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Toxoplasma gondii–derived heat shock protein HSP70 functions as a B cell mitogen

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Abstract We have investigated the role of *Toxoplasma gondii*–derived heat shock protein 70 (*Tg*HSP70) as a B cell mitogen by measuring proliferative responses in vitro. *Tg*HSP70 induced prominent proliferative responses in murine B cells derived not only from *T gondii*–infected but also from uninfected mice. Nude mice responded to *Tg*HSP70; however, severe combined immunodeficiency, RAG1$^{-/-}$ B6, and μMT mice failed to respond. B220^{+} spleen cells showed marked proliferation after stimulation with *Tg*HSP70, but neither CD4^{+} nor CD8^{+} population responded. This unresponsiveness of CD4^{+} and CD8^{+} T cells to *Tg*HSP70 was antigen presenting cells independent. These data indicate that *Tg*HSP70 induced the proliferation of B cells but not T cells. Polymyxin B, a potent inhibitor of lipopolysaccharide (LPS), did not eliminate *Tg*HSP70-induced proliferation. C3H/HeN mice responded well to *Tg*HSP70 stimulation; however, C3H/HeJ mice carrying a point mutation in the Toll-like receptor (TLR) 4 failed to respond. This indicates that TLR4 is required for *Tg*HSP70-induced B cell activation. The involvement of TLR4 in the *Tg*HSP70-induced proliferative responses of spleen cells was also shown by the use of TLR4$^{-/-}$ mice. But *Tg*HSP70-induced, but not LPS-induced, spleen cell proliferation was observed in MyD88$^{-/-}$ mice, indicating that the MyD88 molecule was involved in LPS-induced proliferation but not in *Tg*HSP70-induced proliferation.

INTRODUCTION

The 70-kDa heat shock proteins (HSP70), a ubiquitous class of molecular chaperones, are highly immunogenic proteins with an exceptional degree of evolutionary conservation. Until recently, the primary function ascribed to HSP was as intracellular molecular chaperones of naive, aberrantly folded, or mutated proteins as well as in cytoprotection after a wide range of stressful stimuli. But novel functions of HSP70 as both chaperone and cytokine (chaperokine) in the extracellular milieu or expressed on the cell surface have recently been reported (Asea et al 2000). Immunization with HSP-peptide complexes elicited CD8^{+} cytotoxic T lymphocyte (CTL) and CD4^{+}Th cell-mediated responses. This demonstrated the effect of HSP70 as an adjuvant, eliciting immune responses as a carrier protein (Castellino et al 2000). Actually, bacterial HSPs are known to stimulate a host immune response, eg, as a component in Freund's complete adjuvant. This evidence suggests a strategy for immunization against infectious organisms and cancers, using HSP proteins and bound peptides isolated from infected tissues or tumors (Ciupitu et al 1998; Moroi et al 2000).

We have previously shown that human heat shock cognate protein (HSC) 71–associated peptides function as epitopes for *Toxoplasma gondii*–specific CD4^{+}CTL using a human melanoma cell line pulsed with *T gondii*–infected melanoma-derived HSC71 as a target (Yang et al 1997). After cloning *T gondii* HSP70 (*Tg*HSP70) complementary deoxyribonucleic acid (cDNA) (GenBank accession number AB109539) (Yano et al 1998), we analyzed the roles of *Tg*HSP70 in host immune responses of *T gondii*–infected mice. We found that *Tg*HSP70 was recognized by B cells from *T gondii*–infected mice despite high homology between *Tg*HSP70 and mouse HSP70 (mHSP70) (Mun et al 1999). The anti-*Tg*HSP70 antibodies produced by *T gondii*–infected mice cross-reacted with mHSP70,

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and anti-mHSP70 autoantibodies were produced by B-1 cells in *T gondii*–infected mice (Chen et al 2000).

In this study, we analyzed the effect of *Tg*HSP70 as a B cell mitogen by measuring proliferative responses in vitro. The investigation of proliferative responses in Toll-like receptors (TLR), TLR2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice revealed the involvement of TLR4 in *Tg*HSP70-induced proliferative responses of spleen cells, similar to *Escherichia coli*–derived lipopolysaccharide (LPS)-induced responses. On the other hand, *Tg*HSP70, but not LPS, induced spleen cell proliferation in MyD88$^{-/-}$ mice, indicating the involvement of MyD88 molecule in LPS-induced, but not in *Tg*HSP70-induced, proliferative responses.

MATERIALS AND METHODS

Mice and *T gondii* strain

Sex-matched 8-week-old C57BL/6 (B6) (H-2^b), BALB/c (H-2^d), C3H/HeN, and C3H/HeJ mice were purchased from SLC (Hamamatsu, Japan). TLR2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice were generated by Drs O. Takeuchi and S. Akira (Osaka University, Japan). ICR nude and severe combined immunodeficiency (SCID) mice were purchased from Charles River Japan Inc (Yokohama, Japan). B cell–deficient mice (μMT: H-2^b) were kindly provided by Drs A. Hashimoto, G. Matsuzaki (Kyushu University, Japan), and Kitamura (Science University of Tokyo, Japan). RAG1$^{-/-}$ B6 mice and FcγR$^{-/-}$ B6 mice were kindly provided by Drs T. Nakayama and T. Saito (Chiba University, Japan). Preparation and maintenance of *T gondii* Fukaya strain cysts and *T gondii* RH strain tachyzoites were described previously (Yano et al 1989; Luo et al 1997). Mice were perorally infected with 5 cysts of *T gondii* Fukaya strain as previously described (Yano et al 1989; Chen et al 2000).

Recombinant proteins and sodium dodecyl sulfate–polyacrylamide gel electrophoresis

There are 2 *T gondii* stages, which are encountered in mice and human: tachyzoites and bradyzoites. *Tg*HSP70 molecule is specifically expressed on tachyzoites and *Tg*HSP30 is expressed on bradyzoites. Cloning and expression of recombinant *Tg*HSP70 and *Tg*HSP30 were described previously (Mun et al 1999). Recombinant proteins were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie blue as previously described (Yang et al 1997; Mun et al 1999).

Fractionation of cells and flow cytometry analysis

Spleen cells from *T gondii*–infected and uninfected mice were fractionated to separate B220$^+$, CD4$^+$, and CD8$^+$ cell populations or B220$^+$ and CD90$^+$ cell populations. These cell populations were isolated by incubation with anti-mouse B220, CD4, CD8, or CD90 monoclonal antibody (mAb)–conjugated microbeads followed by separation using a magnetic cell separator system (Vario-MACS separator system; Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's protocol. The fractionated B220$^+$ population was stained with phycoerythrin (PE)-conjugated anti-mouse CD90 mAb and fluorescein isothiocyanate (FITC)–conjugated anti-mouse B220 mAb. CD4$^+$ and CD8$^+$ populations were stained with PE-conjugated anti-mouse CD8 mAb and FITC-conjugated anti-mouse CD4 mAb (Pharmingen, San Diego, CA, USA). The stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Tokyo, Japan).

Proliferation assay

Whole or fractionated B220$^+$, CD4$^+$, or CD8$^+$ spleen cells from uninfected or *T gondii*–infected mice were cultured in a round-bottomed 96-well plate (10^5 cells/well) at 37°C in a 5% CO_2 incubator. Each well contained 200 μL of Roswell Park Memorial Institute-1640 culture medium supplemented with 5% fetal calf serum, 2-mercaptoethanol, and antibiotics, with or without *Tg*HSP70. Cells were harvested at 24-hour intervals from day 1 to day 7, and the proliferation of the responding cells was measured by the degree of incorporation of [^{3}H] methyl-thymidine (^{3}H-TdR; specific activity 16 Ci/mmol, New England Nuclear, Boston, MA, USA) as described previously (Yano et al 1989). Microbead-fractionated B220$^+$ or CD90$^+$ spleen cells from B6 mice were treated with either anti-I-A^b (25-5-6) mAbs (a generous gift from Dr Shinohara, Kitazato University, Japan) plus complement (C) or C alone and were cultured with or without *Tg*HSP70 as described previously (Yano et al 1987). Spleen cells (10^5) from uninfected syngeneic mice treated with 50 μg/mL of mitomycin C (Kyowa Hakko, Tokyo, Japan) for 1 hour were added to the culture as antigen presenting cells (APC). Proliferative responses of the responder cells to 1 μg/mL of Concanavarin A (Con A) (Sigma, St Louis, MO, USA) or 25 μg/mL of LPS from E. coli 026:B6 (Difco Laboratories, Detroit, MI USA) were also tested. Five or 10 μg/mL of Polymyxin B (Sigma) was added to the assay culture with *Tg*HSP70 or LPS. Data were expressed as mean count per minute (cpm) of ^{3}H-TdR incorporation or as the difference between antigen-stimulated and control responses (Δcpm).

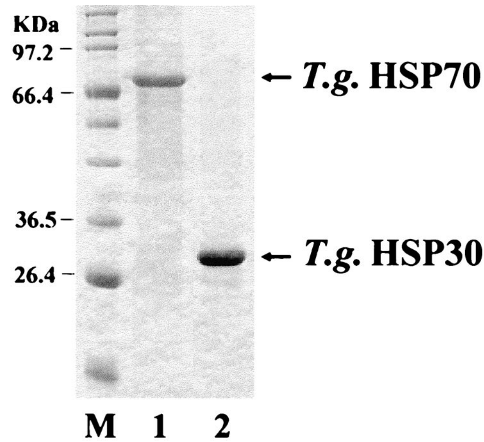

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of recombinant *Tg*HSP70 and *Tg*HSP30. Recombinant *Tg*HSP70 (lane 1) and *Tg*HSP30 (lane 2) were run on a 12% SDS-PAGE with a molecular size marker (M).

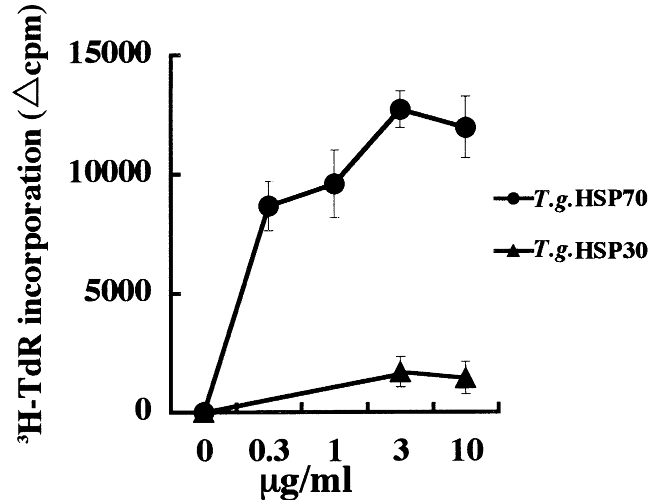

Fig. 2. Dose-response analysis. Spleen cells of uninfected B6 mice were cultured with the indicated amounts of *Tg*HSP70 (closed circle) or *Tg*HSP30 (closed triangle). Data are expressed as Δcpm of [^{3}H]-thymidine incorporation. Representative data of 2 independent experiments are shown. Cpm, counts per minute.

Reverse transcriptase–polymerase chain reaction

Total RNA was extracted from B220$^+$ spleen cells of B6 and TLR4$^{-/-}$ mice cultured for 36 hours with or without 25 μg/mL of LPS, 3 μg/mL of *Tg*HSP70 or *Tg*HSP30 by a guanidium isothiocyanate-phenol-chroloform extraction method (Trizol™, GIBCO BRL, Gaithersburg, MD, USA) and reverse transcribed using avian myeloblastosis virus reverse transcriptase (Takara Shuzo Co., Kyoto, Japan). The cDNA products were amplified with polymerase chain reaction (PCR). The primer sequences are available on request (K. Miyake, personal communication). As an internal control, glyceraldehyde 3-phosphate dehydrogenase was used. PCR products were run on a 1% agarose gel with ethidium bromide staining.

RESULTS

*Tg*HSP70 induced proliferative responses in murine spleen cells

The stimulation ability of *Tg*HSP70 in spleen cells from *T gondii*–infected B6 (a susceptible strain) and BALB/c (a resistant strain) mice was examined. As a control, stimulation ability of *Tg*HSP30, a marker of *T gondii* bradyzoites, was tested. Molecular sizes of recombinant protein of *Tg*HSP70 and *Tg*HSP30 were 74 kDa and 30 kDa, respectively (Fig 1). *Tg*HSP70 induced prominent proliferative responses of spleen cells from B6 mice, whereas *Tg*HSP30 did not induce this proliferative response (Fig 2). Therefore, this proliferative response of spleen cells was specifically induced by *Tg*HSP70. *Tg*HSP70-induced proliferative responses were observed from 0.3 μg/mL and plateaued at 3 μg/mL of *Tg*HSP70 (Fig 2). Following experiments were carried out by using 3 μg/mL of *Tg*HSP70.

In the next experiment, the kinetic studies of stimulation effects of *Tg*HSP70 on spleen cells from *T gondii*–infected and uninfected B6 and BALB/c mice were tested. The spleen cells from *T gondii*–infected and uninfected B6 and BALB/c mice also proliferated markedly after stimulation with *Tg*HSP70 but not with *Tg*HSP30 (Fig 3A,B). The *Tg*HSP70-induced proliferative responses peaked on day 3 and subsided by day 6 (Fig 3A,B). Spleen cells of B6 mice proliferated at a higher rate than those of BALB/c mice (Fig 3A,B).

*Tg*HSP70 induced proliferation of B cells but not T cells

To analyze the proliferating cells stimulated with *Tg*HSP70, whole spleen cells of *T gondii*–infected B6 mice were subfractionated to B220$^+$, CD4$^+$CD8$^-$, and CD4$^-$CD8$^+$ populations by a positive sorting with microbeads conjugated with anti-B220 mAb, anti-CD4 mAb, and anti-CD8 mAb. The purity of B220$^+$, CD4$^+$CD8$^-$, and CD4$^-$CD8$^+$ populations were 96.1%, 95.9%, and 93.3%, respectively (Fig 4A–C). These fractionated cells were cultured with or without *Tg*HSP70. Stimulation with *Tg*HSP70 induced marked proliferation in the B220$^+$ population (Fig 5). CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells did not respond to *Tg*HSP70 with or without APC (Fig 5). The proliferative responses of the B220$^+$ population to *Tg*HSP70 and LPS subsided when major histocompatibility complex (MHC) class II positive cells were eliminated from the responder population (Fig 6). CD90$^+$ T cells did not respond to *Tg*HSP70 with or without APC, but they responded well to Con A with APC (Fig 6). These data

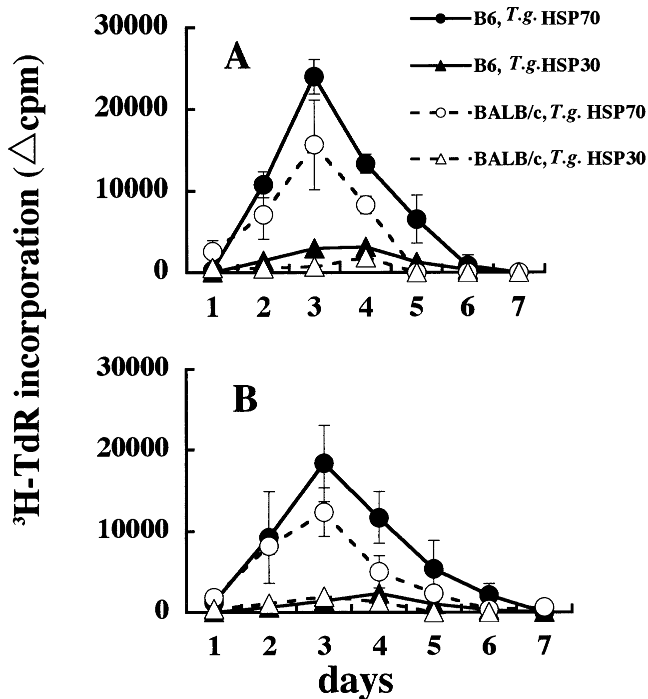

Fig. 3. Kinetic study of the proliferative responses of spleen cells to *Tg*HSP70. 1 × 10^5 spleen cells from *Toxoplasma gondii*–infected (A) or uninfected (B) B6 (a susceptible strain) and BALB/c (a resistant strain) mice were cultured with 3 μg/mL of either *Tg*HSP70 or *Tg*HSP30 and were harvested on days 1–7. Proliferative responses are expressed as Δcpm of [^{3}H]-thymidine incorporation. Symbols: spleen cells of B6 mice cultured with *Tg*HSP70 (closed circle with solid line) or *Tg*HSP30 (closed triangle with solid line), spleen cells of BALB/c mice cultured with *Tg*HSP70 (open circle with dotted line) or *Tg*HSP30 (open triangle with dotted line). The data are representative of 5 independent experiments. Cpm, counts per minute.

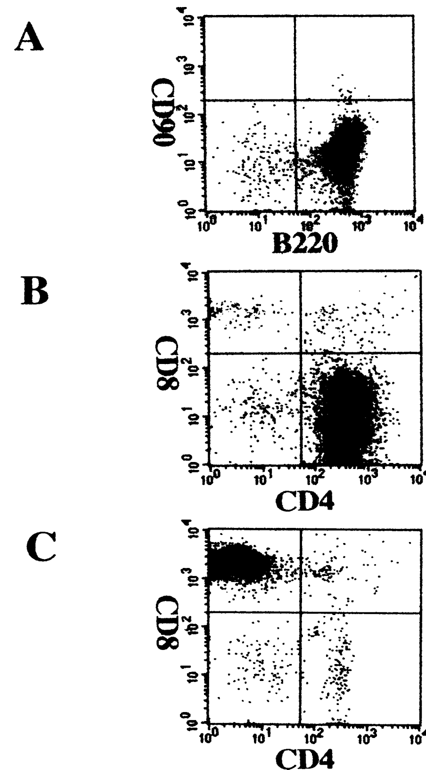

Fig. 4. Fractionation of spleen cells. Whole spleen cells of uninfected B6 mice were fractionated to B220$^+$, CD4$^+$, and CD8$^+$ populations by a positive sorting with microbeads conjugated with anti-B220, anti-CD4, or anti-CD8 mAb. (A) B220$^+$ population was stained with PE-conjugated anti-CD90 mAb and FITC-conjugated anti-B220 mAb. (B) CD4$^+$ and (C) CD8$^+$ populations were stained with PE-conjugated anti-CD8 mAb and FITC-conjugated anti-CD4 mAb. The purity of B220$^+$, CD4$^+$, and CD8$^+$ populations by flow cytometry analysis was 96.1%, 95.9%, and 93.3%, respectively. PE, phycoerythrin; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate.

indicated that *Tg*HSP70 induced proliferation of B cells but not T cells.

To confirm the mitogenic activity of *Tg*HSP70 on B cells, *Tg*HSP70-induced proliferative responses of spleen cells from uninfected FcγR$^{-/-}$ B6, ICR nude, μMT, RAG1$^{-/-}$ B6, and SCID mice were compared (Fig 7). LPS and Con A were used as mitogen controls for B cells and T cells, respectively. FcγR$^{-/-}$ B6 mice responded to *Tg*HSP70 as well as to LPS and Con A, indicating that FcγR was not involved in exogenous *Tg*HSP70-induced spleen cell proliferation. ICR nude mice responded to *Tg*HSP70 and LPS but not to Con A. μMT mice did not respond to *Tg*HSP70 and LPS but responded to Con A. RAG1$^{-/-}$ B6 mice and SCID mice failed to respond to *Tg*HSP70, LPS, or Con A. These results confirmed that *Tg*HSP70 induced proliferation of B cell but not T cell populations.

Polymyxin B did not inhibit *Tg*HSP70-induced proliferation

The mitogenic activity of *Tg*HSP70 was compared with that of LPS. Polymyxin B, an LPS-specific inhibitor that binds to the lipid A portion of LPS in the cell membrane of Gram-negative bacteria, was added to the *Tg*HSP70- or LPS-induced spleen cell proliferation assay. LPS-induced proliferative responses of spleen cells were almost completely inhibited by the addition of Polymyxin B. But *Tg*HSP70-induced proliferative responses were not significantly inhibited by Polymyxin B. These results showed that the *Tg*HSP70-induced proliferative responses of spleen cells were not due to endotoxin contamination (Fig 8).

Involvement of TLR4 but not MyD88 in *Tg*HSP70-induced proliferation

As TLR4 is known as a receptor for LPS, the messenger ribonucleic acid (mRNA) expression of TLR4 in B220$^+$ spleen cells unstimulated or stimulated with LPS, *Tg*-HSP70, and *Tg*HSP30 was comparatively analyzed. In B6 mice, compared with the B cells without stimulation, mRNA expression of TLR4 in *Tg*HSP70-stimulated B cells

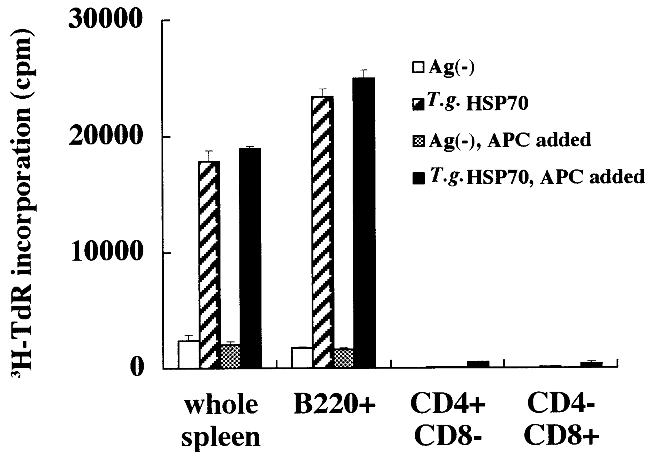

Fig. 5. *Tg*HSP70 induced proliferation of B cells but not T cells. 1 $\times$ 10^5 whole spleen cells and fractionated B220$^+$, CD4$^+$, or CD8$^+$ population were cultured for 3 days with the absence (open bars) or presence (striped bars) of *Tg*HSP70. 1 $\times$ 10^5 mitomycin C–treated spleen cells were added as APC to the culture in the absence (shaded bars) or presence (filled bars) of *Tg*HSP70. Data are representative of 5 independent experiments and are expressed as cpm of [^{3}H]-thymidine incorporation. APC, antigen presenting cells; cpm, counts per minute.

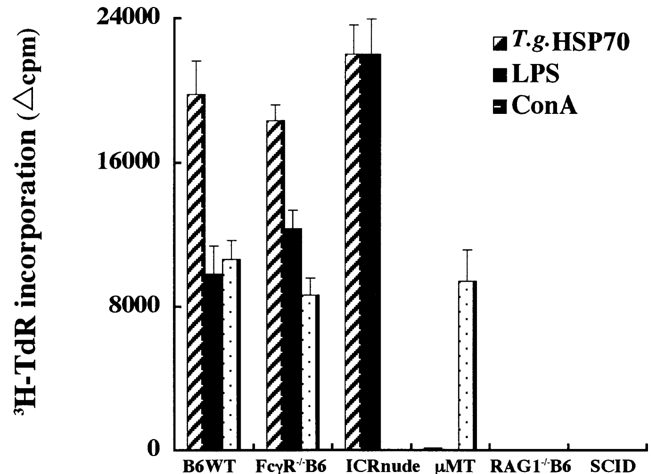

Fig. 7. *Tg*HSP70-induced proliferative responses of spleen cells from various strains of mice. *Tg*HSP70-induced (striped bars) proliferative responses of 1 $\times$ 10^5 spleen cells from uninfected B6 WT mice, FcγR$^{-/-}$ B6 mice, ICR nude mice, μMT mice, RAG1$^{-/-}$ B6 mice, and SCID mice were compared with LPS-induced (filled bars) or Con A–induced (dotted bars) proliferative responses. Data are representative of at least 2 independent experiments and are expressed as Δcpm of [^{3}H]-thymidine incorporation. SCID, severe combined immunodeficiency; LPS, lipopolysaccharide; Con A, Concanavarin A; cpm, counts per minute.

was slightly enhanced, whereas that of LPS-stimulated B cells was slightly reduced and that of *Tg*HSP30-stimulated B cells was unchanged (Fig 9).

In the next experiment, we examined the involvement of TLR4 and MyD88 molecules in *Tg*HSP70-induced B cell proliferation. Spleen cells of C3H/HeJ mice, which carry a point mutation in the cytoplasmic region of TLR4 and are hyporesponsive to LPS, responded poorly to *Tg*HSP70 or LPS. Spleen cells of C3H/HeN mice proliferated as well as those of B6 mice, indicating that not only LPS- but also *Tg*HSP70-induced B cell activation requires the TLR4 molecule as a receptor (Fig 10A). The involvement of TLR4 in *Tg*HSP70-induced spleen cell proliferation was also shown by the low responsiveness of TLR4$^{-/-}$ mice (Fig 10B). Involvement of MyD88 molecules in *Tg*HSP70-induced B cell proliferation was examined by using MyD88$^{-/-}$ mice. *Tg*HSP70-induced, but not LPS-induced, spleen cell proliferation was observed in MyD88$^{-/-}$ mice (Fig 10B). Therefore, the MyD88 molecule is involved in LPS-induced proliferation but not in *Tg*HSP70-induced proliferation. No proliferative response was observed in TLR2$^{-/-}$ mice.

DISCUSSION

Previous studies have shown 2 major properties of *Tg*HSP70 in host immune responses of *T gondii*–infected

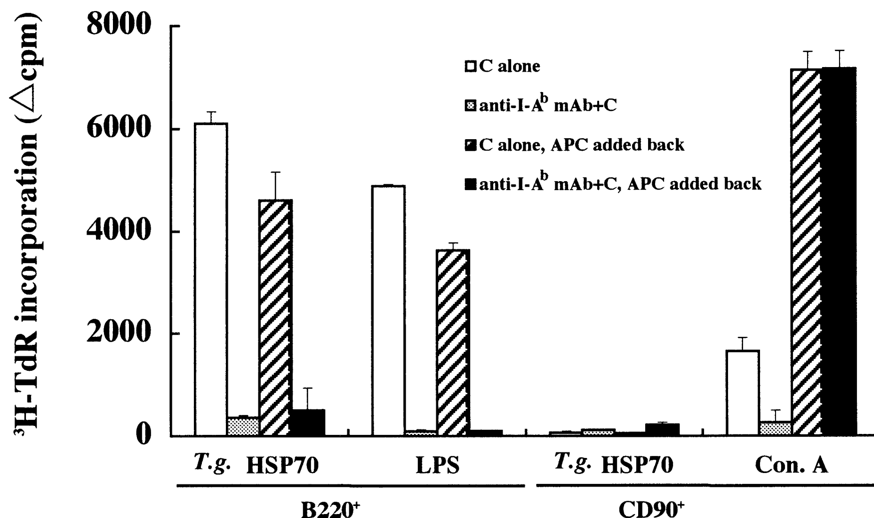

Fig. 6. *Tg*HSP70-induced B cell proliferative responses were subsided by the elimination of MHC class II positive cells. 1 $\times$ 10^5 of B220$^+$ or CD90$^+$ spleen cells of B6 mice were treated with either complement (C) alone (open bars) or anti-I-A^b mAb plus C (shaded bars) and were cultured without or with *Tg*HSP70, LPS, or Con A. 1 $\times$ 10^5 of APC were added back to the culture treated with either C alone (striped bars) or anti-I-A^b mAb plus C (filled bars). Data are representative of 3 independent experiments and are expressed as Δcpm of [^{3}H]-thymidine incorporation. MHC, major histocompatibility complex; mAb, monoclonal antibody; LPS, lipopolysaccharide; Con A, Concanavarin A; cpm, counts per minute.

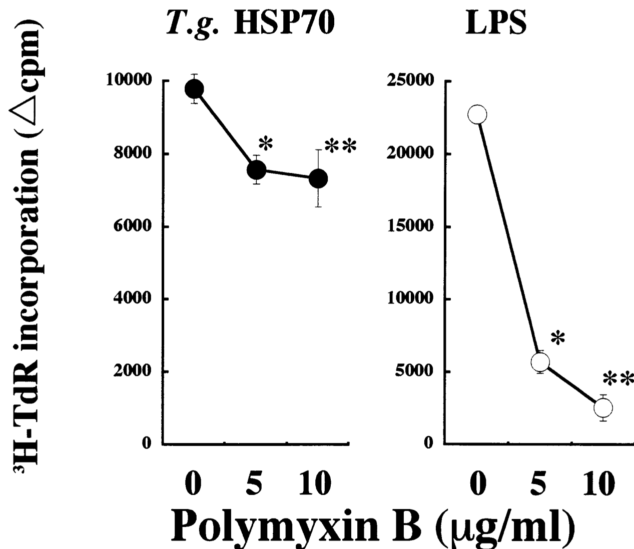

Fig. 8. Polymyxin B did not inhibit *Tg*HSP70-induced proliferative responses. Polymyxin B was added at 10 μg/mL and 5 μg/mL in the culture of *Tg*HSP70-induced (closed circle) or LPS-induced (open circle) spleen cell proliferation assay. Data are expressed as Δcpm of [^{3}H]-thymidine incorporation. Representative data from 3 independent experiments are shown. Differences of mean values between control and groups were determined with the unpaired Student's *t*-test. *, $P < 0.003$; **, $P < 0.002$; cpm, counts per minute.

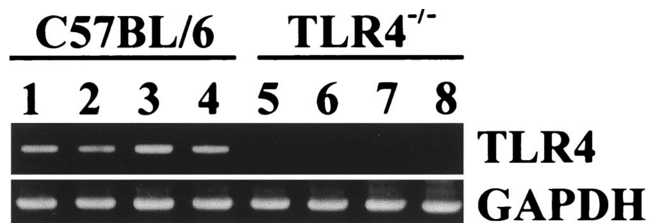

Fig. 9. TLR4 expression of *Tg*HSP70-stimulated B cells. B220$^+$ spleen cells of B6 and TLR4$^{-/-}$ mice were cultured for 36 hours in the absence or presence of either LPS, *Tg*HSP70, or *Tg*HSP30. The mRNA expression of TLR4 on B220$^+$ spleen cells of B6 (lanes 1–4) and TLR4$^{-/-}$ (lanes 5–8) mice unstimulated (lanes 1 and 5) or stimulated with LPS (lanes 2 and 6), *Tg*HSP70 (lanes 3 and 7), and *Tg*HSP30 (lanes 4 and 8) was examined by RT-PCR. As an internal control, GAPDH mRNA was tested. LPS, lipopolysaccharide; mRNA, messenger ribonucleic acid; RT-PCR, reverse transcriptase–polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

mice. First, *Tg*HSP70 was shown to deteriorate the host defense by down-regulating nitric oxide release of peritoneal macrophages in the *T gondii*–infected host (Mun et al 2000). Second, *Tg*HSP70 induced anti-mHSP70 autoantibody formation by B-1 cells in *T gondii*–infected mice (Chen et al 2000).

In this study, we have demonstrated that *Tg*HSP70 functions as a B cell mitogen. The kinetic patterns of *Tg*HSP70-induced proliferative responses of spleen cells resembled those of antigen-specific secondary responses; however, *Tg*HSP70 induced prominent proliferative responses in spleen cells from uninfected mice. The analysis of responder cells revealed that *Tg*HSP70 induced proliferation of B cells but not T cells, and *Tg*HSP70 was shown to induce proliferation of the irrespective-antigen–primed B cells (data not shown), indicating that *Tg*HSP70 possessed B cell mitogenic properties. The effects of microorganism-derived HSP70 on B cells have previously been reported. Bonorino et al (1998) found an increase of B cells in mice after in vitro incubation with mycobacterial HSP70. The immunogen-specific early strong IgG antibody response after immunization with HSP70 is responsible for this proliferation. Rico et al (1999) reported that spleen cells from unprimed mice were stimulated to proliferate with *Leishmania* HSP70, and they speculated that B cells might be involved in HSP70-induced proliferation of spleen cells. This study is the first to report proliferative responses of B cells to HSP70 derived from *T gondii*.

The proliferative response of spleen cells of B6 mice was higher than that of BALB/c mice, indicating that the degree of *Tg*HSP70-induced proliferation is related to the genetically restricted susceptibilities of mice to *T gondii* infection. We previously reported the differential anti-*Tg*HSP70 and anti-*Tg*HSP30 antibody formations in B6 and BALB/c mice after *T gondii* infection. The levels of anti-*Tg*HSP70 IgG antibody production in B6 mice were higher than those in BALB/c mice, whereas the levels of anti-*Tg*HSP30 IgG antibody production in B6 were lower than those of BALB/c mice (Mun et al 1999). We speculate that levels of *Tg*HSP70-induced B cell proliferation in B6 and BALB/c mice may correlate with anti-*Tg*HSP70 antibody producing responses in those mice.

Arnold-Schild et al (1999) showed that HSPs bound specifically to the surface of professional APC and were internalized spontaneously by receptor-mediated endocytosis, demonstrating the existence of specific receptors for HSP on the professional APC. Furthermore, recent studies demonstrated another function of HSP70 to induce dendritic cell (DC) maturation after binding to immature DC and suggested the abundant receptor expression for HSP70 on immature DC (Todryk et al 1999; Basu et al 2000; Kuppner et al 2001). Functional analyses on activation or inactivation of APC by *Tg*HSP70 stimulation are now underway.

In our study, the level of proliferation of spleen cells from *T gondii*–infected mice was slightly higher than that from uninfected mice. At least 2 receptors are involved in B cell activation by *Tg*HSP70. One is immunoglobulins on B cells because antibody production for *Tg*HSP70 was observed in mice infected with *T gondii* or immunized with *Tg*HSP70 (Mun et al 1999; Chen et al 2000). The other one is TLR4 as reported in this article. Thus, it may be speculated that B cells from *T gondii*–infected mice proliferated at a higher rate than B cells from uninfected mice.

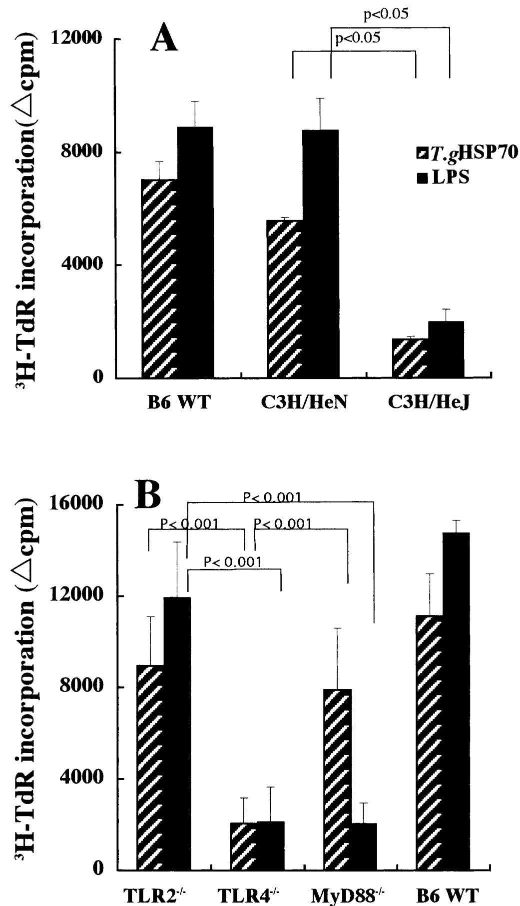

Fig. 10. Involvement of TLR4 but not MyD88 molecules in *Tg*HSP70-induced spleen cell proliferation. (A) *Tg*HSP70-induced (striped bars) or LPS-induced (filled bars) proliferative responses of 1×10^5 spleen cells from B6 WT, C3H/HeN, and C3H/HeJ mice were compared. (B) *Tg*HSP70-induced (striped bars) or LPS-induced (filled bars) proliferative responses of 1×10^5 spleen cells from TLR2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice were compared with those of B6 WT mice. Differences between mean values were analyzed with the unpaired Student's *t*-test. *P* values less than 0.05 were considered statistically significant. Data are representative of 5 independent experiments and are expressed as Δcpm of [^{3}H]-thymidine incorporation. LPS, lipopolysaccharide; cpm, counts per minute.

*Tg*HSP70 induced proliferative responses in ICR nude mice but not in μMT, RAG1$^{-/-}$ B6, or SCID mice. Nude mice are known to possess certain T-independent antigen-specific B cells that contain mitogenic compounds such as polysaccharides. Therefore, we speculate that *Tg*HSP70 stimulates B cells in a manner similar to LPS.

Because *Tg*HSP70 was revealed to be a B cell mitogen, the functions of *Tg*HSP70 and LPS have been comparatively analyzed. *Tg*HSP70-induced proliferative responses were not significantly inhibited by Polymyxin B, which almost completely inhibited LPS-induced proliferative responses. Similarly, mycobacterial HSP70–induced proliferation of spleen cells from BALB/c mice was reduced by approximately 50% by the addition of Polymyxin B, whereas LPS-induced proliferation was almost completely inhibited (Bonorino et al 1998). Also, HSP70-induced DC maturation (Kuppner et al 2001) and up-regulation of secretion of cytokines such as interleukin (IL)-1β, tumor necrosis factor α, and IL-6 from DC or monocytes (Asea et al 2000; Moroi et al 2000) were not inhibited by Polymyxin B in mouse and human systems. But induction of these responses by LPS was completely inhibited. The interaction between Polymyxin B and LPS involves ionic forces between amino groups in Polymyxin B and phosphate and carboxyl groups in the lipid A portion of LPS, with hydrophobic interactions between the respective acyl groups. B cell mitogenic chemical moieties of *Tg*HSP70 remain to be analyzed.

It is well known that TLR4, a member of the TLR family, is a critical receptor and signal transducer for LPS. LPS down-regulated surface expression of the TLR4-MD2 complex on peritoneal macrophages (Akashi et al 2000; Sato et al 2000). As the cell-surface expression of TLR4 in association with MD-2 on B cells in spleen was hardly detectable by the staining with anti-TLR4 mAb MTS510 (Akashi et al 2000; K. Miyake, personal communication), we examined the mRNA expression of TLR4 in splenic B220$^+$ cells. The level of mRNA expression of TLR4 in *Tg*HSP70-stimulated B cells was slightly enhanced, whereas that of LPS-stimulated B cells was slightly reduced. Analyses of differential control mechanisms by LPS and *Tg*HSP70 in expression of TLR4 on macrophages and B cells are underway.

The cytoplasmic adaptor molecule, MyD88, is associated with various TLRs including TLR4. The involvement of TLR4 and MyD88 molecules in *Tg*HSP70- and LPS-induced B cell proliferation was investigated by using TLR4$^{-/-}$ and MyD88$^{-/-}$ mice. The results with C3H/HeJ and TLR4$^{-/-}$ mice demonstrated that both *Tg*HSP70- and LPS-induced proliferative responses of spleen cells required TLR4 molecules as a receptor. Although Moroi et al (2000) reported that mHSP70 induced a small amount of IL-1β and IL-6 release from DC in TLR4-deficient LPS-resistant C57BL/10ScN mice, they acknowledged that the mice are not absolutely resistant to LPS. Thus, their data did not necessarily conflict with our results. On the other hand, although LPS induced low responsiveness in MyD88$^{-/-}$ mice, *Tg*HSP70 induced apparent proliferative responses in spleen cells of MyD88$^{-/-}$ mice. Additional experiments such as analyses of direct binding of *Tg*HSP70 to TLR4 and its signal transduction remain to be analyzed to confirm the TLR4 involvement in *Tg*HSP70-induced proliferative responses, whereas TLR4 on B cells are hardly detected. Also molecular-based analyses are required to examine the participation of a MyD88-independent pathway downstream of TLR4 in *Tg*HSP70-induced proliferative responses. Recently, a cytoplasmic adapter protein for TLR4, called Toll-IL-1 receptor (TIR)

domain–containing adapter protein (TIRAP) or MyD88-adapter–like (MAL) molecule that activates downstream signal transduction events in the absence of MyD88, has been identified (Fitzgerald et al 2001; Horng et al 2001; Kaisho et al 2001). Further studies will be required to examine whether the TIRAP- or MAL-dependent signaling pathway is involved in *Tg*HSP70-induced B cell proliferation.

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