Toll-Like Receptor 4 Mediates Tolerance in Macrophages Stimulated with Toxoplasma gondii-Derived Heat Shock Protein 70

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Peritoneal macrophages (PMs) from toll-like receptor 4 (TLR4)-deficient and wild-type (WT) mice were responsive to recombinant Toxoplasma gondii-derived heat shock protein 70 (rTgHSP70) and natural TgHSP70 (nTgHSP70) in NO release, but those from TLR2-, myeloid differentiation factor 88 (MyD88)-, and interleukin-1R-associated kinase 4 (IRAK4)-deficient mice were not. Polymyxin B did not inhibit PM activation by TgHSP70 and nTgHSP70 from WT and TLR4-deficient mice, while it inhibited PM activation by lipopolysaccharide. Pretreatment of PMs from WT but not from TLR4-deficient mice with rTgHSP70 resulted in suppression of NO release on restimulation with rTgHSP70. Similarly, pretreatment of PMs from WT but not nTgHSP70 from WT resulted in suppression of NO release on restimulation with nTgHSP70. Polymyxin B did not inhibit rTgHSP70- and nTgHSP70-induced tolerance of PMs from TLR4-deficient mice. Furthermore, PMs from WT mice increased suppressor of cytokine-signaling-1 (SOCS-1) expression after restimulation with rTgHSP70, while those from TLR4-deficient mice did not. Phosphorylation of JNK and IκBα occurred in rTgHSP70-induced tolerance of PMs from TLR4-deficient mice, but not in that from WT mice. These data indicated that TgHSP70 signaling mechanisms were mediated by TLR2, MyD88, and IRAK4, but not by TLR4. On the other hand, signaling of TgHSP70-induced tolerance was mediated by TLR4, and the expression of SOCS-1 suppressed the TLR2 signaling pathway.

Members of the heat shock protein (HSP) family have been shown to have important functions as (i) intracellular detergents for aggregated and denatured molecules formed as a result of exposure of cells to physical stressors and (ii) molecular chaperones in peptide and protein transport between cell organelles (13, 34, 42, 43). Of the HSP family members, HSP70 has been shown to be a major immunodominant antigen in bacterial and parasite infections, as well as the preferred target of the host from developing a shock syndrome caused by hyperinflammation (10, 45). This mechanism may protect the host from the deleterious effects of inflammation by endotoxin tolerance (10, 45).

TLRs play a critical role in the innate immune responses in mammals (15, 37). TLR4 and TLR2 are essential for recognition of lipopolysaccharide (LPS) and lipoteichoic acids, respectively (15, 37). The current understanding of TLR2- and TLR4-associated signaling complexes is based on the signal transduction cascade shared by interleukin-1 receptor (IL-1R) and other TLRs where, upon specific ligand binding, the receptor associates with the intracellular adaptor protein MyD88, which recruits IL-1R-associated kinases (IRAKs) to the receptor complex (20, 44). Among these IRAK proteins, IRAK4 appears to be critical for the transduction of IL-1R/TLR signals (35, 36).

Prior exposure to LPS induces a transient state of cellular hyporesponsiveness to subsequent stimulation with LPS known as endotoxin tolerance (10, 45). This mechanism may protect the host from developing a shock syndrome caused by hyperactivation of monocytes and macrophages with persistent bact-
teria and LPS. Animals pretreated with LPS showed reduced febrile responses and mortality rates after a second challenge with LPS. Some articles have reported that suppressor of cytokine-signaling 1 (SOCS-1) is a negative regulator of LPS-induced macrophage activation to protect the host from harmful overresponses to LPS (18, 28). On the other hand, it has recently been postulated that suppressed IL-12 production by monocytes and dendritic cells associated with endotoxin tolerance may result in an inability to respond appropriately to secondary infections in survivors of sepsis (16).

This report for the first time demonstrates that TgHSP70-induced NO release is mediated via the TLR2/MyD88 signal transduction pathway and that prior exposure to TgHSP70 induces a hyporesponse to subsequent stimulation with TgHSP70 by expression of SOCS-1 via TLR4.

MATERIALS AND METHODS

Experimental mice. TLR2-, TLR4-, MyD88-, and IRAK4-deficient mice (15, 35–37) with a C57BL/6 background and WT C57BL/6 mice (SLC, Hamamatsu, Japan) were used at 8 to 12 weeks of age.

Preparation of recombinant protein. Preparation and expression of recombinant TgHSP70 (rTgHSP70), rTgHSP30, and rSAG1 were previously described (26).

Preparation of rTgHSP70 protein. Natural TgHSP70 (nTgHSP70) was purified from T. gondii strain RH by affinity chromatography on CNBr-activated Sepharose 4B-bound anti-TgHSP70 monoclonal antibody (TgNCR C2) (5, 6). The purified nTgHSP70 C2 was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Tachyzoites (10⁵) 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, the nTgHSP70 bound to TgNCR C2 was eluted by using 0.1 M glycine-HCl containing 0.5 M NaCl buffer, pH 2.5. The eluted nTgHSP70 was dialyzed against phosphate-buffered saline and lyophilized.

Preparation of PMs. Peritoneal exudate cells (PECs) from TLR2-, TLR4-, MyD88-, and IRAK4-deficient and WT mice were harvested and added to 96-well plates at a density of 5 × 10⁵ cells/100 μl/well and incubated for 2 h at 37°C in RPMI 1640 containing 10% fetal calf serum. Nonadherent cells were then removed by gentle washing (3, 17). Adherent cells were collected with a cell lifter. The adherent cells were stained with fluorescein isothiocyanate-conjugated anti-mouse F4/80 antibody and then analyzed by FACScan (Becton Dickinson, Tokyo, Japan). Over 97% of these adherent cells were macrophages.

Determination of NO. Serial dilutions of rTgHSP70, rTgHSP30, or rSAG1 (2 μg/ml), nTgHSP70 (2 μg/ml), or LPS (30 ng/ml) (Escherichia coli O55:B5; Sigma, St. Louis, MO) were simultaneously added to the culture of 2 × 10⁵ PMs and were present for the entire cultivation time of 24 h. Iron in PMs was measured by using 2 μg/ml of rTgHSP70, rTgHSP30, or rSAG1, NO₂⁻ was measured 24 h posttreatment by Griess reaction. A P value of <0.05 was taken as significant. Three to four mice comprised each group in each experiment, which was performed in triplicate. The error bars indicate standard deviations.

RESULTS

TgHSP70-MEDIATED TOLERANCE VIA TLR4

FIG. 1. TgHSP70 induces NO release. (A) PMs from WT mice were harvested. Serial dilutions of rTgHSP70, rTgHSP30, or rSAG1 were added to the culture of PMs and were present for the entire cultivation time of 24 h. (B) PMs from TLR4-, TLR2-, MyD88-, and IRAK4-deficient and WT mice were stimulated for 24 h in the presence of 2 μg/ml of rTgHSP70, rTgHSP30, or rSAG1. NO₂⁻ was measured 24 h posttreatment by Griess reaction. A P value of <0.05 was taken as significant. Three to four mice comprised each group in each experiment, which was performed in triplicate. The error bars indicate standard deviations.
FIG. 2. TLR2 regulates TgHSP70-induced NO release. (A and B) PMs from TLR4-deficient (closed circles), TLR2-deficient (closed triangles), MyD88-deficient (closed diamonds), and IRAK4-deficient (open squares) and WT (closed squares) mice were harvested. Serial dilutions of rTgHSP70 (A) or LPS (B) were simultaneously added to the culture of PMs and were present for the entire cultivation time of 24 h. NO2/HO2 was measured 24 h posttreatment by Griess reaction. (C and D) PMs from TLR4-, TLR2-, and MyD88-deficient and WT mice were stimulated for 8 h in the presence of rTgHSP70 (2 μg/ml; C) or LPS (1 ng/ml; D), and iNOS mRNA expression was determined by RT-PCR. The data are representative of three independent experiments. (E) Ten micrograms per milliliter of polymyxin B (PB) was added to the culture with rTgHSP70 (2 μg/ml) or LPS (1 ng/ml) and was present for the entire cultivation time of 24 h posttreatment by Griess reaction. (F) Ten micrograms per milliliter of polymyxin B (PB) was added to the culture with rTgHSP70 (2 μg/ml) or LPS (1 ng/ml) and was present for the entire cultivation time of 24 h posttreatment by Griess reaction. (G) Ten micrograms per milliliter of polymyxin B (PB) was added to the culture with rTgHSP70 (2 μg/ml) or LPS (1 ng/ml) and was present for the entire cultivation time of 24 h posttreatment by Griess reaction. (H) SDS-PAGE analysis of rTgHSP70 and nTgHSP70. Recombinant TgHSP70 (lane 1) and nTgHSP70 (lane 2) were run on a 10% SDS-PAGE with a molecular size marker (M; kDa). P < 0.05 was taken as significant. Three to four mice comprised each group in each experiment, which was performed in triplicate. The error bars indicate standard deviations.
FIG. 3. TLR4 induces TgHSP70-induced NO release tolerance. PMs from TLR4-, TLR2-, and MyD88-deficient and WT mice were stimulated for 24 h with rTgHSP70 (2 μg/ml) (A), LPS (30 ng/ml) (A), or nTgHSP70 (2 μg/ml) (B). Ten micrograms per milliliter of polymyxin B was added to the culture with rTgHSP70, LPS, or nTgHSP70 and was present for the entire cultivation time of 24 h posttreatment. Then, the cells were washed twice and restimulated for an additional 24 h with the same concentration of rTgHSP70 (A), LPS (A), or nTgHSP70 (B). Polymyxin B was added to the culture with rTgHSP70, LPS, or nTgHSP70 and was present for the entire cultivation time of 24 h posttreatment by Griess reaction. A P value of <0.05 was taken as significant. Three to four mice comprised each group in each experiment, which was performed in triplicate.
and rSAG1. rTgHSP70 induced the release of NO, and the expression of iNOS was dependent on TLR4 and MyD88, but not on TLR4 (Fig. 2A and C). On the other hand, PMs from TLR2-deficient and WT mice, but not from TLR4- and MyD88-deficient mice, responded to LPS challenge with NO production and INOS mRNA expression (Fig. 2B and D). To determine whether the contaminating LPS in rTgHSP70 was responsible for its NO production, we used polymyxin B and TgNCR C2 in cultures of PMs stimulated with rTgHSP70, rTgHSP70, and LPS (Fig. 2E to G). Polymyxin B did not inhibit rTgHSP70 activation of PMs, in contrast to its effect on LPS, and TgNCR C2 inhibited rTgHSP70 activation of PMs.

**TLR4-dependent NO tolerance induced by TgHSP70.** PMs from WT mice released NO by primary stimulation with LPS. Then, the cells were washed and restimulated with the same concentration of LPS, and a more than 90% reduction in the level of NO release was observed (Fig. 3A and Table 1). Similarly, PMs from WT mice released NO by primary stimulation with rTgHSP70, but the level of NO release was reduced by more than 90% upon secondary stimulation with the same concentration of rTgHSP70. Thus, like LPS tolerance, a secondary rTgHSP70 challenge induced tolerance in PMs from WT mice. On the other hand, when PMs from TLR4-deficient mice were pretreated with rTgHSP70, no reduction was observed in the level of NO release upon secondary stimulation with rTgHSP70. Furthermore, PMs from WT mice released NO by primary stimulation with rTgHSP70, but the level of NO release was reduced by more than 90% upon secondary stimulation with the same concentration of rTgHSP70 (Fig. 3B). On the other hand, when PMs from TLR4-deficient mice were pretreated with rTgHSP70, no reduction was observed in the level of NO release upon secondary stimulation with rTgHSP70. Thus, TLR4-mediated signaling activation by secondary TgHSP70 challenge induces tolerance of NO release in mouse PMs by primary TgHSP70 challenge through TLR2.

**Cross-reactive NO tolerance by TgHSP70 and LPS.** We next analyzed whether rTgHSP70 treatment induced cross-reactive tolerance to LPS and vice versa. rTgHSP70 pretreatment resulted in a significant decrease in NO release in response to LPS, and LPS pretreatment resulted in a significant decrease in NO release in response to rTgHSP70 in PMs from WT mice (Fig. 3A).

NO release from PMs of TLR2-deficient mice stimulated by rTgHSP70/LPS (primary stimulation/secondary stimulation) was induced only by the secondary stimulation of LPS via TLR4. Primary stimulation by rTgHSP70 had no effect on NO release by TLR2-deficient mice. NO release from PMs of TLR4-deficient mice stimulated by LPS/rTgHSP70 was induced only by the secondary stimulation of rTgHSP70 via TLR2. Primary stimulation by LPS did not affect NO release by TLR4-deficient mice. As PMs were washed after primary stimulation, NO release was not observed from PMs either from TLR2-deficient mice participating in LPS/rTgHSP70 stimulation or from TLR4-deficient mice receiving rTgHSP70/LPS stimulation. Polymyxin B did not affect rTgHSP70 tolerance of PMs, in contrast to its effect on LPS.

These results indicate that rTgHSP70 treatment cross-reactively induced tolerance to LPS-induced NO production, and vice versa.

**TgHSP70-stimulated TNF-α expression of PMs via TLR2.** PMs from WT mice responded to produce TNF-α expression by primary rTgHSP70 challenge (Fig. 4). PMs from TLR4-deficient and WT mice, but not from TLR2- and MyD88-deficient mice, responded to produce TNF-α expression by primary rTgHSP70 challenge. To determine whether the contaminating LPS in rTgHSP70 was responsible for its TNF-α expression, polymyxin B was added to cultures of PMs stimulated with rTgHSP70. Polymyxin B did not inhibit the rTgHSP70 activation of PMs.
TLR4-dependent TNF-α expression tolerance induced by TgHSP70. PMs from WT mice expressed TNF-α by primary stimulation with rTgHSP70, but the level of TNF-α expression decreased upon secondary stimulation with the same concentration of rTgHSP70 (Fig. 4B). On the other hand, when PMs from TLR4-deficient mice were pretreated with rTgHSP70, no reduction was observed in the level of TNF-α expression upon secondary stimulation with rTgHSP70. Thus, TLR4-mediated signaling activation by secondary TgHSP70 challenge induces the tolerance of TNF-α expression of PMs by primary TgHSP70 challenge through TLR2.

To determine whether the contaminating LPS in rTgHSP70 was responsible for its TNF-α expression tolerance, polymyxin B was added to cultures of PMs stimulated with rTgHSP70. Polymyxin B did not inhibit the rTgHSP70 activation of PMs.

SOCS-1-mediated tolerance by TgHSP70 through TLR4.

Next, the role of SOCS-1 in TgHSP70-mediated signaling activation for NO release was examined in the PMs from mice. A
high level of SOCS-1 expression was detected in the PMs from TLR2- and MyD88-deficient and WT mice after rTgHSP70/ rTgHSP70, rTgHSP70/LPS, LPS/rTgHSP70, and LPS/LPS stimulation (Fig. 5). On the other hand, SOCS-1 was not expressed at a high level in PMs from TLR4-deficient mice after rTgHSP70/rTgHSP70, rTgHSP70/LPS, LPS/rTgHSP70, and LPS/LPS stimulation, suggesting that a TLR4-dependent signal was required for SOCS-1 expression and TgHSP70-induced tolerance. Furthermore, a high level of IFN-β expression was detected in TgHSP70-pretreated PMs from TLR2- and MyD88-deficient and WT mice after rTgHSP70/rTgHSP70, rTgHSP70/LPS, LPS/rTgHSP70, and LPS/LPS stimulation, whereas IFN-β was not expressed at a high level in PMs from TLR4-deficient mice. Tolerance was shown to be maintained for up to 24 h (data not shown). Thus, these data indicated that SOCS-1 and IFN-β induced TgHSP70 tolerance via TLR4.

**Lack of phosphorylation of JNK and I-κBα in rTgHSP70-induced tolerance.** To examine whether degradation, as well as phosphorylation, of JNK and I-κBα occurred in rTgHSP70-induced tolerance, we performed immunoblotting analysis of JNK and I-κBα in PMs after primary and secondary stimulation with rTgHSP70 (Fig. 6). A high level of phosphorylation of JNK and I-κBα was detected in the PMs from TLR4-deficient and WT mice after primary stimulation with rTgHSP70. In contrast, the levels of phosphorylation of JNK and I-κBα decreased in the TgHSP70-pretreated PMs from WT mice after primary and secondary stimulation with rTgHSP70. On the other hand, when PMs from TLR4-deficient mice were pretreated with rTgHSP70, no reductions in the levels of phosphorylation of JNK and I-κBα were observed upon secondary stimulation with rTgHSP70. These results suggest that the absence of phosphorylation of JNK and I-κBα contributes to the downregulated NO release and TNF-α mRNA expression in rTgHSP70-tolerant cells.

**DISCUSSION**

In this study, we showed that TgHSP70-induced NO release is dependent on TLR2, MyD88, and IRAK4, but not on TLR4 (Fig. 7). Furthermore, TLR4-mediated signaling activation by TgHSP70 challenge induced the tolerance of NO release in mouse PMs by TLR2-mediated TgHSP70 challenge. On the other hand, rhHSP70-1, a highly purified rhHSP70, did not induce TNF-α release from murine macrophages, and the TNF-α-inducing activity of rhHSP70-2, a less purified rhHSP70, was entirely due to the contaminating LPS (11, 40). In the present study, however, polymyxin B did not inhibit rTgHSP70 and rTgHSP70 activation of PMs. TgHSP70-induced NO release was dependent on TLR2, whereas LPS-induced NO release was dependent on TLR4.

There was an impression that greater NO release occurred in WT mice than in TLR4-deficient mice when stimulated with TgHSP70 and also that greater NO release occurred in WT mice than in TLR2-deficient mice when stimulated with LPS. The precise mechanisms to cause such lower levels of NO release by TLR4- and TLR2-deficient mice compared with WT mice are not known.

Recent evidence supports the hypothesis that HSP activates the innate immune system via TLRs, the sensors of innate immunity. TLRs are transmembrane proteins with an extracellular domain consisting of leucine-rich repeats involved in recognition of microbial components. Asea et al. addressed the signal transduction cascade stimulated by exogenous HSP70, revealing that HSP70-induced NF-κB promoter activity was MyD88 dependent and was transduced via both TLR2 and TLR4 (2). Also, Cabanas et al. reported that human HSP70 acts as an endogenous stimulus for the Toll/IL-1 receptor signal pathway that engages TLR2 and TLR4 (41). However, Dybdahl et al. reported that both TLR-4 and CD14 are involved in the HSP70-mediated proinflammatory response (8). Triantafilou et al. presented evidence of a CD14-independent LPS receptor cluster that includes the constitutive forms of HSP70 and HSP90, among others (39).

Aosai et al. reported that TgHSP70 induced spleen B-cell proliferation in TLR2- and MyD88-deficient mice, but not in TLR4-deficient mice, indicating the involvement of TLR4 in TgHSP70-induced proliferative responses of B cells (1). These data and the present study indicated that TgHSP70-induced tolerance in NO production of PMs by secondary challenge, as well as TgHSP70-induced proliferation of B cells, was mediated via TLR4, but TgHSP70-induced NO production in PMs by primary challenge was mediated via TLR2 and MyD88.

Previous studies have reported that production of NO was induced by LPS even in LPS-tolerant cells (9, 22). That result seems contradictory to our findings and those of others (29, 31); however, in those studies, macrophages had been pretreated with a low concentration of LPS (<20 ng/ml). Several articles had previously reported that induction of LPS tolerance was not severe when stimulated with a low concentration of LPS (29, 31). In the present study, we used a 30-ng/ml concentration of LPS for LPS tolerance by PMs.

In our study, PMs from WT mice expressed SOCS-1 and IFN-β after stimulation with rTgHSP70, while those from TLR4-deficient mice did not. LPS has been demonstrated to induce the expression of IFN-β in macrophages (33).
induction of SOCS-1 mRNA by LPS requires autocrine/paracrine factors that include IFN-β (7). In this report, we suggested that primary TgHSP70 stimulation initiated the expression of SOCS-1, which could not inhibit NO production by primary TgHSP70 stimulation. SOCS-1 activated by primary TgHSP70 stimulation via TLR4 inhibits the NO synthesis signaling activated by secondary stimulation of TgHSP70 via TLR2. Kinjo et al. reported that SOCS-1 is a negative regulator of TLR signaling, suggesting that SOCS-1 might be involved in the induction of endotoxin tolerance (18). SOCS-1 overexpression suppressed LPS-induced NF-κB transcrip- tional activity in RAW cells, as well as in fibroblasts expressing both TLR4 and MD2 (18). Therefore, the requirement for SOCS-1 suggests that the mechanism for LPS signal suppression by SOCS-1 is different from that of the cytokine-dependent JAK/STAT pathway. SOCS-1 also inhibited NF-κB activation by IRAKs and TNF receptor-associated factor 6 (TRAF6) but not by I-κB kinase-β (IKK-β) when these molecules were overexpressed in fibroblasts, indicating that the inhibition by SOCS-1 occurs downstream of TRAF6 and upstream of IKK-β (18).

Overall, these results indicated that the TgHSP70-induced NO release was mediated via the TLR2/MyD88 signal transduction pathway and that prior exposure to TgHSP70 induced a tolerance of NO production for subsequent stimulation with TgHSP70 via TLR4.

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FIG. 7. Mechanism of TgHSP70 tolerance in P388D1 cells. TgHSP70 stimulation leads to TLR2 activation and the pathway of TLR2-MyD88-IRAK4-TRAF6-IKK-mediated phosphorylation of I-κBα when these molecules were overexpressed in fibroblasts expressing both TLR4 and MD2 (18). Therefore, the requirement for SOCS-1 suggests that the mechanism for LPS signal suppression by SOCS-1 is different from that of the cytokine-dependent JAK/STAT pathway. SOCS-1 also inhibited NF-κB activation by IRAKs and TNF receptor-associated factor 6 (TRAF6) but not by I-κB kinase-β (IKK-β) when these molecules were overexpressed in fibroblasts, indicating that the inhibition by SOCS-1 occurs downstream of TRAF6 and upstream of IKK-β (18). Overall, these results indicated that the TgHSP70-induced NO release was mediated via the TLR2/MyD88 signal transduction pathway and that prior exposure to TgHSP70 induced a tolerance of NO production for subsequent stimulation with TgHSP70 via TLR4.
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