor mycobacterial HSP65 could activate B cells (Fig. 3).

The administration of HSP60 and its variants was found to arrest the destructive inflammation responsible for various models of autoimmune diseases (9, 38–44). These beneficial effects of HSP60 were marked by a shift in the autoimmune response from a damaging Th1 phenotype to a healing Th2 phenotype. Some explanation may be found in our recent discovery that HSP60 can directly affect T cells via TLR2 to down-regulate T cell chemotaxis (8). HSP60 was also found to significantly down-regulate the secretion of IFN-γ and TNF-α and enhance the secretion of IL-10 upon activation by mitogenic anti-CD3 (9). Another possible explanation for the ability of HSP60 to down-regulate autoimmune inflammation, as we show in this study, could be by way of the IL-10 produced by HSP60-activated B cells. It has become apparent that B cells mediate a variety of functions within the immune system in addition to Ab production, which could play a regulatory role in inflammation and autoimmunity. B cells were shown to down-regulate inflammatory bowel disease (45), arthritis (46), diabetes (47), and experimental autoimmune encephalomyelitis (46, 48). In most cases, IL-10 secreted from B cells was shown to be critical for the regulatory effect. It is conceivable that the effects of HSP60 on B cells could enhance, in part, such regulation.

Acknowledgments

We thank Danielle Sabah-Israel for excellent secretarial assistance. We thank Prof. A. Horovitz (Weizmann Institute) for providing recombinant GroEl, and Prof. Shizuo Akira (Osaka University, Osaka, Japan) for kindly providing the TLR2- and MyD88-deficient mice.

Disclosures

The authors have no financial conflict of interest.

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