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Toxoplasma gondii–derived heat shock protein 70 stimulates maturation of murine bone marrow–derived dendritic cells via Toll-like receptor 4

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Abstract *Toxoplasma gondii*–derived heat shock protein 70 (*T.g.*HSP70) induced maturation of bone marrow–derived dendritic cells (DCs) of wild-type (WT) C57BL/6 mice as evidenced by an increase in surface expression of MHC class I and II molecules and costimulatory molecules such as CD40, CD80, and CD86. Functionally, decreased phagocytic ability and increased alloreactive T cell stimulatory ability were observed in *T.g.*HSP70-stimulated DCs. These phenotypic and functional changes of *T.g.*HSP70-stimulated DCs were demonstrated in Toll-like receptor (TLR) 2- and myeloid differentiation factor 88 (MyD88)-deficient but not TLR4-deficient C57BL/6 mice. DCs from WT and TLR2-deficient but not TLR4-deficient mice produced IL-12 after *T.g.*HSP70 stimulation. *T.g.*HSP70-stimulated DCs from WT, TLR2-deficient, and MyD88-deficient, but not TLR4-deficient mice expressed IFN-β mRNA. Thus, *T.g.*HSP70 stimulates murine DC maturation via TLR4 through the MyD88-independent signal transduction cascade.

INTRODUCTION

Toxoplasma gondii, an obligate intracellular parasite, is the causal agent of significant morbidity and mortality worldwide. Pathogenicities of *T. gondii*–derived heat shock protein (HSP) 70 (*T.g.*HSP70) were analyzed in *T. gondii*–infected mice. The expression of *T.g.*HSP70 rapidly increases before death of the host, and *T.g.*HSP70 down-regulates host protective immune responses against *T. gondii* infection by (1) inhibiting nitric oxide (NO) release of peritoneal macrophages (Mun et al 2000), (2) producing anti-HSP70 autoantibody from V_H1-J_H1 B-1 cells (Chen et al 2000, 2003b), and (3) polarizing from Th1 to Th2 immune responses in *T. gondii*–infected mice (Ahmed et al 2004). Thus, *T.g.*HSP70 was revealed to be a ''virulent molecule'' for protective immunity of the host (Dobbin et al 2002; Yano et al 2002).

By using a gene gun, we successfully developed a vaccine with *T.g.*HSP70 gene against *T. gondii* infection, targeting epidermal Langerhans cells (LCs) and dermal dendritic cells (DCs) (Mohamed et al 2003). The induction of protective immunity by *T.g.*HSP70 gene suggested the presence of receptor expression for *T.g.*HSP70 on peripheral immature DCs (LC/DC). DCs are bone marrow–derived 'professional' antigen-presenting cells (APCs) capable of activating naïve antigen-specific T cells and initiating adaptive protective immunity (Banchereau and Steinman 1988). DC maturation is essential to induce T cell activation that plays a central role in protective cellular immunity against intracellular protozoan infection. In peripheral nonlymphoid tissues, DCs exist in an immature form that is highly proficient in acquiring and processing antigens (Sallusto et al 1995). When exposed to microbial infection, DCs take up antigens and migrate to draining lymph nodes (dLNs) with their 'cargo' of an-

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tigens while maturing into highly potent APCs by upregulating the expression of the major histocompatibility complex (MHC) class I and II molecules and T cell–stimulatory surface molecules such as CD40, CD80, and CD86 (Garg et al 2003). During maturation, DCs lose the ability to acquire new antigens (Mellman and Steinman 2001), and mature DCs present antigens as processed peptides to naïve CD4 and CD8 T cells in dLNs.

In microbial infection, specific molecular patterns that exist in microbial components directly stimulate immature DCs in the periphery to differentiate into mature DCs by binding to pattern recognition receptors such as Toll-like receptors (TLRs) that play a critical role in the innate immunity of mammals (Akira and Takeda 2004). We already revealed that TLR2 and myeloid differentiation factor 88 (MyD88) are critical for protective immunity against *T. gondii* infection (Chen et al 2003a; Mun et al 2003; Kudo et al 2004). Also, TLR4 has been found to be essential in *T.g.*HSP70-induced activation of B cells (Aosai et al 2002). B cells work as APCs for T cell activation in acquired immunity, prompting us to analyze the role of *T.g.*HSP70 in DC activation.

Recently, we reported on the maturation of human monocyte-derived DCs by *T.g.*HSP70 stimulation (Kang et al 2004). In the present study, we further analyzed the role of *T.g.*HSP70 in the differentiation/maturation of murine bone marrow–derived DCs (BMDCs) and defined the involvement of TLRs and signal molecules in *T.g.*HSP70-induced murine BMDC maturation.

MATERIALS AND METHODS

Mice and recombinant *T. gondii* HSP70

Sex-matched 8-week-old wild type (WT) C57BL/6 (B6) (H-2^b) and BALB/c (H-2^d) mice were purchased (SLC, Hamamatsu, Japan). TLR2-, TLR4-, and MyD88-deficient mice with B6 background were maintained in our laboratory (Aosai et al 2002; Chen et al 2003a; Mun et al 2003) and their genomic DNA was screened by polymerase chain reaction (PCR) for TLR2, TLR4, and MyD88 mutation as reported (Adachi et al 1998; Hoshino et al 1999; Takeuchi et al 1999; personal communication). *T.g.*HSP70 was prepared as described previously (Mun et al 1999).

Generation of BMDCs

BMDCs were generated as reported previously (Inaba et al 1992) with minor alterations. Briefly, bone marrow cells were flushed from the tibiae and femurs of WT, TLR2-, TLR4-, and MyD88-deficient B6 mice and cultured to differentiate in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (FCS), antibiotics, 5 ng/ml granulocyte/macrophage colony-stimulating factor (GM-CSF) (e-Bioscience; Rikaken Co., Nagoya, Japan) and 5 ng/ml interleukin 4 (IL-4) (e-Bioscience) in a 6-well plastic plate (Nunc, Roskilde, Denmark). At day 3, nonadherent cells were washed out after gentle swirling of the plate, and the remaining adherent cells were cultured further in fresh medium containing the same ingredients. At day 5, nonadherent cells were again gently washed out and the remaining loosely adherent cells were used as immature BMDCs.

Stimulation of immature BMDCs and flow cytometry analysis

Immature BMDCs obtained at day 5 were cultured with or without 1 μg/ml *T.g.*HSP70 or 100 ng/ml lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 (Sigma-Aldrich, Tokyo, Japan) for another 48 hours. At day 7, $CD11c^+$ population was positively purified from loosely adherent cells with microbead-conjugated anti-CD11c monoclonal antibody (mAb) by a magnetic cell sorting system (MACS) (Miltenyi Biotec, Auburn, CA, USA), and $CD11c^+$ BMDCs were stained with phycoerythrin (PE)-conjugated anti-CD40, -CD80, or -CD86 mAb (e-Bioscience), or fluorescein isothiocyanate (FITC)-conjugated anti-I-A^b or -K^b mAb (e-Bioscience) at 4°C for 30 minutes. After washing in chilled phosphate buffered saline containing 0.05% NaN_3 and 1% FCS, the surface expression of these molecules on BMDCs was analyzed by FACScan flow cytometer (Becton Dickinson, Tokyo, Japan). Inhibition effects of Polymyxin B (Sigma, St Louis, MO, USA) on phenotypic analysis of BMDCs were examined by adding 10 μg/ml of Polymyxin B to the culture of immature BMDCs with or without *T.g.*HSP70 or LPS. The effect of *T.g.*HSP70 denatured by boiling (100°C, 1 hour) on phenotypic analysis of BMDCs was also examined.

Uptake of FITC-dextran

Phagocytic activity of BMDCs was measured by assaying for uptake of FITC-conjugated dextran (molecular weight [M.W.], 40 000; Sigma) as described (Sallusto et al 1995; Kang et al 2004). Briefly, $CD11c^+$ BMDCs cultured with or without *T.g.*HSP70 or LPS for 48 hours were harvested at day 7 and incubated in a 96-well microtiter plate with or without FITC-conjugated dextran at a concentration of 1 mg/ml for 1 hour at 37°C. After washing, the uptake of FITC-conjugated dextran was analyzed by FACScan.

Alloreactive T cell stimulation

$CD11c^+$ BMDCs cultured with or without *T.g.*HSP70 for 48 hours were harvested at day 7 and irradiated with 20 Gy. Thereafter, 1×10^4 $CD11c^+$ BMDCs were cocultured as stimulator cells with 1×10^5 responder allogeneic T

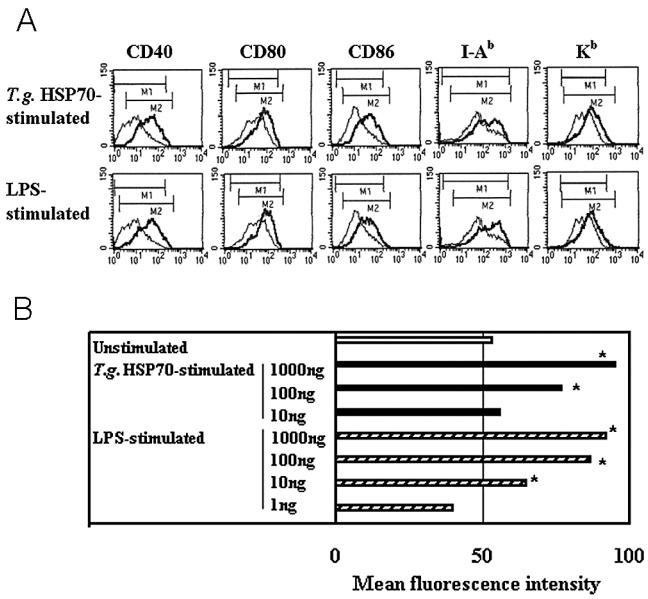

Fig 1. (A) Flow cytometric analyses of CD40, CD80, CD86, and MHC expression on *T.g.*HSP70-stimulated BMDCs. Immature BMDCs from WT mice were cultured with or without 1 μg/ml *T.g.*HSP70 or 100 ng/ml LPS for 48 hours and stained with PE-conjugated anti-CD40, -CD80, or -CD86 mAb, or FITC-conjugated anti-I-A^b or -K^b mAb. Surface expressions of the costimulatory molecules (CD40, CD80, and CD86) and MHC class I and II molecules of BMDCs stimulated with (bold lines) or without (thin lines) *T.g.*HSP70 or LPS were comparatively analyzed by FACScan. Representative data from three independent experiments are shown. (B) Dose-response analysis. Immature BMDCs from WT mice were cultured with the indicated amounts of *T.g.*HSP70 (black bars) or LPS (striped bars), or medium alone (open bar) for 48 hours. Surface expressions of CD86 were shown as mean fluorescence intensities. Differences of fluorescence intensities between *T.g.*HSP70- or LPS-stimulated and unstimulated BMDCs were determined by Kolmogorov-Smirnov (K-S) two-sample test. *, $P < 0.001$.

cells from spleens of BALB/c mice, which were positively purified with microbead-conjugated anti-CD90 antibody by MACS (Miltenyi Biotec), in a 96-well microtiter plate in RPMI 1640 medium containing 5% FCS, antibiotics, and 2-mercaptoethanol. Proliferation of responder cells was measured by [^{3}H]thymidine incorporation as described previously (Aosai et al 2002). Inhibition effects of Polymyxin B were examined by adding 10 μg/ml Polymyxin B to the culture of immature BMDCs with or without *T.g.*HSP70. Effects of denatured *T.g.*HSP70 were also examined by boiling (100°C, 1 hour).

Reverse transcriptase-polymerase chain reaction

The expression of interferon-β (IFN-β) mRNA from BMDCs cultured with or without *T.g.*HSP70 for 48 hours was investigated by reverse transcriptase-polymerase chain reaction (RT-PCR) as previously described (Mun et al 2000). The sequences of the forward and reverse IFN-β primers used were 5′-TCCAAGAAAGGACGAACATT CG-3′ and 5′-TGAGGACATCTCCCACGTCAA-3′, respectively (Toshchakov et al 2002). As an internal control, the mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was tested.

IL-12 production

BMDCs cultured with or without *T.g.*HSP70 or LPS for 48 hours were stained with PE-conjugated anti-mouse IL-12 mAb (PharMingen, San Diego, CA, USA) by Cytofix/Cytoperm kit (PharMingen) according to the manufacturer's instructions. After washing, the intracellular expression of IL-12 of BMDCs was analyzed by FACScan.

RESULTS

*T.g.*HSP70 stimulates murine BMDC maturation

Immature BMDCs from B6 mice induced by GM-CSF and IL-4 were cultured with or without 1 μg/ml *T.g.*HSP70 or 100 ng/ml LPS. Compared to DCs cultured in medium alone, the levels of surface expression of costimulatory molecules for T cell activation such as CD40, CD80, and CD86, and MHC class I and II molecules on DCs cultured with *T.g.*HSP70 were markedly up-regulated, as well as those cultured with LPS, a known DC maturation inducer (Fig 1A; Table 1). Thus, from phenotypic analysis, *T.g.*HSP70 was capable of inducing the maturation of murine BMDCs.

Table 1 Surface expression of CD40, CD80, CD86, and MHC molecules on BMDCs

	CD40		CD80		CD86		I-A^b		K^b	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Unstimulated	15.67	21.79	40.51	38.29	23.19	27.09	130.03	188.71	53.95	47.13
*T.g.*HSP70-stimulated	*53.91	51.71	*76.71	60.12	*56.98	52.49	*225.86	231.47	*107.46	97.68
LPS-stimulated	*55.29	54.95	*79.17	57.89	*49.58	47.55	*250.86	249.67	*99.27	99.67

Mean fluorescence intensities and standard deviations (SD) of CD40, CD80, CD86, and MHC expression on unstimulated (shown as M1 in Fig 1A) or *T.g.*HSP70- or LPS-stimulated (shown as M2 in Fig 1A) BMDCs of B6 mice are presented.

* Difference of fluorescence intensity between *T.g.*HSP70- or LPS-stimulated and unstimulated BMDCs was statistically significant ($P < 0.001$) by Kolmogorov-Smirnov (K-S) two-sample test.

Table 2 Surface expression of CD40, CD80, CD86, and MHC molecules on BMDCs

		CD40		CD80		CD86		I-A^b		K^b	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TLR2-deficient	Unstimulated	23.05	16.49	47.49	71.72	39.37	80.09	130.07	166.07	56.11	70.28
	*T.g.*HSP70-stimulated	*33.68	19.70	*163.29	169.96	*122.72	151.05	*307.08	268.91	*131.80	132.95
TLR4-deficient	Unstimulated	24.61	26.76	76.13	86.74	58.95	65.96	42.49	58.10	53.53	51.69
	*T.g.*HSP70-stimulated	15.04	15.02	89.98	89.49	70.01	72.63	42.36	58.12	43.69	31.83
MyD88-deficient	Unstimulated	19.27	24.97	49.29	51.04	33.21	39.66	86.42	143.18	47.08	46.69
	*T.g.*HSP70-stimulated	*44.36	43.22	*60.63	51.1	*62.91	53.89	*201.56	255.79	*114.23	74.92

Mean fluorescence intensities and SD of CD40, CD80, CD86, and MHC expression on unstimulated (shown as M1 in Fig 2) or *T.g.*HSP70- or LPS-stimulated (shown as M2 in Fig 2) BMDCs from TLR2-, TLR4-, and MyD88-deficient mice are presented.

* Difference of fluorescence intensity between *T.g.*HSP70- or LPS-stimulated and unstimulated BMDCs was statistically significant ($P < 0.001$) by K-S two-sample test.

Table 3 Uptake of FITC-dextran by BMDCs

	WT		TLR2-deficient		TLR4-deficient		MyD88-deficient	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Unstimulated	93.66	119.99	37.46	42.60	66.37	98.51	65.05	65.85
*T.g.*HSP70-stimulated	*20.45	37.08	*19.74	24.45	60.22	85.11	*25.75	38.23

Mean fluorescence intensities and SD of FITC-conjugated dextran uptake by unstimulated (shown as M1 in Fig 3) or *T.g.*HSP70- or LPS-stimulated (shown as M2 in Fig 3) BMDCs of WT, TLR2-, TLR4-, or MyD88-deficient B6 mice are presented.

* Difference of fluorescence intensity between *T.g.*HSP70-stimulated and unstimulated BMDCs was statistically significant ($P < 0.001$) by K-S two-sample test.

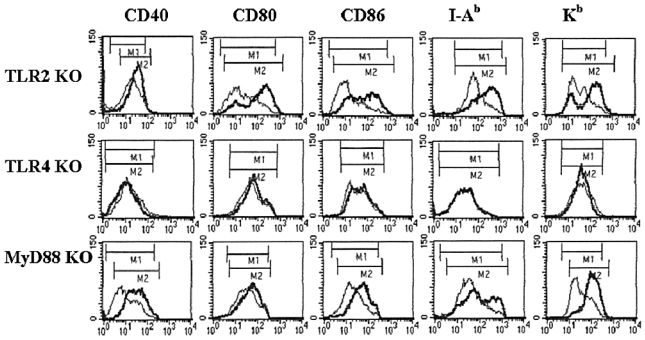

Fig 2. Involvement of TLR in *T.g.*HSP70-stimulated BMDC maturation. Immature BMDCs from TLR2-, TLR4-, and MyD88-deficient B6 mice were cultured with or without *T.g.*HSP70 for 48 hours, and surface expressions of CD40, CD80, CD86, and MHC class I and II molecules of BMDCs cultured with (bold lines) or without (thin lines) *T.g.*HSP70 were comparatively analyzed by FACScan. Representative data from three independent experiments are shown.

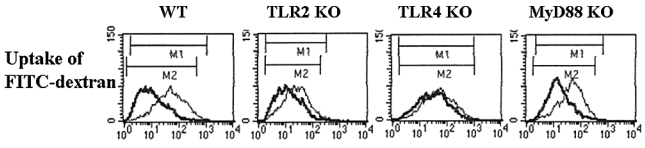

Fig 3. Uptake of FITC-dextran by *T.g.*HSP70-stimulated BMDCs. Immature BMDCs from WT, TLR2-deficient, TLR4-deficient, and MyD88-deficient B6 mice were cultured with (bold lines) or without (thin lines) *T.g.*HSP70 for 48 hours and were incubated in a 96-well microtiter plate with FITC-dextran at a concentration of 1 mg/ml for 1 hour at 37°C. Uptake of FITC-dextran by BMDCs was analyzed by FACScan. Representative data from three independent experiments are shown.

The effects of *T.g.*HSP70 and LPS to induce *T.g.*HSP70 maturation were dose-dependent (Fig 1B). A concentration of 1 μg/ml *T.g.*HSP70 or 100 ng/ml LPS was used for the following experiments.

Involvement of TLR4 in *T.g.*HSP70-stimulated BMDC maturation

In the next experiment, the involvement of TLRs in *T.g.*HSP70-stimulated maturation of BMDCs was examined. Immature DCs from TLR2- and TLR4-deficient B6 mice were cultured with or without 1 μg/ml *T.g.*HSP70. Expression levels of CD40, CD80, and CD86 molecules and MHC class I and II molecules of DCs from TLR2-deficient mice were up-regulated (Fig 2; Table 2) as well as those from WT B6 mice (Fig 1A; Table 1), whereas those of DCs from TLR4-deficient mice were unchanged after *T.g.*HSP70 stimulation (Fig 2; Table 2). These data indicated that TLR4 but not TLR2 was involved in *T.g.*HSP70-stimulated DC maturation.

Furthermore, *T.g.*HSP70 induced the up-regulation of the molecules of BMDCs from MyD88-deficient B6 mice (Fig 2; Table 2), indicating that the signal pathway downstream of TLR4 in *T.g.*HSP70-stimulated DC maturation was MyD88 independent.

Dextran uptake by *T.g.*HSP70-stimulated BMDCs

In the next experiment, the phagocytic ability of BMDCs cultured with or without *T.g.*HSP70 was assessed by flow

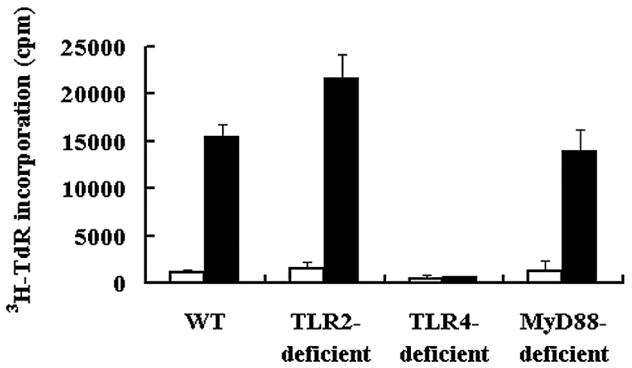

Fig 4. Alloreactive T cell stimulatory activity by *T.g.*HSP70-stimulated BMDCs. Immature BMDCs from WT, TLR2-deficient, TLR4-deficient, and MyD88-deficient B6 mice were cultured with (filled bars) or without (open bars) *T.g.*HSP70 for 48 hours and irradiated with 20 Gy. 1×10^4 BMDCs from each mouse strain were cocultured as stimulator cells with 1×10^5 responder T cells from spleens of BALB/c mice. Proliferation of responder cells is expressed as counts per minute (cpm) of [^{3}H]thymidine incorporation. Data are representative of three independent experiments.

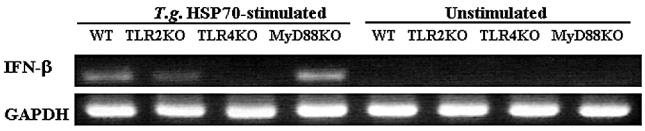

Fig 5. IFN-β mRNA expression of *T.g.*HSP70-stimulated BMDCs. Immature BMDCs from WT (lanes 1 and 5), TLR2-deficient (lanes 2 and 6), TLR4-deficient (lanes 3 and 7), and MyD88-deficient (lanes 4 and 8) B6 mice were cultured with *T.g.*HSP70 for 48 hours, and expressions of IFN-β mRNA in BMDCs stimulated with (lanes 1–4) or without (lanes 5–8) *T.g.*HSP70 were investigated by RT-PCR. The sequences of forward and reverse IFN-β primers used were 5′-TCCAAGAAAGGACGAACATTCG-3′ and 5′-TGAGGACATCTC CCACGTCAA-3′, respectively. GAPDH was used for internal control. Data are representative of three independent experiments.

cytometry of dextran-FITC phagocytosis. By stimulation with *T.g.*HSP70, the phagocytic ability of DCs from WT and TLR2-deficient mice decreased, whereas that from TLR4-deficient mice did not change (Fig 3; Table 3). Thus, the involvement of TLR4 in *T.g.*HSP70-stimulated DC maturation was confirmed.

The phagocytic ability of BMDCs from MyD88-deficient mice decreased by stimulation with *T.g.*HSP70 compared with that of unstimulated DCs (Fig 3; Table 3). Thus, MyD88 was not involved in *T.g.*HSP70-stimulated DC maturation.

Alloreactive T cell stimulatory activity by *T.g.*HSP70-stimulated BMDCs

Alloreactive T cell stimulatory abilities of BMDCs cultured with or without *T.g.*HSP70 were comparatively analyzed by using WT, TLR2-deficient, and TLR4-deficient mice. *T.g.*HSP70-stimulated DCs from WT and TLR2-deficient B6 mice induced significant proliferative responses of allogeneic splenic T cells from BALB/c mice, whereas those from TLR4-deficient B6 mice did not (Fig 4). DCs from WT, TLR2-deficient, and TLR4-deficient mice cultured in medium alone did not stimulate alloreactive T cell proliferation (Fig 4). Thus, TLR4 was necessary to induce the alloreactive T cell stimulatory ability by *T.g.*HSP70-stimulated DCs.

Furthermore, *T.g.*HSP70-stimulated BMDCs from MyD88-deficient mice induced proliferative responses of allogeneic T cells (Fig 4). Thus, MyD88 was not involved in the signal pathway downstream of TLR4 to induce the alloreactive T cell-stimulatory ability of DCs.

IFN-β expression by MyD88-independent pathway

IFN-β is produced by the MyD88-independent TIR-domain-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM)-dependent signal transduction cascade downstream of TLR4 (Akira and Takeda 2004). To analyze the involvement of the MyD88-independent signal pathway in *T.g.*HSP70-stimulated DC maturation, the expression of IFN-β mRNA in BMDCs from WT, TLR2-deficient, TLR4-deficient, and MyD88-deficient mice was examined. *T.g.*HSP70-stimulated DCs from WT, TLR2-deficient, and MyD88-deficient mice expressed IFN-β mRNA, but DCs from TLR4-deficient mice did not (Fig 5). DCs from WT, TLR2-deficient, TLR4-deficient, and MyD88-deficient mice cultured in medium alone did not express IFN-β mRNA (Fig 5). These results indicated the involvement of the TRAM/TRIF-dependent signal pathway downstream of TLR4 in *T.g.*HSP70-stimulated BMDC maturation.

Specificity of *T.g.*HSP70 to stimulate BMDC maturation

To ascertain that *T.g.*HSP70-stimulated BMDC maturation was not due to contamination of the *T.g.*HSP70 preparation by endotoxin, the inhibition effect of Polymyxin B on *T.g.*HSP70- or LPS-stimulated BMDC maturation of WT B6 mice was examined. The addition of 10 μg/ml Polymyxin B, an LPS-specific inhibitor that binds to the lipid A portion of LPS, did not inhibit up-regulation of the surface expression of MHC class II molecules and the stimulatory ability of alloreactive T cells of *T.g.*HSP70-stimulated DCs, whereas it completely inhibited those of LPS-stimulated DCs (Fig 6 A,B). The LPS-induced DC maturation was revealed to be inhibited even by a much lower concentration (0.5 μg/ml) of Polymyxin B (data not shown). Furthermore, denatured *T.g.*HSP70 by boiling (100°C, 1 hour) failed to up-regulate surface MHC class II molecule expression or induce stimulatory ability of alloreactive T cells by DCs, whereas boiling did not affect LPS-induced DC maturation (Fig 6 C,D). These results

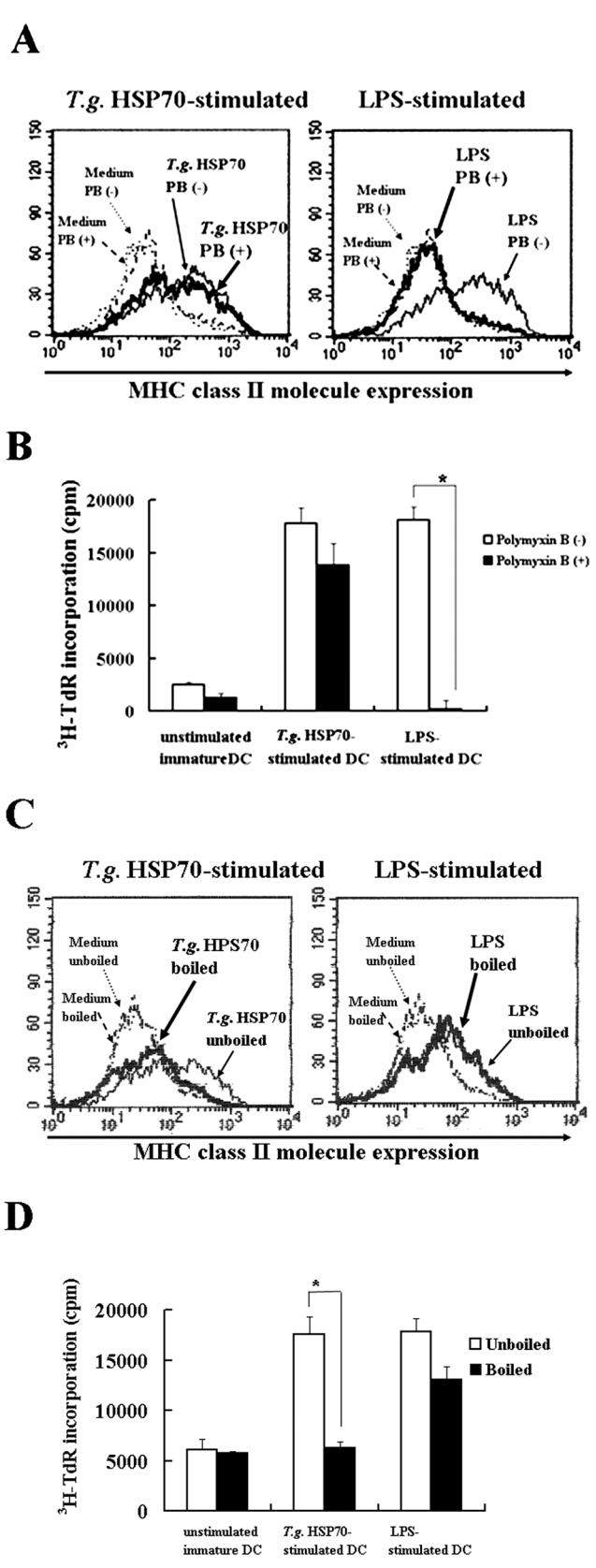

Fig 6. Specificity of *T.g.*HSP70 to stimulate DC maturation. Effects of Polymyxin B on up-regulation of MHC class II molecule expression (A) and on alloreactive T cell stimulatory ability (B) of *T.g.*HSP70- or LPS-stimulated BMDCs were examined by adding 10 μg/ml Polymyxin B to the culture of immature BMDCs of WT mice with or without *T.g.*HSP70 or LPS. Effects of boiled (100°C, 1 hour) *T.g.*HSP70 or LPS on immature BMDCs to induce surface MHC class II molecule expression (C) or alloreactive T cell stimulatory ability (D) were also examined. In FACScan analysis (A and C), MHC class II molecule expressions of BMDCs are shown as follows: medium without Polymyxin B or boiling (thin dotted lines), medium with Polymyxin B or boiling (bold dotted lines), *T.g.*HSP70- or LPS-stimulated without Polymyxin B or boiling (thin solid lines) and *T.g.*HSP70- or LPS-stimulated with Polymyxin B or boiling (bold solid lines). In alloreactive T cell stimulation (B and D), cultures with (filled bars) or without (open bars) treatment by Polymyxin B or boiling were compared. Differences of mean values between untreated and treated groups were determined by unpaired Student's *t*-test. *, $P < 0.01$; cpm, counts per minute. Data are representative of three separate experiments.

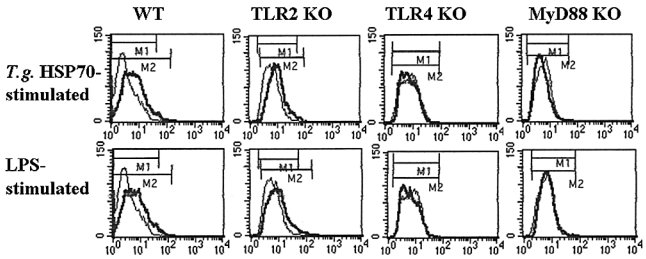

Fig 7. IL-12 production by *T.g.*HSP70-stimulated BMDCs. Immature BMDCs from WT, TLR2-deficient, TLR4-deficient, and MyD88-deficient B6 mice were cultured with (bold lines) or without (thin lines) *T.g.*HSP70 or LPS for 48 hours and stained with PE-conjugated anti-IL-12 mAb by Cytofix/Cytoperm kit (PharMingen). After washing, the intracellular expression of IL-12 of BMDCs was analyzed by FACScan. Representative data from three independent experiments are shown.

revealed that the *T.g.*HSP70-stimulated BMDC maturation was not due to endotoxin contamination.

Defect of IL-12 production by *T.g.*HSP70-stimulated BMDCs from TLR4-deficient mice

Production of IL-12 from mature DCs is required for initiating Th1 type responses to *T. gondii* infection. Therefore, the effects of *T.g.*HSP70-stimulation on IL-12 production by BMDCs were comparatively analyzed with those of LPS-stimulation. *T.g.*HSP70-stimulated DCs from WT and TLR2-deficient mice produced IL-12, whereas those from TLR4-deficient mice did not (Fig 7; Table 4). Failure of IL-12 production from DCs of TLR4-deficient mice was also observed after LPS stimulation (Fig 7; Table 4). These data indicated that *T.g.*HSP70-stimulated DCs were capable to produce IL-12 via TLR4 and modulate Th polarization.

*T.g.*HSP70-stimulated DCs from MyD88-deficient mice did not produce IL-12 (Fig 7; Table 4). Thus, it was suggested that signaling of IL-12 production by *T.g.*HSP70-

Table 4 Intracellular IL-12 production by BMDCs

	WT		TLR2-deficient		TLR4-deficient		MyD88-deficient	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Unstimulated	4.56	4.35	7.24	4.70	8.23	5.46	6.79	3.97
*T.g.*HSP70-stimulated	*9.46	12.53	*11.69	9.66	8.29	5.58	5.48	3.67
LPS-stimulated	*9.42	11.87	*12.52	13.61	7.27	5.45	7.52	4.59

Mean fluorescence intensities and SD of intracellular IL-12 expression of unstimulated (shown as M1 in Fig 7) or *T.g.*HSP70- or LPS-stimulated (shown as M2 in Fig 7) BMDCs of WT, TLR2-deficient, TLR4-deficient, and MyD88-deficient mice are presented.

* Difference of fluorescence intensity between *T.g.*HSP70- or LPS-stimulated and unstimulated BMDCs was statistically significant ($P < 0.001$) by K-S two-sample test.

stimulated DCs was transduced via TLR4/MyD88-signal pathway.

DISCUSSION

Mature DCs are distinct from immature DCs in terms of phenotype and in biological function. Induction of DC differentiation/maturation is rather important for the induction of acquired protective cellular immunity against intracellular microbial infection. Maturation of human monocyte-derived DCs by *T. gondii* infection or excreted-secreted soluble antigens (Subauste et al 2000; Wei et al 2002; Diana et al 2004) has been reported. Also, maturation of murine splenic DCs by *T. gondii* infection or soluble *T. gondii* lysate (Reis e Sousa et al 1997; Aliberti et al 2000; Straw et al 2003; McKee et al 2004) has been extensively analyzed. However, the exact molecule that stimulates DC maturation in *T. gondii* infection has not been previously identified. Our data clearly demonstrate the capability of *T.g.*HSP70 to induce phenotypic and functional differentiation/maturation of murine BMDCs. We have already reported the *T.g.*HSP70-induced differentiation/maturation of human monocyte-derived DCs (Kang et al 2004). Thus, by triggering DC activation, *T.g.*HSP70 plays a pivotal role in the induction of protective immunity both in humans and mice against toxoplasmosis.

It is known that the initiation of immune responses by DCs is promoted by various exogenous or endogenous stimuli including LPS, TNF-α, and HSP (Sallusto et al 1994; Basu et al 2000; Kaisho et al 2001). Because *T.g.*HSP70 is generated from *E. coli*, it is necessary to confirm that *T.g.*HSP70-induced maturation of DCs is not due to endotoxin contamination. The *T.g.*HSP70-induced maturation of DCs was not inhibited even by 10 μg/ml Polymyxin B, which inhibited LPS-induced DC maturation even at only 0.5 μg/ml (data not shown). Also, treatment by boiling (100°C, 1 hour) abrogated the *T.g.*HSP70-induced but not the LPS-induced DC maturation. Thus, *T.g.*HSP70-induced DC maturation was confirmed to be different from endotoxin-induced DC maturation.

Receptor-mediated endocytosis of HSP70 by DC has been reported (Arnold-Schild et al 1999; Todryk et al 1999; Castellino et al 2000). Murine BMDCs pulsed with HSP70 elicited a partial maturation signal, ie, modest stimulation of surface expression of CD86 but not CD80, nor MHC class II or CD40 (Basu et al 2000). Also, HSP70 bound to human monocyte-derived immature DCs and induced DC maturation as evidenced by an increase in CD40, CD86, and CD83 expression (Kuppner et al 2001). Together with these reports, our data from both murine BMDCs and human monocyte-derived DCs (Kang et al 2004) strongly suggested the abundant receptor expression for *T.g.*HSP70 on immature DCs. As expected, FITC-conjugated *T.g.*HSP70 specifically bound to the cell surface of immature DCs (data not shown).

We have analyzed the TLR involvement in *T.g.*HSP70-induced DC maturation. Lipsker et al (2002) previously reported the specific internalization of human HSP70 into $CD14^-$, $TLR4^-$ monocyte-derived human DCs by receptor-mediated endocytosis. They also described that TLR2- and TLR4-transfected cells did not bind to biotinylated hHSP70. However, several reports demonstrated the binding of HSP70 to CD91 (Basu et al 2001), CD14 (Asea et al 2000), and to both TLR2 and TLR4 (Asea et al 2002; Vabulas et al 2002). By using TLR-transfected cells, Asea et al (2002) reported that exogenous HSP70-induced proinflammatory cytokine production was transduced via both TLR2 and TLR4 in a CD14-dependent fashion. Also, Vabulas et al (2002) reported that human HSP70 acts as an endogenous stimulus for the Toll/IL-1 receptor signal pathway that engages TLR2 and TLR4. Similarly, we have already reported that TLR2 is essential for host-protective immunity against intraperitoneal *T. gondii* infection (Mun et al 2003; Kudo et al 2004), and that TLR4 is also essential as a receptor molecule for *T.g.*HSP70-induced B cell activation (Aosai et al 2002). Therefore, in this study, the role of TLR2 or TLR4 in *T.g.*HSP70-induced DC maturation was investigated. As described, phenotypic and functional analyses revealed that *T.g.*HSP70 induced murine BMDC maturation via TLR4.

Concerning signal molecules downstream of TLR, we

have previously demonstrated the critical role of MyD88 in host protection against *T. gondii* infection (Chen et al 2003a). In addition, nitric oxide production by peritoneal macrophages, and IFN-γ and IL-12 production by peritoneal exudates cells decreased in *T. gondii*–infected MyD88-deficient mice (Mun et al 2003). On the other hand, the signal pathway downstream of TLR4 was MyD88 independent in *T.g.*HSP70-induced B cell activation (Aosai et al 2002). In the present study, we further demonstrated the involvement of the TLR4-dependent/MyD88-independent signal pathway in *T.g.*HSP70-stimulated DC maturation. The expression of IFN-β mRNA was impaired in *T.g.*HSP70-stimulated BMDCs from TLR4-deficient mice, indicating the activation of BMDCs via the MyD88-independent/TRAM/TRIF-dependent signal transduction cascade downstream of TLR4 (Akira and Takeda 2004).

IL-12 is a key cytokine for the induction of Th1-type protective immunity against *T. gondii* infection (Aliberti et al 2000). DC activation can be monitored from a ''T cell perspective'' by assessing the ability of DCs to provide T cells with T cell receptor ligands (signal 1), costimulation (signal 2), and signals that direct T helper polarization (signal 3) (Kalinski et al 1999). Recently, Sporri and Sousa (2005) demonstrated that direct contact with pathogen components, i.e., pattern recognition by DC, was necessary for full DC activation that promoted T helper responses, whereas inflammatory mediators generated DCs that supported $CD4^+$ T cell clonal expansion but failed to direct T helper cell differentiation. As demonstrated here, *T.g.*HSP70 stimulation induced IL-12 production from BMDCs of WT and TLR2-deficient but not TLR4-deficient B6 mice (Fig 7). Aliberti et al (2004) recently reported that they were unable so far to attribute the soluble tachyzoite extract (STAg)–induced IL-12 response of DC to a single TLR despite extensive testing of different TLR transfectants and knockout mice. This might have been due to their use of crude STAg of *T. gondii*. This is the first time to define the TLR that is essential in the initiation of immune responses with IL-12 by DCs after *T.g.*HSP70 stimulation. We also demonstrated that the IL-12 production from *T.g.*HSP70-stimulated BMDCs from MyD88-deficient mice was impaired (Fig 7), indicating that this IL-12 production was transduced via the TLR4/MyD88-signal pathway. The lack of IL-12 production from *T.g.*HSP70-stimulated BMDCs of MyD88-deficient mice was consistent with previous reports of Th2 dominance in MyD88-deficient mice (Kaisho et al 2002; Sporri and Sousa 2005). Thus, MyD88 dependency was different between the maturation induction of DCs (signals 1 and 2) and the IL-12 production from DCs (signal 3) after *T.g.*HSP70 stimulation. Similar to the present study, Asea et al (2002) reported that a combination of TLR2 and TLR4 plus CD14 induced the MyD88-independent synergistic augmentation of HSP70-induced proinflammatory cytokine production, whereas HSP70-induced nuclear factor (NF)–κB activation was mediated via MyD88 signal transduction, suggesting that HSP70 might signal by both MyD88-dependent and MyD88-independent signal pathways under certain conditions. Kaisho et al (2001) also reported that LPS stimulated DC maturation via the TLR4-dependent/MyD88-independent pathway, although it activated cytokine production through TLR4 in an MyD88-dependent manner. Mechanisms of functional heterodimers of TLR2 and TLR4 together with MyD88-dependent and -independent signal transduction cascades in *T.gondii* infection remain to be analyzed.

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