

Anaphylactic reaction induced by *Toxoplasma gondii*-derived heat shock protein 70

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Abstract

Toxoplasma gondii-derived heat shock protein 70 (*T.g.HSP70*) is a virulent molecule specific for tachyzoites of *T. gondii*. The expression of *T.g.HSP70* rapidly increases just before death of the host, indicating that *T.g.HSP70* functions as a danger signal during lethal acute *T. gondii* infection. In the present study, *T.g.HSP70* was proven to be capable of inducing lethal anaphylactic reaction in *T. gondii*-infected wild-type (WT) mice. Anaphylactic reaction appeared within the first hour after intraperitoneal injection of *T.g.HSP70* and was characterized by a series of consequent symptoms until death. *T.g.HSP70*-induced anaphylactic reaction was not observed in IFN- γ knockout (GKO) mice, indicating the involvement of IFN- γ in the reaction. The anaphylactic reaction was transferable to GKO mice by splenocytes but not serum from infected WT mice. Also, this reaction occurred in B cell-deficient mice, indicating that *T.g.HSP70*-induced anaphylactic reaction occurred through an Ig-independent pathway. The messenger RNA (mRNA) expression of IFN- γ increased significantly in splenocytes from *T. gondii*-infected WT mice after *T.g.HSP70* injection. Furthermore, the mRNA expression of platelet-activating factor (PAF) acetylhydrolase in WT, but not GKO mice, distinctly increased during the occurrence of *T.g.HSP70*-induced anaphylactic reaction, indicating the involvement of PAF in *T.g.HSP70*-induced anaphylactic reaction. Treatment with PAF receptor antagonist rescued WT mice from the anaphylactic reaction. These data demonstrated the involvement of IFN- γ -dependent PAF activation in *T.g.HSP70*-induced anaphylactic reaction.

Introduction

Toxoplasma gondii is one of the obligate intracellular parasites. In intermediate hosts such as humans and mice, perorally (p.o.) infected encysted *T. gondii* bradyzoites undergo stage conversion to rapidly dividing tachyzoites that are responsible for acute toxoplasmosis (1). The expression of *T. gondii*-derived heat shock protein 70 (*T.g.HSP70*) increased rapidly just before the death of *T. gondii*-infected host, indicating that *T.g.HSP70* functioned as a danger signal during lethal, acute *T. gondii* infection (1, 2). *T.g.HSP70* was shown to cause deterioration of the host defense by down-regulating nitric oxide (NO) release by peritoneal macrophages (2, 3), producing anti-HSP70 autoantibody from V_H1-J_H1 B1 cells (4, 5) and polarization from T_H1 to T_H2 immune responses in *T. gondii*-infected host (6). Furthermore, *T.g.HSP70* is capable of activating B cells and dendritic cells (7–9). Thus, *T.g.HSP70* has been revealed to be a key

molecule in the modulation of host immune responses in *T. gondii* infection (1–10).

Anaphylaxis is an acute, life-threatening allergic reaction in which a physiologic process that normally acts in a local and limited manner to protect against infection occurs massively and systemically. Although it is classically mediated by histamine released in response to antigen cross-linking of IgE bound to Fc ϵ RI on mast cells, both human and rodent studies indicate that this classical pathway does not account for all anaphylactic responses (11–16). In particular, several rodent studies suggest that a pathway involving IgG, Fc γ RIII, granulocytes, macrophages and platelets and platelet-activating factor (PAF) may be more important than the classical pathway in animals challenged with antigen rather than with anti-IgE antibody (17, 18). In the PAF-dependent pathway, the secretion of PAF acts on target cells to increase vascular

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permeability, which causes hemoconcentration and depletes the intravascular volume. The resulting decrease in vital organ perfusion is the primary cause of the symptoms that characterized murine anaphylactic reaction (11, 12, 18).

In the present study, we analyzed a lethal anaphylactic reaction induced by *T.g.*HSP70 injection in *T. gondii*-infected mice. This study demonstrated that *T.g.*HSP70-induced anaphylactic reaction occurred through IFN- γ -dependent PAF activation, but not by Ig-dependent pathway.

Methods

Mice and T. gondii

Eight-week-old wild-type (WT) BALB/c mice were purchased from SLC (Hamamatsu, Japan). IFN- γ knockout (GKO) mice with BALB/c background and B cell-deficient (μ MT) mice with C57BL/6 background were bred and maintained under specific pathogen-free conditions as previously described (19, 20). GKO mice were a generous gift from Yoichiro Iwakura (Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan) and μ MT mice were a generous gift from Daisuke Kitamura (Division of Molecular Biology, Research Institute for Biological Sciences, Tokyo University of Science, Tokyo, Japan). Mice were used at the age of 8 weeks. Genomic DNA from GKO mice was screened by PCR as described (21). Cysts of an avirulent Fukaya strain of *T. gondii* were prepared as previously described (2, 3). Mice were p.o. infected with 10 cysts of Fukaya strain (22, 23).

Induction of anaphylactic reaction by T.g.HSP70

WT, μ MT and GKO mice were p.o. infected with 10 cysts of *T. gondii*. Recombinant *T.g.*HSP70 was prepared as previously described (3). Various doses (10, 30, 100 or 300 μ g) of *T.g.*HSP70 were intraperitoneally (i.p.) injected into mice at 1 week after *T. gondii* infection. As controls, recombinant tachyzoite-specific surface antigen 1 (SAG1) and bradyzoite-specific *T.g.*HSP30 were also i.p. injected into *T. gondii*-infected WT BALB/c mice (3). Survival of mice was monitored every hour after injection of *T.g.*HSP70. Rectal body temperature of mice was measured by digital thermometer (Shibaura Electronics, Tokyo, Japan) every 30 min after *T.g.*HSP70 injection. Peripheral blood samples were collected from tails of *T. gondii*-infected WT BALB/c mice every 30 min after injection of *T.g.*HSP70 and hematocrit (Ht) was measured as follows. Peripheral blood samples were filled into a microtube (Modulohm, Herlev, Denmark) and centrifuged at $10\,000 \times g$ for 5 min, and the percentage of the length of RBCs in the length of whole blood was calculated as Ht (18).

For histopathological examination, organs such as liver, spleen, lung and kidney from *T. gondii*-infected and *T.g.*HSP70-injected WT BALB/c mice were removed 3 h post-*T.g.*HSP70 injection. Organs were fixed in 15% buffered PFA and 4 μ m paraffin sections were stained with hematoxylin and eosin. Organs from naive WT BALB/c mice, *T. gondii*-infected WT BALB/c mice without *T.g.*HSP70 injection and uninfected WT BALB/c mice with *T.g.*HSP70 injection were examined as controls.

Effect of polymyxin B on T.g.HSP70-induced anaphylactic reaction

*T.g.*HSP70 (100 μ g) and LPS (10 μ g) from *Escherichia coli* 026:B6 (Sigma-Aldrich, Tokyo, Japan) were pre-incubated with or without 100 μ g of polymyxin B (PMB) (Sigma-Aldrich) at 37°C for 1 h and then injected to *T. gondii*-infected WT BALB/c mice at 7 days post-infection (p.i.). Survival of mice injected with *T.g.*HSP70 or LPS with or without treatment of PMB was checked hourly after injection of *T.g.*HSP70 or LPS.

Treatment of GKO mice with exogenous recombinant IFN- γ

Infected WT or GKO BALB/c mice were i.p. injected with or without 2 μ g of murine recombinant IFN- γ (rIFN- γ) (Wako, Tokyo, Japan) in 0.2 ml saline daily from day 0 to day 7 p.i. Both rIFN- γ -treated and -untreated mice were i.p. injected with 100 μ g of *T.g.*HSP70 at day 7 p.i. The survival of mice was monitored every hour after injection of *T.g.*HSP70.

Transfer of serum and splenocytes

Serum or splenocytes were prepared at 7 days p.i. from *T. gondii*-infected WT BALB/c mice, and transferred to naive GKO BALB/c mice. Splenocytes were fractionated by VarioMACS separator system (Miltenyi Biotec, Auburn, CA, USA) as follows. CD90⁺ cells were positively purified from splenocytes of *T. gondii*-infected WT BALB/c mice with microbead-conjugated anti-CD90 mAb according to the company's instructions. Subsequently, B220⁺ cells were positively enriched from CD90-depleted splenocytes with microbead-conjugated anti-CD45R (B220) mAb. Finally, CD11b⁺ cells and CD11c⁺ cells were purified from CD90-depleted and B220-depleted splenocytes with microbead-conjugated anti-CD11b⁺ mAb or anti-CD11c⁺ mAb. CD4⁺ and CD8⁺ T subsets were also purified from splenocytes of *T. gondii*-infected WT BALB/c mice with microbead-conjugated anti-CD4⁺ mAb or anti-CD8⁺ mAb. All microbead-conjugated mAbs were purchased from Miltenyi Biotec. The purity of the recovered cells was >93% as analyzed by flow cytometry (Becton Dickinson, Tokyo, Japan). Each fraction of cells was washed three times in chilled PBS and i.p. transferred into GKO BALB/c mice at 1 week p.i. Each fraction of cells or serum obtained from one WT mouse was transferred to one GKO mouse.

Reverse transcriptase-PCR

The expression of messenger RNA (mRNA) from peritoneal exudate cells (PECs) or splenocytes of WT or GKO BALB/c mice with or without *T. gondii* infection or *T.g.*HSP70 injection was investigated by reverse transcriptase (RT)-PCR as previously described (24, 25). PCR was carried out with the following specific IL-4 (26), IL-10 (27), IL-6 (26), IFN- γ (26), IL-12 p40 (28), tumor necrosis factor (TNF)- α (26), platelet-activating factor acetylhydrolase (PAF-AH) (29) and platelet-activating factor receptor (PAF-R) (29) primers: mouse IL-4 sense (5'-ATG GGT CTC AAC CCC CAG CTA GT-3') and anti-sense (5'-GCT CTT TAG GCT TTC CAG GAA GTC-3'), mouse IL-10 sense (5'-CGG GAA GAC AAT AAC TG-3') and anti-sense (5'-CAT TTC CGA TAA GGC TTG G-3'), mouse IL-6 sense (5'-ATG AAG TTC CTC TCT GCA AGA GAC T-3') and

anti-sense (5'-CAC TAG GTT TGC CGA GTA GAT CTC-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'), mouse IL-12 p40 sense (5'-CGT GCT CAT GGC TGG TGC AAA G-3') and anti-sense (5'-GAT GAA GAA GCT GGT GCT G-3'), mouse TNF- α sense (5'-ATG AGC ACA GAA AGC ATG ATC CGC-3') and anti-sense (5'-CCA AAG TAG ACC TGC CCG GAC TC-3'), 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'), PAF-R sense (5'-CAA CGA GGG CGA CTG GAT T-3') and anti-sense (5'-GAC ACC CAA AAA GGC CAC ACT-3'). Glyceraldehyde-3-phosphate dehydrogenase was used for internal control.

Platelet counting

Peripheral blood was obtained from the tail of *T. gondii*-infected WT BALB/c mice at 3 h after *T.g.*HSP70 injection. Smears of peripheral blood were stained using the Giemsa method, and platelet numbers were counted under a microscope at $\times 200$. Data were shown as the ratio of platelet number to 1000 RBCs (PLT:1000 RBC) according to the Fono method (30).

Effects of PAF-R antagonist against *T.g.*HSP70-induced anaphylactic reaction

One hundred, 50 or 25 μ g of PAF-R antagonist CV6209 (Wako) was i.p. administered to *T. gondii*-infected WT BALB/c mice at 10 min before injection of 100 μ g of *T.g.*HSP70. Survival of mice injected with CV6209 or PBS was checked every hour post injection of *T.g.*HSP70.

Statistics

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

Results

Induction of anaphylactic reaction in WT mice by *T.g.*HSP70

Anaphylactic reaction by i.p. administering *T.g.*HSP70 was induced in *T. gondii*-infected but not in uninfected WT BALB/c mice (Fig. 1A). Anaphylactic reaction was not observed in *T. gondii*-infected mice without administration of *T.g.*HSP70 (Fig. 1A). *T.g.*HSP70-induced anaphylactic reaction was similarly observed in *T. gondii*-infected WT C57BL/6 mice (data not shown). The effect of *T.g.*HSP70 to induce anaphylactic reaction was dose dependent (Fig. 1B). A dose of 100 μ g *T.g.*HSP70 was used for the following experiments. Neither SAG1 nor *T.g.*HSP30 induced anaphylactic reaction in *T. gondii*-infected WT mice, indicating that the reaction was *T.g.*HSP70 specific (Fig. 1C). The reaction appeared within the first hour after *T.g.*HSP70 injection and was characterized by staggering, ruffling fur, crawling and prostration and seizure and convulsion. The mice died within 3 h. As indices of anaphylactic reaction, rectal body temperature and Ht were measured after the i.p. challenge of *T.g.*HSP70. Rectal body temperature gradually declined from 37.5 to 31.3°C while Δ Ht

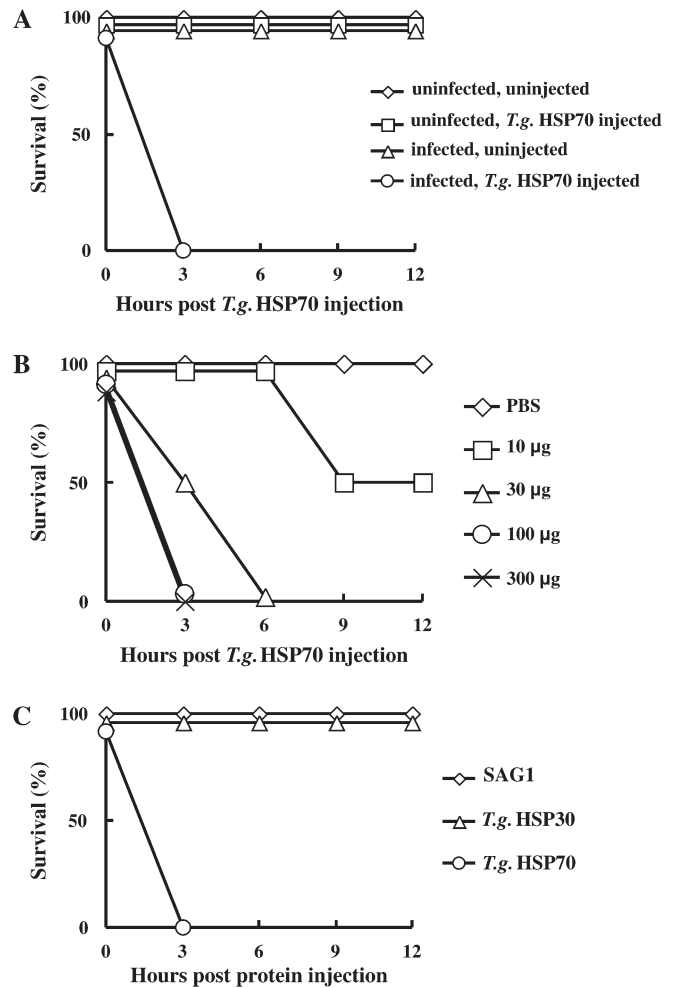


Fig. 1. *T.g.*HSP70-induced anaphylactic reaction in *Toxoplasma gondii*-infected mice. (A) *T.g.*HSP70 (100 μ g) was injected to uninfected (squares) or *T. gondii*-infected (circles) WT BALB/c mice at 7 days p.i. with 10 cysts of Fukaya strain. Uninfected (diamonds) or *T. gondii*-infected (triangles) WT BALB/c mice without injection of *T.g.*HSP70 were used as controls. Survival of mice was checked every hour after injection of *T.g.*HSP70. (B) Various doses of *T.g.*HSP70 (300, 100, 30 or 10 μ g) were i.p. injected to WT BALB/c mice at 7 days p.i. Survival of mice was checked every hour after 300 μ g (crosses), 100 μ g (circles), 30 μ g (triangles), 10 μ g (squares) of *T.g.*HSP70 or PBS (diamonds) injection. (C) To clarify the specificity of *T.g.*HSP70-induced anaphylactic reaction (circles), 300 μ g of SAG1 (diamonds) or *T.g.*HSP30 (triangles) was injected to *T. gondii*-infected WT BALB/c mice at 7 days p.i. Survival of mice was checked every hour after injection of SAG1, *T.g.*HSP30 or *T.g.*HSP70. Three to four mice were used in each experimental group. Data are representative of three separate experiments.

increased from 0 to 27% after the challenge of *T.g.*HSP70 injection until death (Fig. 2A). The most prominent histopathological changes were necrosis and diffuse parenchymal degeneration in the liver of *T. gondii*-infected and *T.g.*HSP70-injected mice (Fig. 2B, d). Moderate lymphofollicular hyperplasia and inflammatory cell infiltration in red pulp were also observed in the spleen (data not shown). Inflammatory cell infiltration in perivascular area was seen in *T. gondii*-infected mice (Fig. 2B, b and d). Apparent histopathological changes were not found in uninfected and *T.g.*HSP70-injected mice (Fig. 2B, a and c).

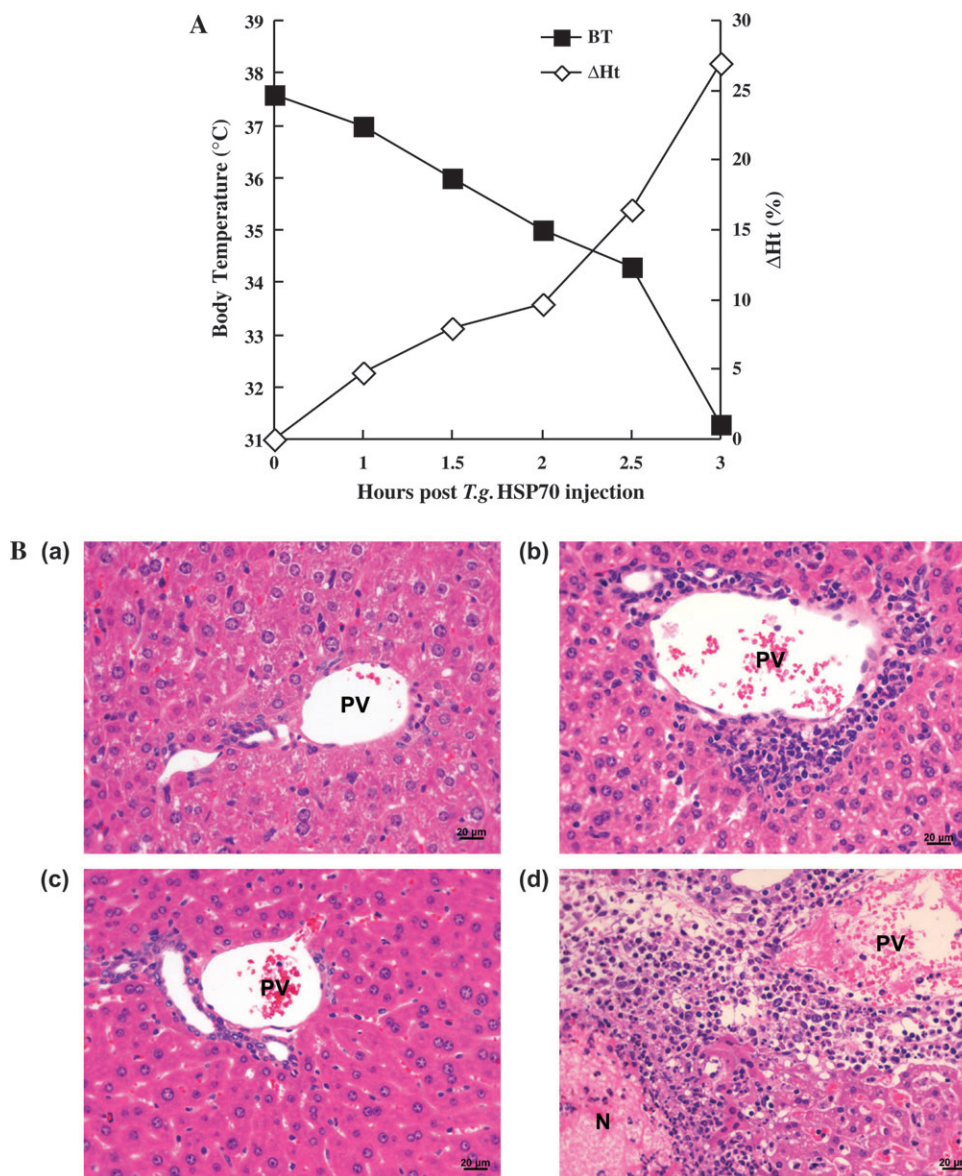


Fig. 2. Clinical indices and histopathological changes in *T.g.*HSP70-induced anaphylactic reaction. (A) Body temperature and Ht changes in *T.g.*HSP70-induced anaphylactic reaction. *T.g.*HSP70 (100 μ g) was i.p. injected to WT BALB/c mice at 7 days p.i. Rectal body temperature (squares) and Ht were checked every half hour. Δ Ht (diamonds) was calculated as follows: [(value at post-challenge – value at pre-challenge)/value at pre-challenge] $\times$ 100%. Three mice were used in each experimental group. Data are representative of three separate experiments. (B) Histopathology of the livers from naive WT BALB/c mice (a), *Toxoplasma gondii*-infected WT BALB/c mice without *T.g.*HSP70 injection (b), uninfected WT BALB/c mice with *T.g.*HSP70 injection (c), and *T. gondii*-infected and *T.g.*HSP70-injected WT BALB/c mice (d) was examined (hematoxylin and eosin staining, original magnification $\times$ 40). PV: portal vein; N: necrosis. Bar, 20 μ m.

To ascertain that *T.g.*HSP70-induced anaphylactic reaction was not due to contamination of the *T.g.*HSP70 preparation by endotoxin of *E. coli*, the inhibition effect of PMB, an LPS-specific inhibitor that binds to the Lipid A portion of LPS, on *T.g.*HSP70- or LPS-induced reaction was examined in WT BALB/c mice. LPS (10 μ g) was chosen according to a dose-response study of LPS (data not shown). Both 100 μ g of *T.g.*HSP70 and 10 μ g of LPS were pre-incubated with or without 100 μ g of PMB at 37°C for 1 h and then injected to *T. gondii*-infected mice. Mice injected with either PMB-treated or PMB-untreated *T.g.*HSP70 died within 3 h after injection (Fig. 3). On the other hand, mice injected with PMB-untreated

LPS died within 3 h after injection, while the mice injected with PMB-treated LPS survived (Fig. 3). These data indicated that *T.g.*HSP70-induced anaphylactic reaction was not caused by contamination of LPS.

Absence of *T.g.*HSP70-induced anaphylactic reaction in GKO mice

As IFN- γ is a key molecule of the host defense against *T. gondii* infection, GKO mice were also challenged by i.p. injection of *T.g.*HSP70 at 7 days p.i. Interestingly, *T.g.*HSP70 did not induce anaphylactic reaction in GKO mice, indicating that IFN- γ

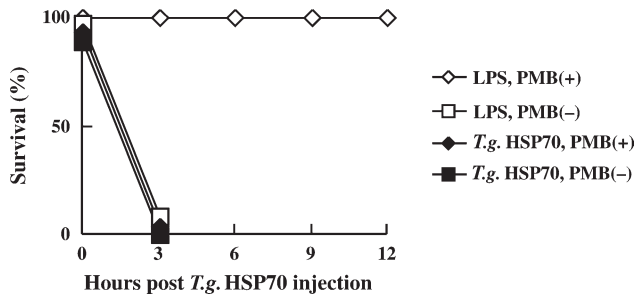


Fig. 3. Specificity of *T.g.*HSP70 to induce anaphylactic reaction. *T.g.*HSP70 (100 µg) and LPS (10 µg) were pre-incubated with or without 100 µg of PMB at 37°C for 1 h and then injected to *Toxoplasma gondii*-infected WT BALB/c mice. Survival of mice injected with treated *T.g.*HSP70 (closed diamonds), treated LPS (open diamonds), untreated *T.g.*HSP70 (closed squares) or untreated LPS (open squares) was checked every hour after *T.g.*HSP70 or LPS injection. Three mice were used in each experimental group. Experiments were performed twice.

was essential for *T.g.*HSP70-induced anaphylactic reaction. GKO mice usually die within 10 days after *T. gondii* infection, but can be rescued by sulfamethoxazole treatment from day 4 after infection (31, 32). Therefore, we investigated the effect of sulfamethoxazole treatment on *T.g.*HSP70-induced anaphylactic reaction in both WT and GKO mice. Obviously, *T.g.*HSP70 did not induce anaphylactic reaction in GKO mice untreated or treated with sulfamethoxazole, while *T.g.*HSP70 induced anaphylactic reaction in WT mice untreated or treated with sulfamethoxazole within 3 h (Fig. 4A). Thus, sulfamethoxazole treatment did not rescue WT mice from lethal *T.g.*HSP70-induced anaphylactic reaction.

To ascertain the involvement of IFN-γ in *T.g.*HSP70-induced anaphylactic reaction, *T. gondii*-infected GKO BALB/c mice were i.p. administered with 2 µg of murine rIFN-γ daily from day 0 to day 7 p.i. The amount of rIFN-γ used was comparable to the level of IFN-γ in WT mice infected with *T. gondii* (33). Both rIFN-γ-treated and -untreated mice were i.p. injected with 100 µg of *T.g.*HSP70 at day 7 p.i. GKO mice substituted with rIFN-γ died within 9 h after injection of *T.g.*HSP70, while the mice without rIFN-γ treatment survived (Fig. 4B). Thus, the requirement of IFN-γ in *T.g.*HSP70-induced anaphylactic reaction was confirmed.

*T.g.*HSP70-induced anaphylactic reaction can be transferred by splenocytes from WT mice

In order to analyze the effectors participating in *T.g.*HSP70-induced anaphylactic reaction, we i.p. transferred splenocytes, PECs or serum at 7 days p.i. from *T. gondii*-infected WT mice to naive GKO mice, which were then i.p. injected with *T.g.*HSP70 at 2 days after the transfer. Lethal anaphylactic reaction was observed in GKO mice transferred with 1×10^8 or 3×10^7 splenocytes from infected WT mice (Fig. 5A). Similarly, lethal anaphylactic reaction was observed in GKO mice transferred with 1×10^8 PECs from WT mice (data not shown). This anaphylactic reaction was not observed in GKO mice transferred with serum from WT mice (Fig. 5A). These data indicated that lethal *T.g.*HSP70-induced anaphylactic reaction was transferable from cells but not serum from

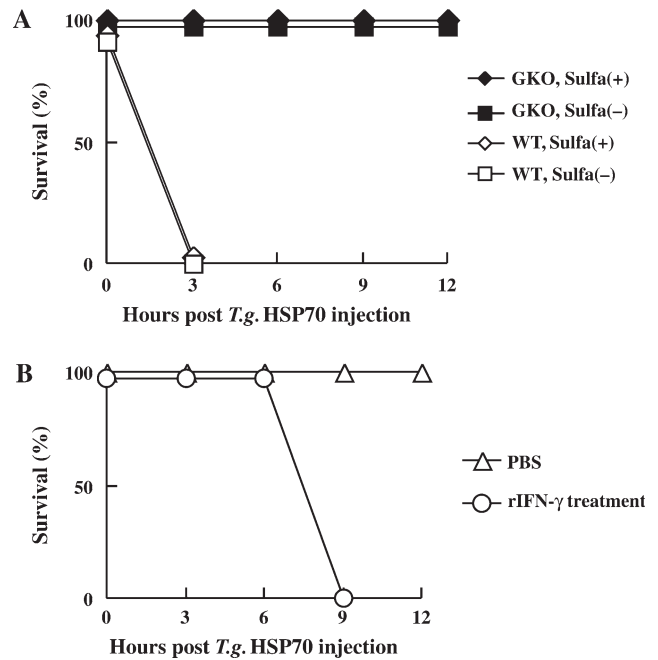


Fig. 4. Absence of *T.g.*HSP70-induced anaphylactic reaction in GKO mice. (A) WT (open legends) and GKO (closed legends) BALB/c mice were treated with (diamonds) or without (squares) sulfamethoxazole from 4 days p.i. as described in Methods. *T.g.*HSP70 (100 µg) was i.p. injected to *Toxoplasma gondii*-infected mice at day 7 p.i., and survival was checked every hour. (B) Infected GKO BALB/c mice were i.p. injected with 2 µg of rIFN-γ in 0.2 ml saline daily from day 0 to day 7 p.i. Both rIFN-γ-treated (circles) and -untreated (triangles) mice were i.p. injected with 100 µg of *T.g.*HSP70 at day 7 p.i. Survival was checked every hour after injection of *T.g.*HSP70. Three to four mice were used in each experimental group. Experiments were performed twice.

T. gondii-infected WT mice. Furthermore, the reaction was also observed in µMT mice, indicating that this anaphylactic reaction occurred through an Ig-independent pathway (Fig. 5B).

In the next experiment, to analyze the cell types involved in *T.g.*HSP70-induced anaphylactic reaction, splenocytes from *T. gondii*-infected WT mice were fractionated into B220⁺, CD90⁺, CD11b⁺ or CD11c⁺ cells and transferred into naive GKO mice. T cells were sub-fractionated into CD4⁺ and CD8⁺ subsets, and then *T.g.*HSP70 was injected at 2 days after the transfer. All GKO mice transferred with CD11b⁺ cells or CD11c⁺ cells died within 24 h, while mice transferred with CD90⁺ died within 48 h, but none transferred with B220⁺ cells died (Fig. 6). GKO mice transferred with CD4⁺ or CD8⁺ splenocytes also died within 48 h (Fig. 6). This proved that CD11b⁺, CD11c⁺ and CD90⁺ (CD4⁺ and CD8⁺), but not B220⁺, cells function as effector cells in *T.g.*HSP70-induced anaphylactic reaction.

It was confirmed that *T.g.*HSP70-induced anaphylactic reaction was absent in GKO mice, so we analyzed cytokine production in WT mice after *T.g.*HSP70 injection. WT mice were divided into four groups as described in Methods. mRNA expressions of IL-4, IL-10, IL-6, IFN-γ, IL-12 p40 and TNF-α in PECs and splenocytes of mice were analyzed by RT-PCR. In PECs, IL-4, IL-6 and IL-10 production, but not IFN-γ, IL-12 p40 or TNF-α production, was detected by *T.g.*HSP70 injection alone. On the other hand, significant

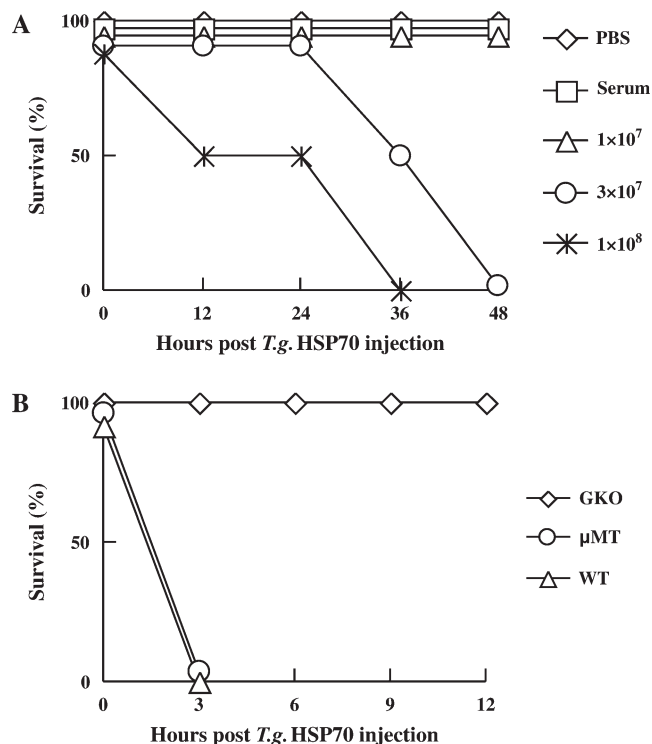


Fig. 5. *T.g.*HSP70-induced anaphylactic reaction was transferable with splenocytes but not with serum from *Toxoplasma gondii*-infected WT mice to GKO mice. (A) GKO BALB/c mice were transferred with various numbers of splenocytes or serum from *T. gondii*-infected WT BALB/c mice. *T.g.*HSP70 (100 μ g) was i.p. injected to GKO mice at 2 days post-transfer. Survival of mice transferred with 1×10^8 (crosses), 3×10^7 (circles) and 1×10^7 (triangles) splenocytes or serum (squares) was checked after injection every 3 h. PBS (diamonds) was injected as a control. (B) *T.g.*HSP70 (100 μ g) was i.p. injected to *T. gondii*-infected GKO (diamonds), μ MT (circles) or WT (triangles) mice at day 7 p.i., and survival was checked every hour. Three to four mice were used in each experimental group. Data are representative of three separate experiments.

increase of IFN- γ production was found in *T. gondii*-infected mice with *T.g.*HSP70 injection, but not in uninfected mice or infected mice without injection (Fig. 7A). Similar cytokine expression was observed in splenocytes from *T. gondii*-infected mice (Fig. 7B). These data proved that *T.g.*HSP70 injection induced remarkable IFN- γ production in *T. gondii*-infected mice.

Involvement of PAF in *T.g.*HSP70-induced anaphylactic reaction

Since it was reported that lethal anaphylactic reaction was accompanied by disseminated intravascular coagulation, the involvement of platelet activation in *T.g.*HSP70-induced anaphylactic reaction was investigated. Platelet numbers relative to RBC numbers were counted in mice before and after *T.g.*HSP70-induced anaphylactic reaction. The platelet numbers dramatically decreased after the reaction occurred (average platelet number was 1/1000 RBC) ($P < 0.001$), whereas the numbers slightly decreased after *T. gondii* infection (153/1000 RBC) ($P < 0.05$) compared with those of uninfected mice (177/1000 RBC) (Fig. 8). These data in-

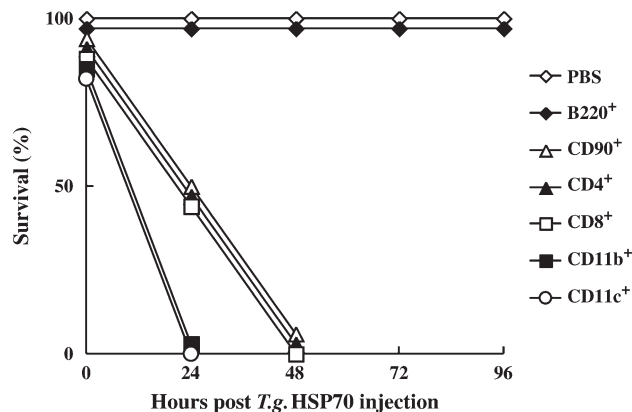


Fig. 6. *T.g.*HSP70-induced anaphylactic reaction in GKO mice transferred with fractionated splenocytes from *Toxoplasma gondii*-infected WT mice. B220⁺, CD90⁺, CD4⁺, CD8⁺, CD11b⁺ or CD11c⁺ splenocytes from *T. gondii*-infected WT BALB/c mice were purified using MACS purification system and transferred to GKO BALB/c mice as described in Methods. *T.g.*HSP70 (100 μ g) was i.p. injected to GKO mice at 2 days post-transfer of fractionated splenocytes. Survival of mice transferred with B220⁺ (closed diamonds), CD90⁺ (open triangles), CD4⁺ (closed triangles), CD8⁺ (open squares), CD11b⁺ (closed squares) or CD11c⁺ (open circles) was checked hourly after injection of *T.g.*HSP70. PBS (open diamonds) was injected as a control. Three to four mice were used in each experimental group. Data are representative of three separate experiments.

dicated that platelets were involved in *T.g.*HSP70-induced anaphylactic reaction.

The expression of mRNA in splenocytes of mice for PAF-R and PAF-AH, the enzyme most responsible for degradation of PAF, was analyzed by RT-PCR. Both WT and GKO mice were divided into four groups as described in Methods. In WT mice, the mRNA expression of PAF-AH from group 4 (*T. gondii*-infected and *T.g.*HSP70-injected mice) ($P < 0.01$) was significantly increased, while the expression of PAF-AH in both group 2 (infection alone) ($P < 0.05$) and group 3 (injection alone) ($P < 0.05$) was slightly higher compared with group 1 (uninfected mice) (Fig. 9A and C). The mRNA expression level of group 4 was significantly higher than those of groups 2 and 3 (Fig. 9A and C). On the other hand, a clear increase was not observed in any of the groups of GKO mice (Fig. 9A and C). Thus, a high level of PAF production was observed in the *T.g.*HSP70-induced anaphylactic reaction in WT mice, indicating the important role of the IFN- γ -dependent PAF pathway in its induction of *T.g.*HSP70-induced anaphylactic reaction. On the other hand, a significant change in the mRNA expression of PAF-R was not observed in any of the four groups of both WT and GKO mice (Fig. 9B and D), whereas differences in the mRNA expression of PAF-R were found between WT and GKO mice, indicating the role of IFN- γ acting on the expression of PAF-R ($P < 0.05$) (Fig. 9B and D).

PAF-R antagonist inhibited *T.g.*HSP70-induced anaphylactic reaction

To confirm the involvement of PAF in *T.g.*HSP70-induced anaphylactic reaction, the PAF-R antagonist CV6209 was i.p. injected to block the reaction. Different doses of CV6209 were administered 10 min before 100 μ g of *T.g.*HSP70 injection in

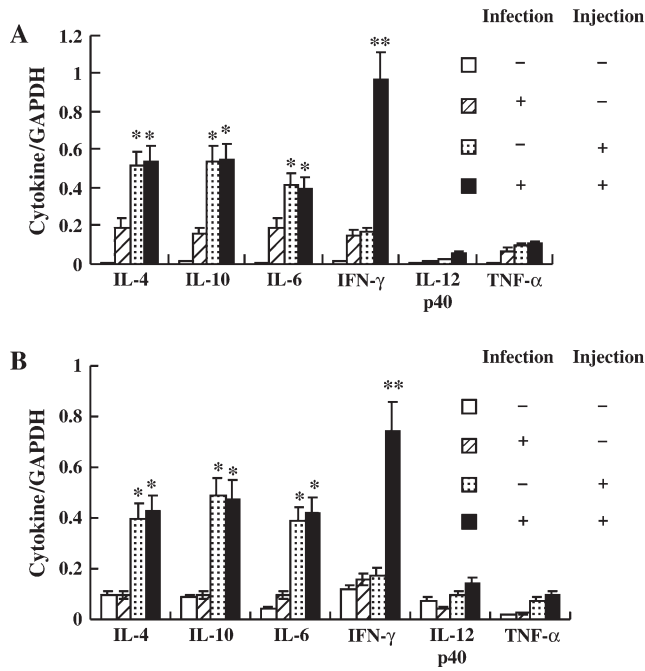


Fig. 7. Cytokine expression in *T.g.*HSP70-injected WT mice. Expressions of IL-4, IL-10, IL-6, IFN- γ , IL-12 p40 and TNF- α in PECs (A) and splenocytes (B) from four experimental groups of WT BALB/c mice were analyzed by RT-PCR as described in Methods. The four experimental groups: group 1 without infection, without *T.g.*HSP70 injection (open bar); group 2 infection, without *T.g.*HSP70 injection (striped bar); group 3 without infection, 3 h post-*T.g.*HSP70 injection (dotted bar); group 4 infection, 3 h post-*T.g.*HSP70 injection (closed bar). The results are expressed as the ratio between optical density of the RT-PCR product of each cytokine and that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Three to four mice were used in each experimental group. Data are representative of three separate experiments. * $P < 0.05$, ** $P < 0.001$ compared with the data of group 1.

T. gondii-infected WT mice. Hundred micrograms of CV6209 blocked the anaphylactic reaction and resulted in survival of the mice. Mice treated with 50 μ g of CV6209 survived 6 h longer than the control group, while mice treated with 25 μ g or without CV6209 died within 3 h (Fig. 10). These results indicated that PAF-R antagonist dose dependently inhibited *T.g.*HSP70-induced anaphylactic reaction *in vivo*.

Expression of PAF-AH and IFN- γ by CD11b⁺, CD11c⁺, CD90⁺ splenocytes from *T. gondii*-infected and *T.g.*HSP70-injected mice

In order to analyze which cell populations produce PAF-AH and/or IFN- γ , expressions of PAF-AH and IFN- γ in fractionated CD11b⁺, CD11c⁺, CD90⁺, CD4⁺ and CD8⁺ splenocytes from *T. gondii*-infected and *T.g.*HSP70-injected WT BALB/c mice were analyzed by RT-PCR. As controls, expressions of PAF-AH and IFN- γ in unfractionated splenocytes from either naive or *T. gondii*-infected and *T.g.*HSP70-injected WT BALB/c mice were examined. Prominent PAF-AH expressions were observed in unfractionated and fractionated CD11b⁺, and CD11c⁺ splenocytes of *T. gondii*-infected and *T.g.*HSP70-injected WT BALB/c mice compared with those of unfractionated splenocytes of naive mice ($P < 0.001$) (Fig. 11A). The

levels of PAF-AH expression in CD90⁺, CD4⁺ and CD8⁺ splenocytes from *T. gondii*-infected and *T.g.*HSP70-injected WT BALB/c mice were not statistically different from those of naive mice (Fig. 11A). On the other hand, high levels of IFN- γ expression were observed in unfractionated and in all fractionated splenocytes from *T. gondii*-infected and *T.g.*HSP70-injected WT BALB/c mice compared with unfractionated splenocytes from naive mice ($P < 0.001$) (Fig. 11B). These data indicated the direct CD11b⁺ and CD11c⁺ and indirect CD90⁺ cell participation in PAF-AH production in *T. gondii*-infected and *T.g.*HSP70-injected WT BALB/c mice.

Discussion

It is known that acute infection of *T. gondii* is associated with the rapid multiplication of tachyzoites stage converted from bradyzoites. Previous data revealed that *T.g.*HSP70 was a tachyzoite-specific virulent molecule for protective immunity of the host (1, 3, 10, 25). On the other hand, the extent of tissue inflammation is often disproportionate to the abundance of parasites in toxoplasmosis (34). Therefore, we investigated the molecular-based mechanisms that induce death of the host. In this study, we demonstrated that *T.g.*HSP70, but not other *T. gondii*-derived antigens such as SAG1 or *T.g.*HSP30, specifically caused lethal anaphylactic reaction in *T. gondii*-infected mice. This study is the first to report the induction of anaphylactic reaction by HSP derived from microorganisms.

Anaphylactic shock has been previously reported in various parasitic infections (35–37). The larval stages of *Echinococcus granulosus* and *Echinococcus multilocularis* are involved in cystic echinococcosis (CE) (hydatid disease) and alveolar echinococcosis (AE), respectively, in humans (35). Anaphylactic reactions, including urticaria, edema, respiratory symptoms and anaphylactic shock due to spontaneous or provoked rupture of the parasitic cyst, are well known in CE. Anaphylactic reactions in AE are observed at the time of metastatic dissemination of parasitic lesions. Murine models of anaphylaxis induced by parasites such as *Anisakis simplex*, *Toxocara canis* and others have been reported (36, 37). All these anaphylactic shocks related to parasitic infection are IgE-dependent type I hypersensitivity reaction. In this study, however, it was clearly shown that the *T.g.*HSP70-induced anaphylactic reaction was caused through an Ig-independent pathway because it was induced in μ MT mice, which are B cell-deficient mice. Platelet activation and PAF activation were confirmed during the occurrence of the reaction, indicating the involvement of PAF in *T.g.*HSP70-induced anaphylactic reaction.

It was reported that PAF was synthesized via two distinct pathways: the *de novo* and the remodeling pathways (38–40). The latter is regulated by extracellular signals, and plays a critical role in stimulus-coupling PAF biosynthesis. In this pathway, the precursor of PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF), is synthesized from 1-*O*-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine and/or 1-*O*-alk-1-enyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine by the action of phospholipase A₂. Subsequently, lyso-PAF is acetylated by acetyl coenzyme A: lyso-PAF acetyltransferase (lyso-PAF acetyltransferase) to form PAF. Another enzyme, PAF-AH, is regarded as the main enzyme involved in PAF

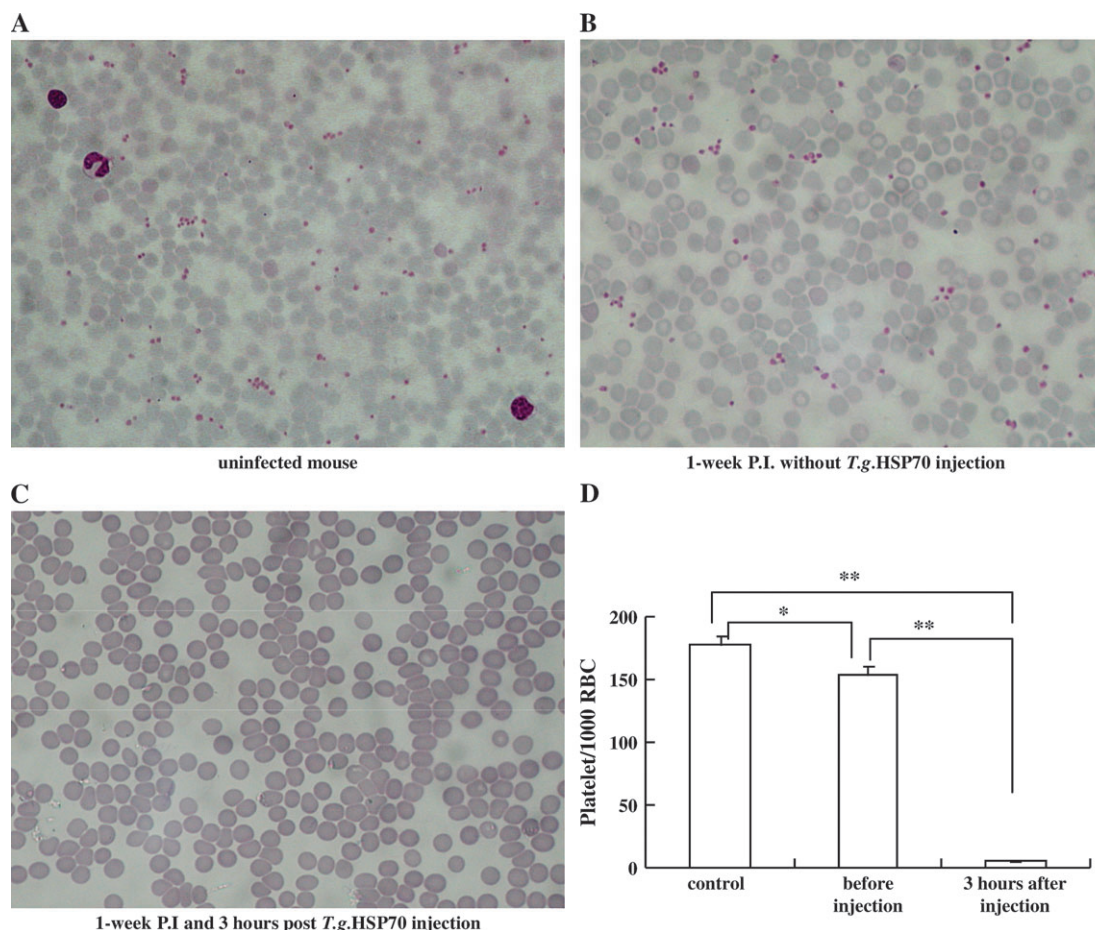


Fig. 8. Decreasing peripheral platelets in *T.g.HSP70*-induced anaphylactic reaction. Peripheral blood was obtained from the tail of mice uninfected and without *T.g.HSP70* injection (A), mice 1 week p.i., without *T.g.HSP70* injection (B) and mice 1 week p.i., 3 h after *T.g.HSP70* injection (C). Peripheral blood smears were stained by Giemsa method (magnification, $\times 200$) (A–C), and platelet numbers are shown as the ratio of PLT:1000 RBC (D). Three mice were used in each experimental group. Data are representative of three separate experiments. * $P < 0.05$, ** $P < 0.001$ compared with the data of uninfected mice.

degradation, and an increase of PAF-AH is considered to be a natural feedback reaction against PAF synthesis (27, 41). It has been proved that lyso-PAF acetyltransferase is responsible for enhanced PAF production by the priming effect of LPS (42). PAF synthesis induced by extracellular signals has also been reported in mouse peritoneal cells stimulated by calcium ionophore (43), human eosinophils stimulated by *N*-formyl-methionyl-leucyl-phenylalanine (44) and human mesangial cells and mouse peritoneal macrophages stimulated by LPS (45, 46). In our study, PAF activation was confirmed in *T.g.HSP70*-induced anaphylactic reaction, which differed from LPS-induced anaphylactic reaction. The reaction was transferable by CD11b⁺ and CD11c⁺ splenocytes from *T.gondii*-infected mice. It might be assumed that *T.g.HSP70* induces the synthesis of PAF by these CD11b⁺ and CD11c⁺ splenocytes.

Han *et al.* (47) reported that Gram-positive bacteria induce NO production using a PAF-R signaling pathway to activate signal transducer and activator of transcription 1 (STAT1) via Janus kinase 2 (Jak2). Their PAF-R/Jak2/STAT1 signaling pathway resembled the type I-IFNR/Jak/STAT1 pathway

described for LPS. We previously reported the *T.g.HSP70*-induced NO production by peritoneal macrophages via Toll-like receptor (TLR) 2 and the tolerance in NO production induced by TLR4 (48). The *T.g.HSP70*-induced TLR2 signaling pathway was suppressed by the expression of suppressor of cytokine-signaling-1 via TLR4 (48). The relation between NO production and *T.g.HSP70*-induced anaphylactic reaction is currently under investigation.

Geffner *et al.* (49) demonstrated that the treatment of polymorphonuclear leukocytes with recombinant human IFN- γ , but not IFN- α or IFN- β , dose dependently enhanced the production of PAF-acether. In our experiment, it was revealed that *T.g.HSP70* significantly induced IFN- γ production and PAF activation in *T.gondii*-infected mice. It could be possible that an over-production of PAF was induced by the IFN- γ -stimulated CD11b⁺ and CD11c⁺ splenocytes in the *T.g.HSP70*-induced anaphylactic reaction.

IFN- γ is produced by activated T lymphocytes and NK cells and exerts its biological activity through the binding of IFN- γ receptors. In our experiment, *T.g.HSP70*-induced anaphylactic reaction was IFN- γ dependent. Similarly, Car *et al.* (50)

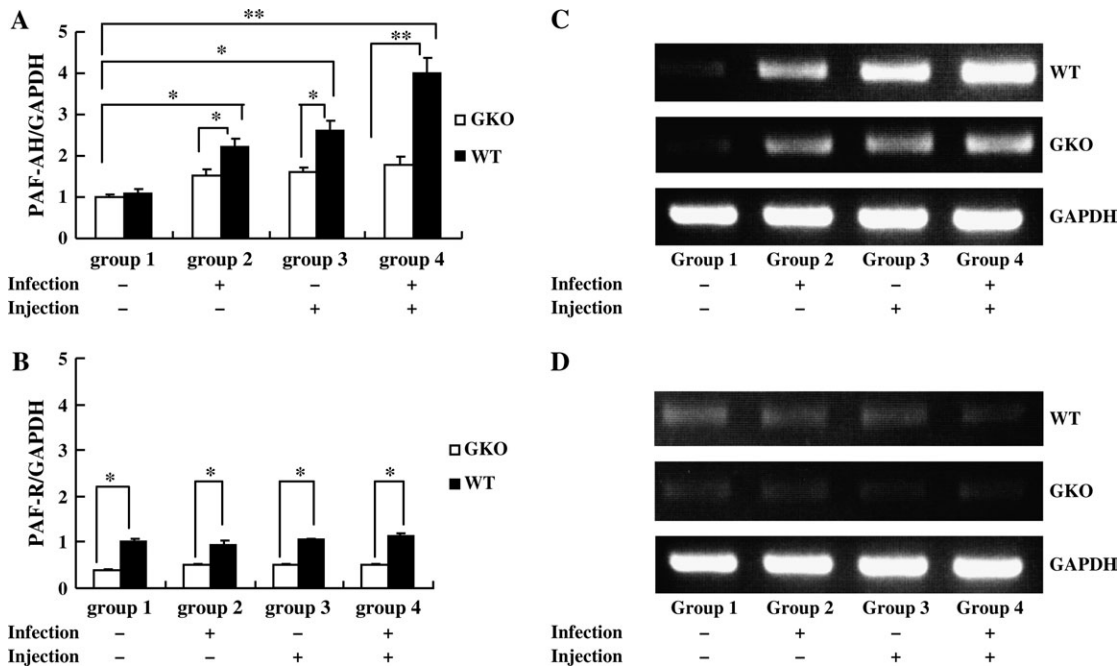


Fig. 9. Involvement of PAF in *T.g.*HSP70-induced anaphylactic reaction. Expressions of PAF-AH (A, C) and PAF-R (B, D) in splenocytes from WT or GKO mice of four experimental groups were analyzed by RT-PCR as described in Methods. WT mice are shown in open bars and GKO mice in closed bars. Four experimental groups: group 1 without infection, without *T.g.*HSP70 injection; group 2 infection, without *T.g.*HSP70 injection; group 3 without infection, 3 h post-*T.g.*HSP70 injection; group 4 infection, 3 h post-*T.g.*HSP70 injection. 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Three mice were used in each experimental group. Data are representative of three separate experiments. * $P < 0.05$, ** $P < 0.001$ compared with the data of group 1.

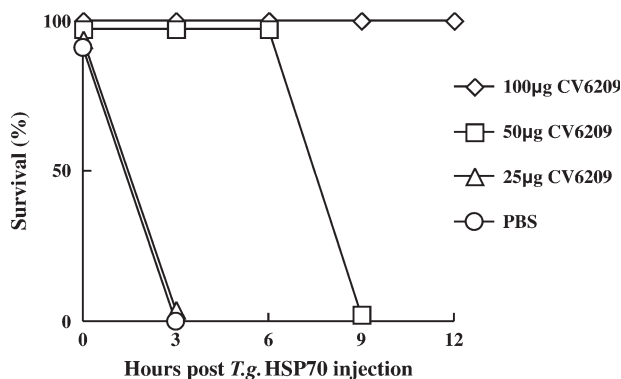


Fig. 10. PAF-R antagonist inhibited *T.g.*HSP70-induced anaphylactic reaction. Hundred micrograms (diamonds), 50 µg (squares), or 25 µg (triangles) of PAF-R antagonist CV6209 was i.p. administered 10 min before 100 µg of *T.g.*HSP70 injection. PBS (circles) was injected as a control. Survival was checked every hour after *T.g.*HSP70 injection. Three mice were used in each experimental group. Data are representative of three separate experiments.

reported that IFN- γ receptor-deficient mice were resistant to endotoxic shock induced by LPS derived from *E. coli*. In our study, the mRNA expression of PAF-R in GKO mice was lower than that of WT mice. Similarly, Ouellet *et al.* (51) also reported that IFN- γ up-regulated PAF-R gene expression in human monocytes by a mechanism suggesting transcriptional regulation. Thus, IFN- γ may act on the expression of PAF-R

during the occurrence of *T.g.*HSP70-induced anaphylactic reaction.

By cell transfer experiment, *T.g.*HSP70-induced anaphylactic reaction was transferred by CD4⁺, CD8⁺, CD11b⁺ and CD11c⁺ splenocytes from *T. gondii*-infected WT mice to naive GKO mice, with the CD11b⁺ and CD11c⁺ splenocytes causing the reaction earlier than the CD4⁺ or CD8⁺ splenocytes in the naive GKO mice. It was revealed that PAF-AH was induced by CD11b⁺ and CD11c⁺ cells, but not by CD4⁺ or CD8⁺ cells, from *T. gondii*-infected WT BALB/c mice after *T.g.*HSP70 injection, whereas IFN- γ was induced by CD11b⁺, CD11c⁺, CD4⁺ and CD8⁺ cells. Therefore, we speculate that IFN- γ , produced by CD4⁺, CD8⁺, CD11b⁺ and CD11c⁺ cells in the presence of *T.g.*HSP70, as a consequence induces PAF production by activating CD11b⁺ and CD11c⁺ splenocytes of naive mice. The direct participation of CD11b⁺ and CD11c⁺ cells and indirect participation of CD90⁺ (CD4⁺ and CD8⁺) cells in PAF-AH production might cause the time differences of the reaction of GKO mice in cell transfer experiments. The exogenous administration of rIFN- γ consistently caused *T.g.*HSP70-induced anaphylactic reaction in naive WT BALB/c mice (Fig. S1, available at *International Immunology Online*). The precise effector mechanisms in *T.g.*HSP70-induced anaphylactic reaction remained to be further analyzed.

Supplementary data

A supplementary figure is available at *International Immunology Online*.

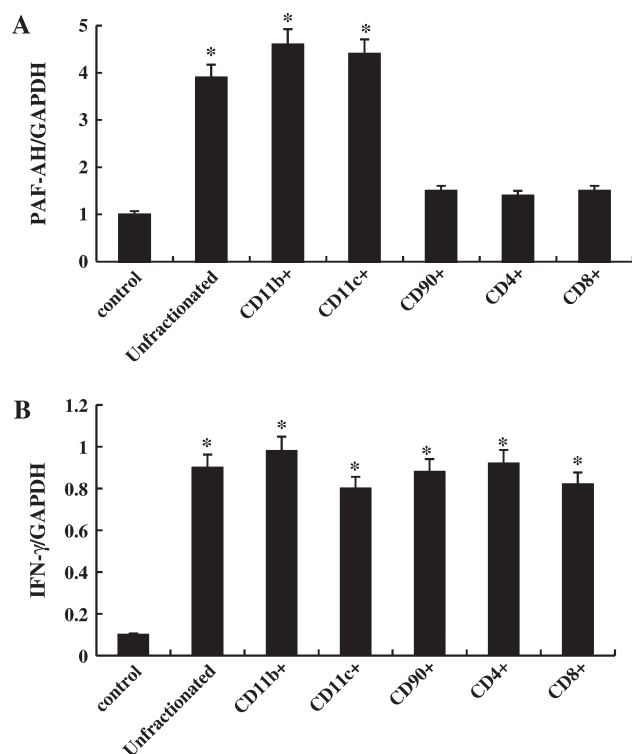


Fig. 11. Expression of PAF-AH and IFN- γ in fractionated splenocytes. Expressions of PAF-AH (A) and IFN- γ (B) were analyzed by RT-PCR in unfractionated or fractionated splenocytes from WT BALB/c mice injected with 100 μ g of *T.g.*HSP70 at 1 week p.i. Splenocytes were fractionated into CD11b⁺, CD11c⁺, CD90⁺, CD4⁺ and CD8⁺ cells using the MACS purification system as described in Methods. Unfractionated splenocytes from naive mice were used as a control. The results are expressed as the ratio between optical density of the RT-PCR product of each cytokine and that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Three to four mice were used in each experimental group. Data are representative of three separate experiments. * $P < 0.001$ compared with the data of group 1.

Acknowledgements

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Abbreviations

AE	alveolar echinococcosis
CE	cystic echinococcosis
GKO	IFN- γ knockout
Ht	hematocrit
i.p.	intraperitoneally
Jak2	Janus kinase 2
lyso-PAF	1-O-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine
lyso-PAF acetyltransferase	acetyl coenzyme A: lyso-PAF acetyltransferase
mRNA	messenger RNA
NO	nitric oxide
μ MT	B cell deficient
PAF	platelet-activating factor
PAF-AH	platelet-activating factor acetylhydrolase
PAFR	platelet-activating factor receptor
PEC	peritoneal exudate cell

p.i.	post-infection
PMB	polymyxin B
p.o.	perorally
rIFN- γ	recombinant IFN- γ
RT	reverse transcriptase
SAG1	surface antigen 1
STAT1	signal transducer and activator of transcription 1
<i>T.g.</i> HSP70	<i>Toxoplasma gondii</i> -derived heat shock protein 70
TLR	Toll-like receptor
TNF	tumor necrosis factor
WT	wild type

References

- Yano, A., Mun, H. S., Chen, M. *et al.* 2002. Roles of IFN- γ on stage conversion of an obligate intracellular protozoan parasite, *Toxoplasma gondii*. *Int. Rev. Immunol.* 21:405.
- Mun, H. S., Aosai, F., Norose, K. *et al.* 2000. *Toxoplasma gondii* Hsp70 as a danger signal in *Toxoplasma gondii*-infected mice. *Cell Stress Chaperones* 5:328.
- Mun, H. S., Aosai, F. and Yano, A. 1999. Role of *Toxoplasma gondii* HSP70 and *Toxoplasma gondii* HSP30/bag1 in antibody formation and prophylactic immunity in mice experimentally infected with *Toxoplasma gondii*. *Microbiol. Immunol.* 43:471.
- Chen, M., Aosai, F., Mun, H. S., Norose, K., Hata, H. and Yano, A. 2000. Anti-HSP70 autoantibody formation by B-1 cells in *Toxoplasma gondii*-infected mice. *Infect. Immun.* 68:4893.
- Chen, M., Aosai, F., Norose, K., Mun, H. S. and Yano, A. 2003. The role of anti-HSP70 autoantibody-forming VH1-JH1 B-1 cells in *Toxoplasma gondii*-infected mice. *Int. Immunol.* 15:39.
- Ahmed, A. K., Mun, H. S., Aosai, F. *et al.* 2004. Roles of *Toxoplasma gondii*-derived heat shock protein 70 in host defense against *T. gondii* infection. *Microbiol. Immunol.* 48:911.
- Aosai, F., Chen, M., Kang, H. K. *et al.* 2002. *Toxoplasma gondii*-derived heat shock protein HSP70 functions as a B cell mitogen. *Cell Stress Chaperones* 7:357.
- Aosai, F., Rodriguez Pena, M. S., Mun, H. S. *et al.* 2006. *Toxoplasma gondii*-derived heat shock protein 70 stimulates maturation of murine bone marrow-derived dendritic cells via Toll-like receptor 4. *Cell Stress Chaperones* 11:13.
- Kang, H. K., Lee, H. Y., Lee, Y. N. *et al.* 2004. *Toxoplasma gondii*-derived heat shock protein 70 stimulates the maturation of human monocyte-derived dendritic cells. *Biochem. Biophys. Res. Commun.* 322:899.
- Dobbin C. A., Smith N. C. and Johnson A. M. 2002. Heat shock protein 70 is a potential virulence factor in murine *toxoplasma* infection via immuno-modulation of host NF- κ B and nitric oxide. *J. Immunol.* 169:958.
- Finkelman, F. D., Rothenberg, M. E., Brandt, E. B., Morris, S. C. and Strait, R. T. 2005. Molecular mechanisms of anaphylaxis: lessons from studies with murine models. *J. Allergy Clin. Immunol.* 115:449.
- Strait, R. T., Morris, S. C., Yang, M., Qu, X. W. and Finkelman, F. D. 2002. Pathways of anaphylaxis in the mouse. *J. Allergy Clin. Immunol.* 109:658.
- Burks, A. W. and Sampson, H. A. 1999. Anaphylaxis and food allergy. *Clin. Rev. Allergy Immunol.* 17:339.
- Sampson, H. A., Mendelson, L. and Rosen, J. P. 1992. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *N. Engl. J. Med.* 327:380.
- Miyajima, I., Dombrowicz, D., Martin, T. R., Ravetch, J. V., Kinet, J. P. and Galli, S. J. 1997. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc γ RIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *J. Clin. Invest.* 99:901.
- Dombrowicz, D., Flamand, V., Miyajima, I., Ravetch, J. V., Galli, S. J. and Kinet, J. P. 1997. Absence of Fc ϵ RI α chain results in upregulation of Fc γ RIII-dependent mast cell degranulation and anaphylaxis. Evidence of competition between Fc ϵ RI and Fc γ RIII for limiting amounts of FcR β and γ chains. *J. Clin. Invest.* 99:915.

- 17 Leir, S. H., Chen, S. H. and Lei, H. Y. 1995. Horse gammaglobulin-induced thrombocytopenia in anaphylaxis involving sequestration and activation of platelets. *Clin. Exp. Allergy* 25:273.
- 18 Choi, I. W., Kim, Y. S., Kim, D. K. *et al.* 2003. Platelet-activating factor-mediated NF- κ B dependency of a late anaphylactic reaction. *J. Exp. Med.* 198:145.
- 19 Chen, M., Mun, H. S., Piao, L. X. *et al.* 2003. Induction of protective immunity by primed B-1 cells in *Toxoplasma gondii*-infected B cell-deficient mice. *Microbiol. Immunol.* 47:997.
- 20 Norose, K., Mun, H. S., Aosai, F. *et al.* 2003. IFN- γ -regulated *Toxoplasma gondii* distribution and load in the murine eye. *Invest. Ophthalmol. Vis. Sci.* 44:4375.
- 21 Tagawa, Y., Sekikawa, K. and Iwakura, Y. 1997. Suppression of concanavalin A-induced hepatitis in IFN- $\gamma^{-/-}$ mice, but not in TNF- $\alpha^{-/-}$ mice: role of IFN- γ in activating apoptosis of hepatocytes. *J. Immunol.* 159:1418.
- 22 Norose, K., Mun, H. S., Aosai, F. *et al.* 2001. Organ infectivity of *Toxoplasma gondii* in interferon- γ knockout mice. *J. Parasitol.* 87:447.
- 23 Luo, W., Aosai, F., Ueda, M. *et al.* 1997. Kinetics in parasite abundance in susceptible and resistant mice infected with an avirulent strain of *Toxoplasma gondii* by using quantitative competitive PCR. *J. Parasitol.* 83:1070.
- 24 Mun, H. S., Aosai, F., Norose, K. *et al.* 2003. TLR2 as an essential molecule for protective immunity against *Toxoplasma gondii* infection. *Int. Immunol.* 15:1081.
- 25 Piao, L. X., Aosai, F., Chen, M. *et al.* 2004. A quantitative assay method of *Toxoplasma gondii* HSP70 mRNA by quantitative competitive-reverse transcriptase-PCR. *Parasitol. Int.* 53:49.
- 26 He, N., Aosai, F., Mun, H. S., Sekiya, S. and Yano, A. 1997. Cytokine production assayed by RT-PCR in pregnant mice infected by *Toxoplasma gondii* as a model of congenital toxoplasmosis. *Jpn. J. Trop. Med. Hyg.* 25:59.
- 27 Svetic, A., Finkelman, F. D., Jian, Y. C. *et al.* 1991. Cytokine gene expression after *in vivo* primary immunization with goat antibody to mouse IgD antibody. *J. Immunol.* 147:2391.
- 28 Hikawa, N., Ishikawa, Y. and Takenaka, T. 2004. Interleukin-12 p40-homodimer production in sensory dorsal root ganglion neurons. *Neuroscience* 129:75.
- 29 Overbergh, L., Decallonne, B., Branisteanu, D. D. *et al.* 2003. Acute shock induced by antigen vaccination in NOD mice. *Diabetes* 52:335.
- 30 Frank, D. M., Margaret, H. and Don, R. M. 1949. Platelets as foci in the coagulation of blood. *Am. J. Physiol. Legacy Content* 158:84.
- 31 Belal, U. S., Norose, K., Aosai, F. *et al.* 2004. Evaluation of the effects of sulfamethoxazole on *Toxoplasma gondii* loads and stage conversion in IFN- γ knockout mice using QC-PCR. *Microbiol. Immunol.* 48:185.
- 32 Norose, K., Aosai, F., Mun, H. S. and Yano, A. 2006. Effects of sulfamethoxazole on murine ocular toxoplasmosis in interferon- γ knockout mice. *Invest. Ophthalmol. Vis. Sci.* 47:265.
- 33 Suzuki, Y., Kang, H., Parmley, S., Lim, S. and Park, D. 2000. Induction of tumor necrosis factor- α and inducible nitric oxide synthase fails to prevent toxoplasmic encephalitis in the absence of interferon- γ in genetically resistant BALB/c mice. *Microbes Infect.* 2:455.
- 34 Kudo, M., Aosai, F., Mun, H. S. *et al.* 2004. The role of IFN- γ and Toll-like receptors in nephropathy induced by *Toxoplasma gondii* infection. *Microbiol. Immunol.* 48:617.
- 35 Vuitton, D. A. Echinococcosis and allergy. 2004. *Clin. Rev. Allergy Immunol.* 26:93.
- 36 Baeza, M. L., Conejero, L., Higaki, Y. *et al.* 2005. *Anisakis simplex* allergy: a murine model of anaphylaxis induced by parasitic proteins displays a mixed Th1/Th2 pattern. *Clin. Exp. Immunol.* 142:433.
- 37 Sa-Nunes, A., Bolzoni, R. M., Medeiros, A. I. *et al.* 2005. Ultrasonically nebulized distilled water prevents exogenous histamine hyperreactivity in *Toxocara canis*-infected mice. *Inflamm. Res.* 54:243.
- 38 Prescott, S. M., Zimmerman, G. A., Stafforini, D. M. and McIntyre, T. M. 2000. Platelet-activating factor and related lipid mediators. *Annu. Rev. Biochem.* 69:419.
- 39 Snyder, F. 1995. Platelet-activating factor and its analogs: metabolic pathways and related intracellular processes. *Biochim. Biophys. Acta* 1254:231.
- 40 Snyder, F. 1995. Platelet-activating factor: the biosynthetic and catabolic enzymes. *Biochem. J.* 305:689.
- 41 Stafforini, D. M., McIntyre, T. M., Zimmerman, G. A. and Prescott, S. M. 1997. Platelet-activating factor acetylhydrolases. *J. Biol. Chem.* 272:17895.
- 42 Shindou, H., Ishii, S., Yamamoto, M., Takeda, K., Akira, S. and Shimizu, T. 2005. Priming effect of lipopolysaccharide on acetyl-coenzyme A: lyso-platelet-activating factor acetyltransferase is MyD88 and TRIF independent. *J. Immunol.* 175:1177.
- 43 Shindou, H., Ishii, S., Uozumi, N. and Shimizu, T. 2000. Roles of cytosolic phospholipase A2 and platelet-activating factor receptor in the Ca-induced biosynthesis of PAF. *Biochem. Biophys. Res. Commun.* 271:812.
- 44 Lee, T., Lenihan, D. J., Malone, B., Roddy, L. L. and Wasserman, S. I. 1984. Increased biosynthesis of platelet-activating factor in activated human eosinophils. *J. Biol. Chem.* 259:5526.
- 45 Camussi, G., Biancone, L., Iorio, E. L. *et al.* 1992. Porins and lipopolysaccharide stimulate platelet activating factor synthesis by human mesangial cells. *Kidney Int.* 42:1309.
- 46 Svetlov, S. I., Liu, H., Chao, W. and Olson, M. S. 1997. Regulation of platelet-activating factor (PAF) biosynthesis via coenzyme A-independent transacylase in the macrophage cell line IC-21 stimulated with lipopoly- saccharide. *Biochim. Biophys. Acta* 1346:120.
- 47 Han, S. H., Kim, J. H., Seo, H. S. *et al.* 2006. Lipoteichoic acid-induced nitric oxide production depends on the activation of platelet-activating factor receptor and Jak2. *J. Immunol.* 176:573.
- 48 Mun, H. S., Aosai, F., Norose, K. *et al.* 2005. Toll-like receptor 4 mediates tolerance in macrophages stimulated with *Toxoplasma gondii*-derived heat shock protein 70. *Infect. Immun.* 73:4634.
- 49 Geffner, J. R., Schattner, M. A., Lazzari, M. A. and Isturiz, M. A. 1991. Interferon- γ enhances PAF-acether production by stimulated human poly-morphonuclear leukocytes. *Scand. J. Immunol.* 33:575.
- 50 Car, B. D., Eng, V. M., Schnyder, B. *et al.* 1994. Interferon- γ receptor deficient mice are resistant to endotoxic shock. *J. Exp. Med.* 179:1437.
- 51 Ouellet, S., Muller, E. and Rola-Pleszczynski, M. 1994. IFN- γ up-regulates platelet-activating factor receptor gene expression in human monocytes. *J. Immunol.* 152:5092.

Fig S1

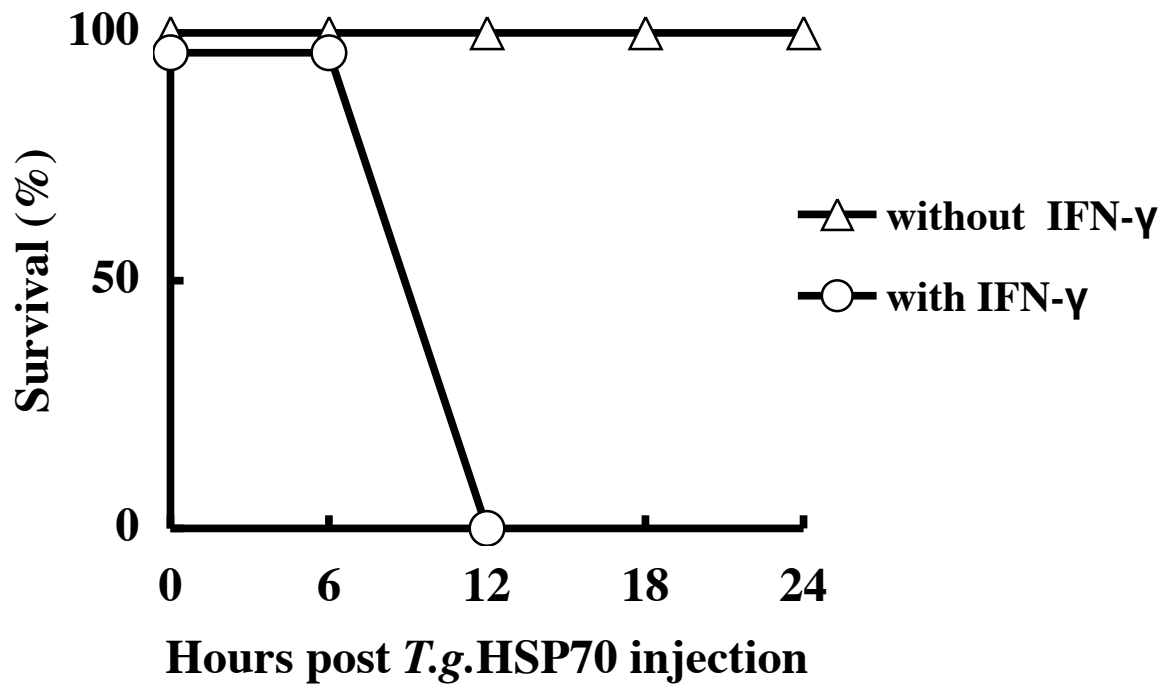


Fig S1. *T.g.HSP70*-induced anaphylactic reaction in rIFN- γ -supplemented WT BALB/c mice. Uninfected WT BALB/c mice were i.p. injected with 10 μ g of rIFN- γ in 0.2ml saline daily from day 0 to day 4. Then 100 μ g of *T.g.HSP70* was i.p. injected into the mice. Mice without rIFN- γ treatment were used as controls. Survival of rIFN- γ treated (circles) and untreated (triangles) mice was checked every hour after injection of *T.g.HSP70*. Three to four mice were used in each experimental group. Data are representative of three separate experiments.

Fig S2

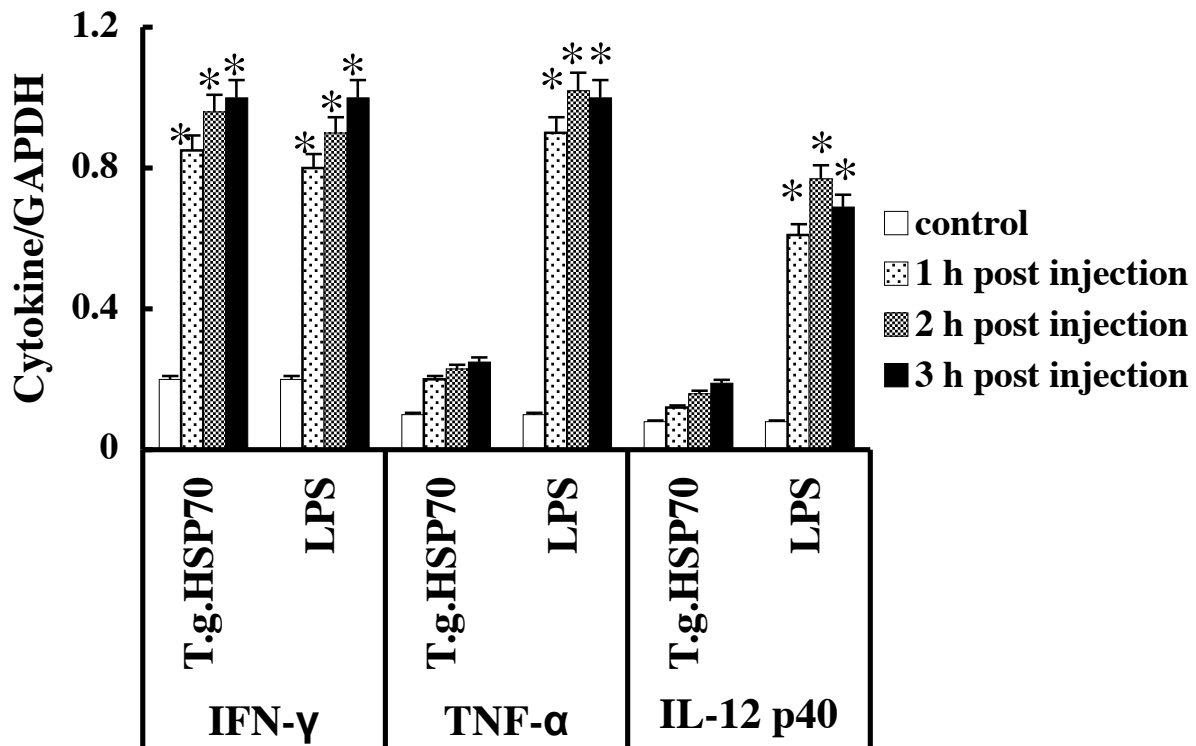


Fig S2. Cytokine expression in T.g.HSP70- or LPS- stimulated splenocytes.

Splenocytes from *T. gondii*-infected WT BALB/c mice were collected 1-week P.I. Then, splenocytes were treated with 10 μ g/ml of T.g.HSP70 or 10 μ g/ml of LPS for 3 hours. Expression of IFN- γ , TNF- α , and IL-12 p40 in T.g.HSP70- or LPS-treated splenocytes was analyzed by RT-PCR at 1 hour (light grey bar), 2 hours (dark grey bar), or 3 hours (black bar) post treatment. Splenocytes without treatment (white bar) was used as a control. The results are expressed as the ratio between optical density (OD) of the RT-PCR product of each cytokine and that of GAPDH. Three to four mice were used in each experimental group. Data are representative of three separate experiments. * p < 0.001 compared with the data of control.