

ORIGINAL ARTICLE

***Toxoplasma gondii*-derived heat shock protein 70 induces lethal anaphylactic reaction through activation of cytosolic phospholipase A₂ and platelet-activating factor via Toll-like receptor 4/myeloid differentiation factor 88**

Hao Fang, Hye-Seong Mun, Akitoshi Kikumura, Yusuke Sayama, Kazumi Norose, Akihiko Yano and Fumie Aosai

Department of Infection and Host Defense, Graduate School of Medicine, Chiba University, Chiba, Japan

ABSTRACT

Toxoplasma gondii-derived heat shock protein 70 (*T.g.HSP70*) was proven to induce IFN- γ -dependent lethal anaphylactic reaction in *T. gondii*-infected mice through an alternative PAF-mediated pathway, but not the classical immunoglobulin (Ig)E-dependent pathway. Although marked IFN- γ production was observed by CD11b⁺, CD11c⁺, CD4⁺ and CD8⁺ splenocytes, CD11b⁺ and CD11c⁺ cells were shown to be the key effector cells which generated pro-inflammatory lipid such as PAF and caused *T.g.HSP70*-induced anaphylactic reaction. In the present study, we found that the *T.g.HSP70*-induced anaphylactic reaction was not observed in TLR 4-deficient ($^{-/-}$) mice, whereas it was observed in WT and TLR2 $^{-/-}$ mice. The mRNA expression of PAF-AH, the main enzyme for PAF degradation, increased in *T. gondii*-infected WT and TLR2 $^{-/-}$ but not in TLR4 $^{-/-}$ mice after *T.g.HSP70* injection. Furthermore, phosphorylation of cPLA₂, which is the key enzyme for pro-inflammatory lipid generation, was detected in CD11b⁺ splenocytes of WT and TLR2 $^{-/-}$ mice but not in TLR4 $^{-/-}$ mice. Subsequently, cPLA₂ activation was suppressed by inhibiting the TLR4-directed p38 and p44/42 MAPK pathways. However, *T.g.HSP70*-induced anaphylactic reaction was observed in TRIF $^{-/-}$ mice, but not in MyD88 $^{-/-}$ mice. These findings indicate the cPLA₂ activated-PAF production via TLR4/MyD88-dependent, but not TRIF-dependent, signaling pathway in *T.g.HSP70*-induced anaphylactic reaction in *T. gondii*-infected mice.

Key words anaphylactic reaction, cytosolic phospholipase A₂, Toll-like receptor 4, *Toxoplasma gondii*-derived heat shock protein 70.

Expression of *Toxoplasma gondii*-derived heat shock protein 70 (*T.g.HSP70*) increases just before death of the host and functions as a danger signal to deteriorate host defense by: (i) downregulating NO release by macrophages (1); (ii)

producing anti-HSP70 autoantibody (2, 3); (iii) inducing B2 suppressor cells (4); and (iv) polarizing from Th1 to Th2 immune responses (5). However, *T.g.HSP70* activated B cells and dendritic cells (DC) (6–8). Thus, *T.g.HSP70* has

Correspondence

Fumie Aosai, Department of Infection and Host Defense, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba, 260-8670, Japan.

Tel: 81 43 226 2073; fax: 81 43 226 2076; email: aosai@faculty.chiba-u.jp

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List of Abbreviations: cPLA₂, cytosolic phospholipase A₂; Ht, hematocrit; IFN, interferon; i.p., intraperitoneally; IRAK, interleukin 1 receptor-associated kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation factor 88; NO, nitric oxide; PI, post-infection; p.o., perorally; PAF, platelet-activating factor; PAF-AH, platelet-activating factor-acetylhydrolase; PAF-R, platelet-activating factor-receptor; PCR, polymerase chain reaction; PMB, polymyxin B; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; TRIF, TIR domain-containing adaptor-inducing IFN- β ; WT, wild-type.

been revealed to be a key molecule in the modulation of host immune responses in *T. gondii* infection (9, 10).

Recently, we proved that *T.g.HSP70* was capable of inducing lethal anaphylactic reaction in *T. gondii*-infected but not uninfected mice, revealing the molecular-based mechanisms of host death in acute toxoplasmosis (11). This anaphylactic reaction was specifically induced by *T.g.HSP70*, but not by other *T. gondii* antigens such as surface antigen 1 or *T. gondii*-derived HSP30. *T.g.HSP70*-induced anaphylactic reaction occurred through the PAF activation-dependent pathway, but not through the classical immunoglobulin (Ig)E-dependent pathway, although the reported anaphylactic reactions by parasite infection were IgE dependent. Furthermore, *T.g.HSP70*-induced anaphylactic reaction was not observed in IFN- γ knockout (GKO) infected mice and the *T.g.HSP70*-induced anaphylactic reaction was transferable by CD11b⁺, CD11c⁺, CD4⁺ and CD8⁺ splenocytes from infected WT to GKO mice. Although IFN- γ production by CD11b⁺, CD11c⁺, CD4⁺ and CD8⁺ splenocytes was remarkable, CD11b⁺ and CD11c⁺ cells were shown to be the main effectors in *T.g.HSP70*-induced PAF generation (11). However, the mechanism of PAF activation in *T.g.HSP70*-induced anaphylactic reaction still remains unknown.

PAF and eicosanoids are the major constituents of pro-inflammatory lipid mediators that play a critical role in inflammation and host defense (12–16). When LPS and other inflammatory stimuli activate macrophages, lipid mediators are synthesized de novo from membrane phospholipids through a cascade of enzymes (12). The initial step, consisting of eicosanoid biosynthesis, which is thought to be the rate-limiting step, is arachidonic acid release from membrane phospholipids by activation of cPLA₂. The liberated arachidonic acid is the common precursor molecule of all eicosanoids and is, in turn, converted to prostaglandins and thromboxanes by the cyclooxygenase pathway and to leukotrienes by the 5-lipoxygenase pathway (12, 13). Concomitant with the release of arachidonate, lysophospholipid is formed and can be enzymatically converted to PAF (17).

TLR play a critical role in innate immune responses in mammals (18–20). TLR2 and TLR4 were involved in recognition of different types of protozoan pathogen-associated molecular patterns (21). TLR2 and TLR4 were also shown to synergize to augment HSP70-induced cytokine production (22). We have already revealed that TLR2 and MyD88 are critical for protective immunity against *T. gondii* infection (23–25). In addition, TLR4 has been found to be essential in *T.g.HSP70*-induced activation of B cells and maturation of DC (6–8). Also, TLR4 mediates tolerance in NO release from macrophages by stimulation with *T.g.HSP70* (26). Thus, *T.g.HSP70* functions as a ligand of TLR2 and TLR4 in *T. gondii* infec-

tion. In contrast, involvement of the TLR4 signaling pathway was reported in PAF generation and production of pro-inflammatory lipid mediators by activating MAPK in LPS-stimulated macrophages, although the involvement of MyD88 and TRIF, the downstream molecules of TLR4, still remained controversial (27, 28).

In the present study, we analyzed the role of TLR as well as their downstream signaling molecules MyD88 and TRIF, in PAF generation in *T.g.HSP70*-induced anaphylactic reaction. We also investigated MAPK and cPLA₂ activation in *T.g.HSP70*-induced PAF generation via TLR.

MATERIALS AND METHODS

Mice and *Toxoplasma gondii*

Eight-week-old wild-type (WT) C57BL/6 mice were purchased from SLC (Hamamatsu, Japan). TLR2-deficient (–/–), TLR4^{–/–} and MyD88^{–/–} mice with C57BL/6 backgrounds were bred and maintained under specific pathogen-free conditions as previously described (8, 23, 24). Original strains of TLR2^{–/–}, TLR4^{–/–} and MyD88^{–/–} mice were generously provided by Dr Shizuo Akira (Graduate School of Medicine, Osaka University). TRIF^{–/–} mice were generously provided by Dr Kiyoshi Takeda (Research Institute for Microbial Disease, Osaka University). Mice were used at the age of 8 weeks. Genomic DNA from TLR2^{–/–}, TLR4^{–/–}, MyD88^{–/–} and TRIF^{–/–} mice was screened by PCR as described (8, 23, 24). Cysts of an avirulent Fukaya strain of *T. gondii* were prepared as previously described (1, 2). Mice were p.o. infected with 10 cysts of *T. gondii* Fukaya strain (29, 30). All animal procedures used in this study complied with guidelines set by the Animal Care and Use Committee of Chiba University.

Preparation of n*T.g.HSP70* protein

Natural *T.g.HSP70* (n*T.g.HSP70*) was purified from *T. gondii* strain RH by affinity chromatography on CNBr-activated Sepharose 4B coupled with anti-*T.g.HSP70* monoclonal antibody (*TgNCR C2*) (2, 3, 24). In brief, the purified *TgNCR C2* was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Tachyzoites (1×10^9) of *T. gondii* strain RH were lysed in lysis buffer and applied to the *TgNCR C2*-binding Sepharose 4B column. After the column was washed, n*T.g.HSP70* bound to *TgNCR C2* was eluted by 0.1 M glycine-HCl buffer containing 0.15 M NaCl (pH 2.5). The eluted n*T.g.HSP70* was dialyzed against phosphate-buffered saline (PBS) and then concentrated for use.

Induction of anaphylactic reaction by *T.g.*HSP70

WT, TLR2^{-/-}, TLR4^{-/-}, MyD88^{-/-} and TRIF^{-/-} mice were p.o. infected with 10 cysts of *T. gondii* Fukaya strain. Recombinant *T.g.*HSP70 (r*T.g.*HSP70) was prepared as previously described (10). n*T.g.*HSP70 or r*T.g.*HSP70 (100 µg) was i.p. injected into mice at 7 days post-*T. gondii* infection (PI). Survival of mice was monitored every hour after i.p. injection of n*T.g.*HSP70 or r*T.g.*HSP70. Rectal body temperature of mice was measured by digital thermometer (Shibaura Electronics, Tokyo, Japan) every 30 min after n*T.g.*HSP70 or r*T.g.*HSP70 injection. Peripheral blood samples were collected from tails of *T. gondii*-infected WT, TLR2^{-/-} and TLR4^{-/-} mice every 30 min after injection of n*T.g.*HSP70 or r*T.g.*HSP70 and Ht was measured as follows. Peripheral blood samples were filled into a microtube (Modulohm, Herlev, Denmark) and centrifuged at 10 000 × g for 5 min, and the percentage of the length of the microtube of red blood cells in the length of the whole blood was calculated as Ht.

Reverse transcription-polymerase chain reaction

Splenocytes were prepared at 7 days PI from *T. gondii*-infected WT, TLR2^{-/-} and TLR4^{-/-} mice at 3 hr after r*T.g.*HSP70 injection. WT, TLR2^{-/-} and TLR4^{-/-} mice were respectively divided into four groups: group 1, naïve mice; group 2, *T. gondii* infection alone; group 3, r*T.g.*HSP70 injection alone; group 4, *T. gondii*-infected and r*T.g.*HSP70-injected mice. Messenger RNA from splenocytes was extracted and reverse transcription (RT)-PCR was carried out with the following specific PAF-AH (31), PAF-R (31), and IFN-γ primers (32): mouse PAF-AH sense (5'-CCT GCA AGC TGG AAT TCT CC-3') and anti-sense (5'-CCC ATT AGA TGC CAA GCC AA-3'), mouse PAF-R sense (5'-CAA CGA GGG CGA CTG GAT T-3') and anti-sense (5'-GAC ACC CAA AAA GGC CAC ACT-3'), mouse IFN-γ sense (5'-TGA ACG CTA CAC ACT GCA TCT TGG-3') and anti-sense (5'-CGA CTC CTT TTC CGC TTC CTG AG-3'). GAPDH was used for internal control.

In vitro stimulation of splenocyte-derived CD11b⁺ cells with *T.g.*HSP70

Splenocytes were prepared at 7 days PI from *T. gondii*-infected WT, TLR2^{-/-} and TLR4^{-/-} mice. CD11b⁺ splenocytes were fractionated by a VarioMACS separator system (Miltenyi Biotec, Auburn, CA, USA) from splenocytes of *T. gondii*-infected mice with microbead-conjugated anti-CD11b⁺ mAb (Miltenyi Biotec) according to the company's instructions. Purity of the recovered cells was >93% as analyzed by flow cytometry (Becton

Dickinson, Tokyo, Japan). Recovered CD11b⁺ splenocytes were stimulated with 10 µg/mL r*T.g.*HSP70 in a 37 °C, 5% CO₂ incubator and then respectively collected at 0, 15, 30, 60 and 120 min after stimulation for immunoblotting analysis.

Immunoblotting

Splenocytes (5 × 10⁶/sample) were lysed in reducing SDS sample buffer and sonicated for 5 sec 50 times at 5-sec intervals. After boiling for 5 min, cellular lysates were separated by 10% SDS-PAGE, and proteins were subsequently electrotransferred onto nitrocellulose membranes (Amersham Bioscience, Buckinghamshire, UK). Membranes were then blocked in 5% non-fat dry milk (Morinaga Co., Tokyo, Japan) containing 0.1% Tween 20 (Sigma-Aldrich, Tokyo, Japan) in Tris-buffered saline, pH 7.5 (TTBS), overnight at 4 °C, followed by incubation with primary rabbit anti-mouse antibody specific for phosphorylated and total cPLA₂, p44/42 (ERK1/2) and p38 MAPK used at the concentrations recommended by the manufacturer (Cell Signaling Technology, Beverly, MA, USA) in 5% BSA in TTBS for 1 hr at room temperature. After washing in TTBS, membranes were incubated with secondary goat anti-rabbit peroxidase antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TTBS for 1 hr at room temperature. After washing in TTBS, membranes were incubated with avidin peroxidase (Sigma-Aldrich) in TTBS for 1 hr at room temperature. After washing in TTBS, bands were visualized using an ECL system (Amersham Bioscience).

Statistics

Significance of differences between groups was determined by Student's *t*-test. Statistically significant differences were reported at *P* < 0.05.

RESULTS

*T.g.*HSP70-induced anaphylactic reaction was observed in WT and TLR2^{-/-} mice, but not in TLR4^{-/-} mice

Anaphylactic reaction induced by i.p. injection of *T.g.*HSP70 was observed in *T. gondii*-infected, but not in uninfected, WT mice (11). The reaction appeared within the first hour after the injection, characterized by staggering, ruffling fur, crawling and prostration and seizure and convulsion. The mice died within 3 hrs. Rectal body temperature of WT mice gradually declined whereas ΔHt increased after the challenge of *T.g.*HSP70 injection until death. In contrast, *T.g.*HSP70 was revealed to be a ligand of TLR2 and TLR4 (7, 8, 23–26). Therefore, we investigated

the involvement of TLR2 and TLR4 in *T.g.*HSP70-induced anaphylactic reaction in *T. gondii*-infected TLR2^{-/-} and TLR4^{-/-} mice by using n*T.g.*HSP70 and r*T.g.*HSP70. As in WT mice, this anaphylactic reaction was observed in *T. gondii*-infected TLR2^{-/-} mice but not in *T. gondii*-infected TLR4^{-/-} mice, after injection of n*T.g.*HSP70 or r*T.g.*HSP70 (Fig. 1a).

*T.g.*HSP70-induced anaphylactic reaction was not due to contamination of the *T.g.*HSP70 preparation by endotoxin of *Escherichia coli* because: (i) purified n*T.g.*HSP70 from *T. gondii* tachyzoites of RH stain by using affinity chromatography coupled with anti-*T.g.*HSP70 monoclonal antibody (*Tg*NCR C2) (2, 3, 24) induced anaphylactic reaction in *T. gondii*-infected WT and TLR2^{-/-}, but not in TLR4^{-/-} mice (Fig. 1a); and (ii) PMB, an LPS-specific inhibitor that binds to the lipid A portion of LPS, inhibited LPS-induced but not *T.g.*HSP70-induced anaphylactic reaction (11). One hundred µg of r*T.g.*HSP70 was used for the following experiments.

As indices of anaphylactic reaction, rectal body temperature and Ht were measured after the i.p. challenge of *T.g.*HSP70. Rectal body temperature of WT and TLR2^{-/-} mice gradually declined (from 37.6 °C to 31.2 °C in WT, from 37.8 °C to 31.1 °C in TLR2^{-/-}) whereas ΔHt increased (from 0% to 27% in WT, from 0% to 33% in TLR2^{-/-}) after *T.g.*HSP70 injection until death (Fig. 1b,c). However, rectal body temperature and Ht did not significantly change in TLR4^{-/-} mice after *T.g.*HSP70 injection (Fig. 1b,c). Thus, TLR4, but not TLR 2, was involved in *T.g.*HSP70-induced anaphylactic reaction.

PAF activation in *T.g.*HSP70-induced anaphylactic reaction was TLR4 dependent but not TLR2 dependent

PAF-AH is known to be the enzyme most responsible for degradation of PAF (33, 34). The mRNA expressions of PAF-AH or PAF-R were analyzed by RT-PCR in splenocytes. All groups of WT, TLR2^{-/-} and TLR4^{-/-} mice were respectively divided into four groups as described earlier. In both WT and TLR2^{-/-} mice, the mRNA expression of PAF-AH from group 4 (*T. gondii*-infected and *T.g.*HSP70-injected mice) ($P < 0.001$) was significantly increased, whereas that in both group 2 (infection alone) ($P < 0.05$) and group 3 (injection alone) ($P < 0.05$) was slightly higher compared with group 1 (naïve mice) (Fig. 2a). The mRNA expression level of group 4 was significantly higher than those of groups 2 and 3 ($P < 0.05$) (Fig. 2a). However, no increase was observed in any of the groups of TLR4^{-/-} mice (Fig. 2a). Thus, a high level of PAF production was noticed in the *T.g.*HSP70-induced anaphylactic reaction in WT and TLR2^{-/-} but not in TLR4^{-/-} mice, indicating the importance of TLR4-dependent PAF production

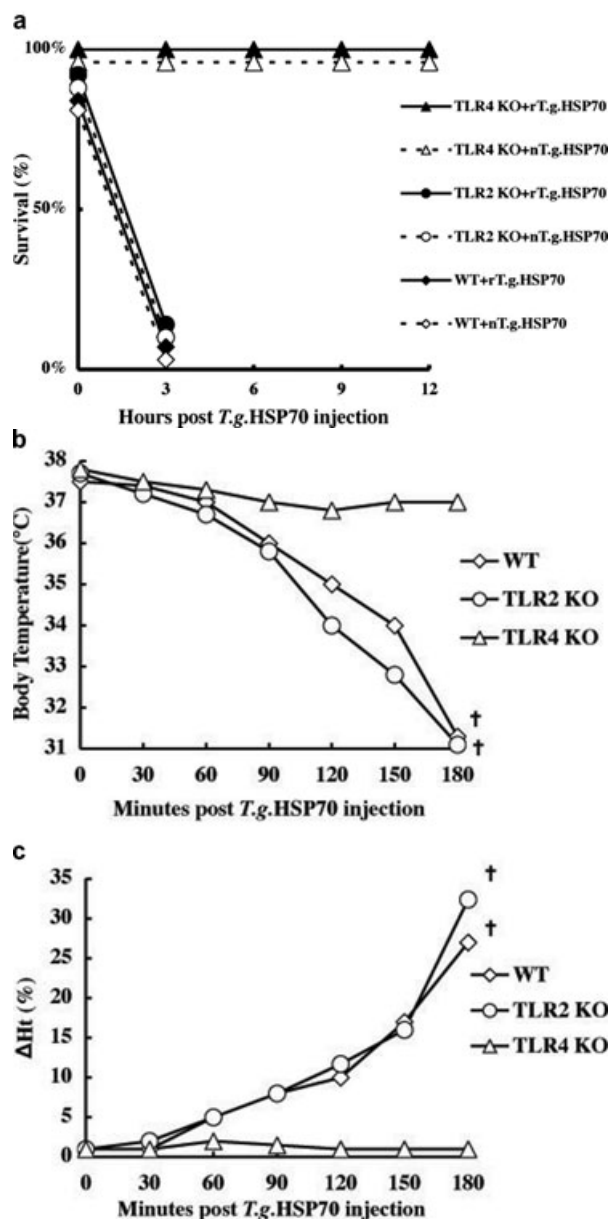


Fig. 1. *T.g.*HSP70-induced anaphylactic reaction in *T. gondii*-infected mice. r*T.g.*HSP70 (100 µg) (closed) or n*T.g.*HSP70 (open) was i.p. injected to *T. gondii*-infected TLR4^{-/-} (triangles), TLR2^{-/-} (circles) and WT (diamonds) C57BL/6 mice at 7 days PI. (a) Survival of mice was monitored every hour after injection of *T.g.*HSP70. (b) Rectal body temperature was checked every 30 min after injection of *T.g.*HSP70. (c) Ht was measured every 30 min after injection of *T.g.*HSP70 and ΔHt was calculated as follows: [(value at post-challenge - value at pre-challenge)/value at pre-challenge] × 100%. Three mice were used in each experimental group. Data are representative of three separate experiments. †Killed.

in *T.g.*HSP70-induced anaphylactic reaction. However, no significant change in the mRNA expression of PAF-R was observed in any of the four groups of WT, TLR2^{-/-} and TLR4^{-/-} mice (Fig. 2b).

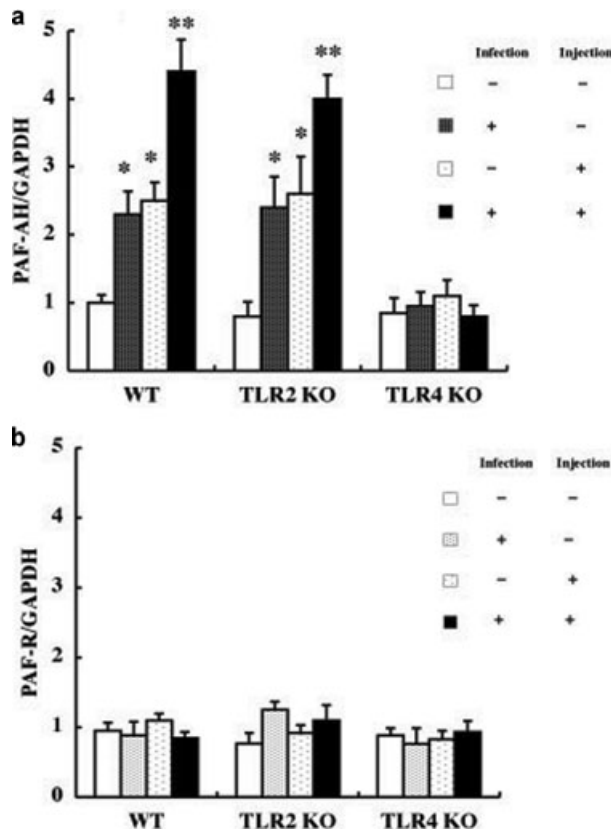


Fig. 2. TLR4-dependent PAF activation in *T.g.*HSP70-induced anaphylactic reaction. Expressions of (a) PAF-AH and (b) PAF-R in splenocytes from WT, TLR2^{-/-} and TLR4^{-/-} mice of four experimental groups were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) as described earlier. Four experimental groups were: group 1, naïve mice; group 2, *T. gondii* infection alone; group 3, *T.g.*HSP70 injection alone; group 4, *T. gondii*-infected and *T.g.*HSP70-injected mice. The results are expressed as the ratio between the optical density of the RT-PCR product of PAF-AH or PAF-R and that of GAPDH. Three mice were used in each experimental group. Data are representative of three separate experiments. **P* < 0.05, ***P* < 0.01 compared with the data of group 1.

IFN- γ was proven to be a crucial cytokine in *T.g.*HSP70-induced anaphylactic reaction, most likely by the mechanism of enhancing PAF generation in CD11b⁺ or CD11c⁺ cells (11). Thus, we also investigated IFN- γ production by RT-PCR in WT, TLR2^{-/-} and TLR4^{-/-} mice during the *T.g.*HSP70-induced anaphylactic reaction. All groups of mice were respectively divided into four groups as described earlier. IFN- γ production was significantly detected in TLR2^{-/-} and TLR4^{-/-} mice of group 4 (*T. gondii*-infected and *T.g.*HSP70-injected mice) compared with mice of group 1 (naïve mice) (*P* < 0.001) (Fig. 3). There were no obvious differences among WT, TLR2^{-/-} and TLR4^{-/-} mice (Fig. 3).

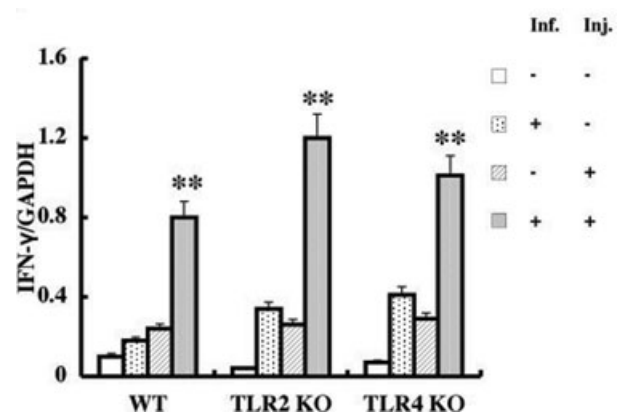


Fig. 3. IFN- γ production in *T.g.*HSP70-induced anaphylactic reaction. Expressions of IFN- γ in splenocytes from WT, TLR2^{-/-} and TLR4^{-/-} mice of four experimental groups were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) as described earlier. Four experimental groups were: group 1, naïve mice; group 2, *T. gondii* infection alone; group 3, *T.g.*HSP70 injection alone; group 4, *T. gondii*-infected and *T.g.*HSP70-injected mice. The results are expressed as the ratio between the optical density of the RT-PCR product of IFN- γ and that of GAPDH. Three mice were used in each experimental group. Data are representative of three separate experiments. ***P* < 0.001 compared with the data of group 1.

TLR4-mediated MAPK pathway was required for *T.g.*HSP70-induced cPLA₂ activation

The MAPK pathway is one of the major downstream pathways in TLR4 signaling (18–21). In addition, it has been shown that cPLA₂ activation, the initial step of eicosanoid biosynthesis and PAF formation from lysophospholipid (35), requires phosphorylation by MAPK and that cPLA₂ is a substrate of p38 and p44/42 MAPK (36, 37). Macrophages were considered to be the main origin of PAF production (35). In the previous article, we already reported that CD11b⁺ splenocytes were the effector cells most responsible for PAF production in *T.g.*HSP70-induced anaphylactic reaction (11). Therefore, we next studied whether the TLR4-mediated MAPK pathway is required for *T.g.*HSP70-induced cPLA₂ activation and PAF production. After stimulation with *T.g.*HSP70, phosphorylation of p38 was detected in CD11b⁺ splenocytes from both WT and TLR2^{-/-} mice, but not from TLR4^{-/-} mice (Fig. 4a). Similarly, phosphorylation of p44/42 was also detected in WT and TLR2^{-/-} mice, but not in TLR4^{-/-} mice (Fig. 4b). These data confirmed that TLR4-mediated MAPK pathway was activated by *T.g.*HSP70 stimulation in CD11b⁺ splenocytes.

Next, phosphorylation of cPLA₂ was investigated by western blotting in CD11b⁺ splenocytes from WT, TLR2^{-/-} and TLR4^{-/-} mice. In CD11b⁺ splenocytes from *T. gondii*-infected WT and TLR2^{-/-} mice,

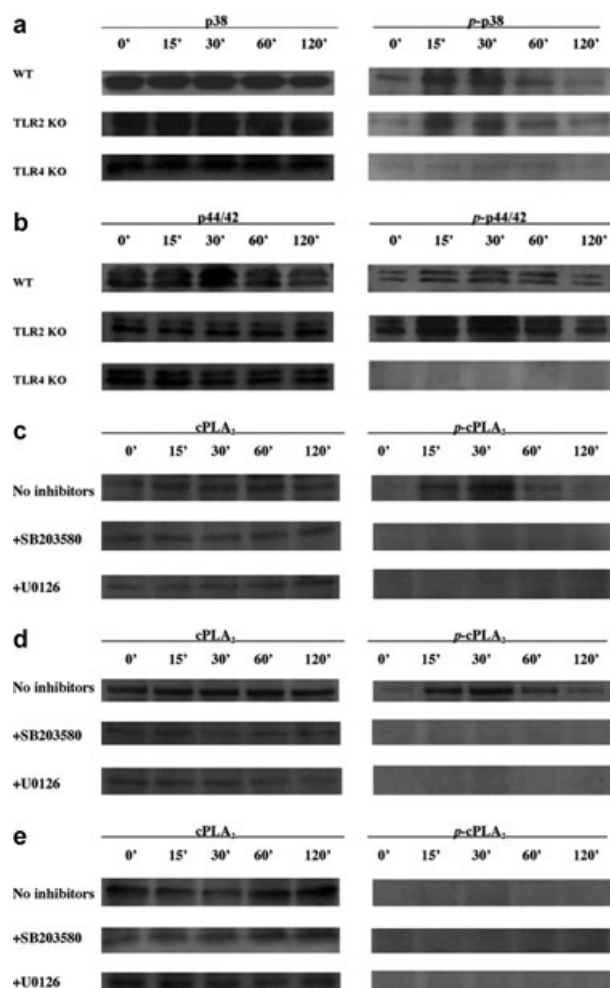


Fig. 4. TLR4-mediated MAPK pathway in *T.g.*HSP70-induced cPLA₂ activation. CD11b⁺ splenocytes were purified by MACS, stimulated with 10 μ g/mL *T.g.*HSP70 and then respectively collected at 0, 15, 30, 60 and 120 min after stimulation for analysis. Phosphorylation of (a) p38 or (b) p44/42 in CD11b⁺ splenocytes of *T. gondii*-infected WT, TLR2^{-/-} or TLR4^{-/-} mice was detected by immunoblotting analysis with antibodies against phosphorylated (p) and total p38 or p44/42, respectively. *T.g.*HSP70-induced cPLA₂ activation with or without MAPK inhibitors (20 μ M SB203580 or 20 μ M U0126) was examined in (c) WT, (d) TLR2^{-/-} and (e) TLR4^{-/-} mice with antibodies against phosphorylated (p) and total cPLA₂, respectively. The results shown are representative of four experiments.

phosphorylation of cPLA₂ started from 15 min, peaked at 30 min and then declined after *T.g.*HSP70 stimulation (Fig. 4c,d). However, no phosphorylation of cPLA₂ was detected in CD11b⁺ splenocytes from *T. gondii*-infected TLR4^{-/-} mice (Fig. 4e). MAPK inhibitors were used to block MAPK phosphorylation. CD11b⁺ splenocytes from WT, TLR2^{-/-} or TLR4^{-/-} mice were respectively treated with SB203580 (inhibitor of p38) or U0126 (inhibitor of p44/42) for 2 hr. Then, these cells were stimulated with

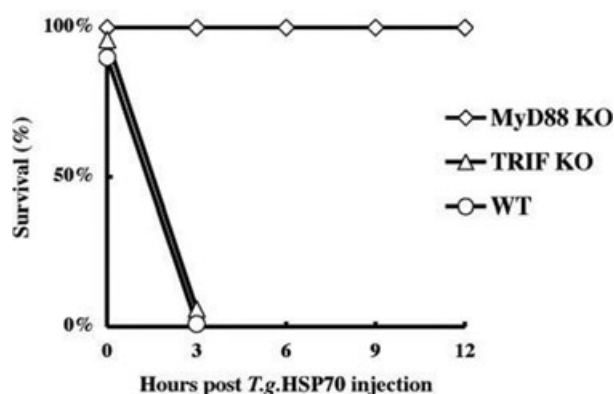


Fig. 5. MyD88-dependent but not TRIF-dependent pathway in *T.g.*HSP70-induced anaphylactic reaction. *T.g.*HSP70 (100 μ g) was i.p. injected into *T. gondii*-infected MyD88^{-/-} ($\diamond$), TRIF^{-/-} ($\triangle$) or WT ($\circ$) C57BL/6 mice at 7 days PI. Survival of mice was checked every hour after injection of *T.g.*HSP70. Data are representative of three separate experiments.

*T.g.*HSP70, and phosphorylation of cPLA₂ was examined at various times after stimulation. Both SB203580 and U0126 inhibited phosphorylation of cPLA₂ in CD11b⁺ splenocytes from WT and TLR2^{-/-} mice, suggesting that the TLR4-mediated MAPK pathway is required for *T.g.*HSP70-induced cPLA₂ activation (Fig. 4c-e). Phosphorylation of cPLA₂ was not observed in uninfected WT, TLR2^{-/-} or TLR4^{-/-} mice (data not shown).

Involvement of MyD88 but not TRIF in *T.g.*HSP70-induced anaphylactic reaction

Both MyD88- and TRAM/TRIF-dependent pathways in TLR4 signaling are capable of activating the downstream MAPK pathway. We next sought to determine whether MyD88 and TRAM/TRIF adaptors are required for *T.g.*HSP70-induced anaphylactic reaction. Similarly, *T.g.*HSP70-induced anaphylactic reaction was examined in MyD88^{-/-} and TRIF^{-/-} mice. The results showed that *T.g.*HSP70-induced anaphylactic reaction occurred in TRIF^{-/-} mice but not in MyD88^{-/-} mice, indicating that MyD88 is essential for *T.g.*HSP70-induced anaphylactic reaction (Fig. 5).

DISCUSSION

*T.g.*HSP70-induced anaphylactic reaction occurred via a PAF activation-dependent pathway but not an IgE-dependent pathway. In the present study, by demonstrating the TLR4/MyD88-dependent PAF generation in *T.g.*HSP70-induced anaphylactic reaction, we could reveal the mechanisms of PAF synthesis in *T. gondii* infection.

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a phospholipid mediator that activates a G-protein-coupled receptor and results in pleiotropic and potent biological effects, including platelet activation, airway constriction, and hypotension (38, 39). PAF is synthesized via two distinct pathways: the de novo and the remodeling pathways (35, 40, 41). The latter pathway, which involves cPLA₂ activation, is regulated by extracellular signals, and plays a critical role in stimulus-coupling PAF biosynthesis.

Cytosolic PLA₂ is the only known PLA₂ activated by receptor-mediated events (42). A wide variety of extracellular stimuli have been found to induce activation or increase synthesis of cPLA₂ in diverse cell models (43–45). Cytosolic PLA₂ phosphorylation is an important mechanism for cPLA₂ activation (46). By using LPS, previous reports have demonstrated that either single or multiple MAPK members are able to activate cPLA₂ upon extracellular stimulation as well as the ability of a specific receptor to trigger the different MAPK family members (47, 48). TLR4 signaling activates multiple members of the MAPK family, including p44/42, p38 kinase, and c-Jun NH₂-terminal kinase (49). *T.g.HSP70*-induced anaphylactic reaction was not due to contamination of LPS (6, 7, 11, 26). The anaphylactic reaction in *T. gondii*-infected mice was confirmed by n*T.g.HSP70*, which was purified from *T. gondii* tachyzoites. In this study, we revealed that either a p38-specific or a p44/42-specific inhibitor was capable of blocking TLR4-mediated cPLA₂ phosphorylation and PAF production after *T.g.HSP70* stimulation. Thus, both the p44/42 and p38 signaling pathways act to activate cPLA₂ in *T.g.HSP70*-induced PAF activation.

In our previous study, *T.g.HSP70*-induced anaphylactic reaction was not observed in GKO mice, indicating the involvement of IFN- γ in the anaphylactic reaction. Furthermore, a large amount of IFN- γ was induced in splenocytes and peritoneal exudate cells by the injection of *T.g.HSP70* in *T. gondii*-infected mice. Also, the exogenous administration of recombinant IFN- γ consistently caused *T.g.HSP70*-induced anaphylactic reaction in GKO BALB/c mice (11). These data demonstrated that the overproduction of IFN- γ played a crucial role in *T.g.HSP70*-induced anaphylactic reaction. Wu *et al.* showed that IFN- γ induced the synthesis and activation of cPLA₂ by using murine and human cell lines (50). Also, Lindbom *et al.* proved that IFN- γ augmented the expression of cPLA₂ (type IVA PLA₂) in the presence of LPS by using human macrophages (51). These data coincided with our data showing the enhancement of cPLA₂ activation in *T.g.HSP70*-induced anaphylactic reaction in infected mice, which produced a large amount of IFN- γ .

We have already revealed that *T.g.HSP70* caused deterioration of the host defense against *T. gondii* infection

via TLR2 by downregulating NO release from peritoneal macrophages (24–26). However, TLR4 was essential in *T.g.HSP70*-induced activation of B cells and maturation of DC (6–8). Thus, *T.g.HSP70* functions as a ligand of both TLR2 and TLR4 in *T. gondii* infection. Similarly, engagement of TLR2 and TLR4 was reported in HSP70-induced cytokine production and Toll/IL-1 receptor signal pathway (22, 52). Therefore, it was speculated that *T.g.HSP70* would stimulate macrophages and/or DC of TLR2^{-/-} and TLR4^{-/-} mice (via either TLR4 or TLR2, respectively) so that IFN- γ would be produced by macrophages, DC, or primed T cells in *T. gondii*-infected TLR2^{-/-} and TLR4^{-/-} as well as WT mice. In the present study, although IFN- γ production was similarly induced by *T.g.HSP70* injection in *T. gondii*-infected TLR2^{-/-} and TLR4^{-/-} mice, anaphylactic reaction was observed in TLR2^{-/-} but not in TLR4^{-/-} mice, indicating that cPLA₂ activation and PAF generation essentially depended on the TLR4 signaling pathway. IFN- γ would enhance the TLR4-dependent cPLA₂ activation.

MyD88, harboring a C-terminal TIR domain and an N-terminal death domain, associates with the TIR domain of TLR. Upon stimulation, MyD88 recruits IRAK 4 to TLR through interaction of the death domains of both molecules, and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Activated IRAK-1 then associates with TNF receptor-associated factor 6, leading to the activation of two distinct signaling pathways. One pathway leads to the activation of AP-1 transcription factors through activation of MAPK (19). Jeyaseelan *et al.* reported that *E. coli*-induced phosphorylation of JNK, ERK, and p38 MAPK was detected in bone marrow-derived macrophages of WT mice, but attenuated in those of TRIF^{-/-} mice, indicating the involvement of TRIF in the activation of MAPK (53). From our data, *T.g.HSP70*-induced anaphylactic reaction was observed in WT and TRIF^{-/-} mice, but not in MyD88^{-/-} mice, indicating the involvement of MyD88 but not TRIF in *T.g.HSP70*-induced anaphylactic reaction (Fig. 6).

Some studies concerning TLR in infectious diseases focused on the mechanism by which TLR mediated the production of pro-inflammatory cytokines. The present study suggested the involvement of the TLR4/MyD88 signaling pathway in the generation of pro-inflammatory lipid mediators such as PAF that caused lethal anaphylactic reaction in *T. gondii*-infected host, although the precise mechanisms of MAPK activation through TLR4/MyD88 pathways for PAF production still need to be analyzed.

PAF and eicosanoids, which include prostaglandins, leukotrienes, thromboxanes, lipoxins etc., are potent modulators of immune responses of hosts. Thardin *et al.* reported the eicosanoid production by mouse peritoneal macrophages during *T. gondii* penetration (54). Aliberti

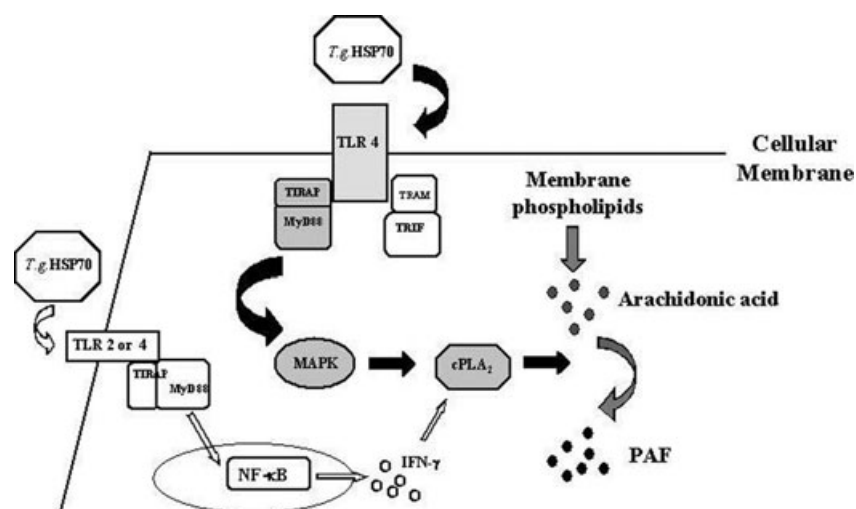


Fig. 6. Model of the signal pathway in CD11b⁺ splenocytes in *T.g.* HSP70-induced anaphylactic reaction. Activation of cPLA₂ involving TLR4, MyD88 and MAPK signals induces PAF generation from arachidonic acid. See text for details.

et al. reported the role of endogenous lipoxins in modulating pro-inflammatory responses during infection with *T. gondii* (55). Our study revealed the pathogenesis of *T.g.* HSP70 that would explain the molecular mechanism of PAF-mediated host death in acute exacerbation of toxoplasmosis in immunocompromised patients. The precise roles of microbial eicosanoids as immune modulators in *T. gondii* infection remain to be analyzed in the future.

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