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Microbial heat shock protein 70 stimulatory properties have different TLR requirements

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Abstract

Members of the HSP70 family have acquired special significance in immunity. Among other receptors, toll like receptor (TLR)-2 and TLR-4 have been suggested to be involved in HSP70-mediated signalling. We have previously shown that recombinant HSP70 from *Trypanosoma cruzi* and from *Plasmodium falciparum* function as adjuvants. In the present study, we have extended the study with other microbial HSPs (mHSPs) and considered of interest to assess the influence of TLR-2 and TLR-4 in mHSP-promoted responses. To test this, we evaluated the adjuvant effect of various mHSP molecules in TLR-$2^{-/-}$, TLR-$4^{-/-}$ and MyD$88^{-/-}$ mice. We show that all the mHSPs tested are strong adjuvants and induced IL-12 production by bone marrow macrophages. However, even within the same family, mHSPs induced different types of immune responses. Furthermore, the mHSPs tested, possess different requirements for signaling through TLRs. Interestingly, Tc70 was found to induce *in vivo* and *in vitro* responses in both TLR-$2^{-/-}$ and TLR-$4^{-/-}$ mice. Possible implications of our findings are taken up in the discussion section.

Keywords: Heat shock proteins; Toll like receptors; Adjuvants

1. Introduction

Heat shock proteins (HSPs) are a large family of proteins with different molecular weights and different intracellular localizations. They are molecular chaperones, undertaking crucial functions in the maintenance of cell homeostasis, which may be the reason for being conserved during evolution [1,2]. Members of the HSP70 family, mainly the microbial HSP70s (mHSP70) have acquired special significance in immunity since they have been shown to be potent activators of the innate immune system [3] and generate specific immune responses against tumours and various infectious agents [4–6]. Their immunostimulatory functions are based on two main features: (a) HSPs are effectively internalized into antigen presenting cells (APCs) by a receptor-mediated endocytosis [7] and once internalized, can traffic into different cellular compartments where chaperoned peptides are released, processed and made available for assembly to MHC molecules [7–10]; (b) HSPs can induce phenotypic and functional maturation of APCs [11,12].

Many studies have described the ability of mHSP70s to enhance the immunogenicity of associated antigens. They serve as a carrier of antigens and effectively induce antigen specific B cells as well as T cell responses without requiring an adjuvant [13–15]. The mechanisms through which HSP70 molecules exert their adjuvant effect have been delineated in

Abbreviations: HSP, heat shock protein; BMM, bone marrow macrophage; IRAK, IL-1 receptor associated kinase; MyD88, myeloid differentiation factor 88; TLR, toll like receptor

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some detail. The interaction of HSP70 with the receptors on APCs lies at the centre of this mechanistic understanding. During the last 3 years, a number of putative HSP70 receptors have been identified. CD91 and LOX-1 are specifically described as endocytic receptors for HSP70 [7,16]. Interaction of HSP70 with CD91 [7] mediates endocytosis that results in cross-presentation of HSP70 associated peptides to both $CD8^+$ and $CD4^+$ T lymphocytes [9,10,17]. HSP70 has also been shown to bind and signal through CD40, triggering the production of CC chemokines and IL-12 [18–20]. It has been reported that recombinant HSP70 could signal through toll like receptor-2 (TLR-2) and TLR-4 [21,22] and was shown to be mediated via the MyD88/NF-κB signal transduction pathway.

TLRs are critical for receiving signals from molecular patterns associated with microbial pathogens to initiate innate immune responses [23,24]. Since bacterial lipopolysaccharide (LPS) is a well-documented ligand for TLR-4 and some of the functional activities of HSPs and LPS are similar, it has been a concern that the stimulatory effects mediated by the HSPs are fundamentally due to the presence of LPS. In fact, studies from Triantafilou et al. have demonstrated a physical association between HSPs and LPS [25]. Following LPS stimulation, HSP70 and HSP90 form multimeric receptor complexes with TLR-4 within lipid microdomains and are implicated as mediators of innate immunity [25]. Furthermore, the same authors demonstrated that HSP70 and HSP90 seemed to be involved in TLR-4/LPS trafficking and targeting to the Golgi apparatus [26].

However, there are evidences in favour of and against the dependency of HSPs on LPS for activation [27]. It is now established that LPS alone cannot mediate the same type of stimulation provided by HSPs, and this is also the case for HSPs that may require the presence of LPS for optimal effects [25,28,29]. Furthermore, in HSP60, macrophage and LPS binding sequences are localized in different places of the molecule [28,30]. This and other works have helped in introducing the notion that HSPs could possess a more important function than it was previously postulated. The growing concept is that HSPs may serve as receptors for LPS and that through TLR-4 would reinforce the initiation of response by other receptors on macrophages.

The involvement of TLR-2 and TLR-4 in HSP-mediated response has been mainly studied in relation to the induction of inflammatory cytokines and the knowledge is restricted to a few HSPs. In our previous studies, we have shown that recombinant HSP70 from *Trypanosoma cruzi* (Tc70) and from *Plasmodium falciparum* (Pf70C) function as adjuvants and greatly enhance the antibody response to ovalbumin (OVA) and other thymus dependent (TD) antigens when coadministered with them [31,32]. In the present study, we extend these studies to other HSP70s to understand the influence of TLR signaling in HSP70 mediated responses both *in vivo* (antibody production) and *in vitro* (IL-12 secretion). To test this, we evaluated the adjuvant effect of various HSP70 molecules in TLR2, TLR4, IRAK4 and MyD88 knockout mice. We show here that HSP70 from different sources function differently and that TLR4 is not always involved in HSP70 induced adjuvanticity.

2. Materials and methods

2.1. Recombinant protein immunogens

The expression and purification of the fusion protein GST-Pf70C has been described in detail before [31]. For the enzymatic cleavage of Pf70C from GST, specific protease factor Xa (Amersham Pharmacia Biotech, Uppsala, Sweden) was used. Factor Xa was removed with *p*-aminobenzamidine beads (Sigma–Aldrich Chemie, Steinheim, Germany). The protein purity was assessed by SDS-PAGE analysis and the gels were either stained with Coomassie Brilliant Blue or used in a Western blotting procedure.

Recombinant *Mycobacterium tuberculosis* HSP70 (MTB70) and its C-terminal part (MTB70C aa 359–610) were provided by LIONEX (Braunschweig, Germany). Recombinant *T. cruzi* HSP70 (Tc70) and *Leishmania brasiliensis* HSP70 (Lb70) were purified as described by Marañón et al. [33]. LPS was a gift from Prof. Tord Holme (Dept of Bacteriology, Karolinska Institute), zymosan A (Zym) and cholera toxin (CT) was purchased from Sigma and List Laboratories (List Biological Laboratories, Campbell, CA, USA), respectively. Monoclonal anti-CD40 antibodies were generated in our laboratory from the hybridoma cell line 1C10. Ovalbumin was obtained from Sigma. Mouse recombinant IL-12 was obtained from R&D system (Minneapolis, USA).

2.2. Immunization of mice

Female mice 6–8 weeks-old C57BL/6 (WT) were purchased from Taconic M & B (DK-8680 RY, Denmark) and kept in the animal facilities of Stockholm University. Breeding pairs from the TLR-$2^{-/-}$, TLR-$4^{-/-}$ and MyD$88^{-/-}$ mice were generated by S. Akira [34]. They had been backcrossed at least six times to C57BL/6 mice and were obtained from Microbiology and Tumour Biology Centre, Karolinska Institute (Sweden) upon permission of S. Akira (Osaka University, Japan) and the animals were kept at the facilities of Stockholm University. Immunization was done twice intra peritoneally (i.p.) with a 4 weeks interval with 50 μg of OVA per animal formulated with different adjuvants. The adjuvant doses per animal and immunization were: 0.5 mg of Zym; 50 μg of MTB70C and Pf70C, 25 μg for MTB70, Tc70 and anti-CD40, and 1 μg for CT and LPS. Animals were bled by retro-orbital plexus puncture 7 days after the last immunization. Sera were stored at −20 °C until assayed.

2.3. Generation of bone marrow-derived macrophages (BMMs)

Bone marrow cells were harvested from femur and tibias of 8–10 weeks-old mice (WT, TLR-$2^{-/-}$, TLR-$4^{-/-}$,

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MyD88$^{-/-}$). Bone marrow macrophages (BMMs) were isolated by flushing the marrow cavities of mouse femora and tibia with cold PBS and then gently mixing the cells by means of a 18-gauge needle to obtain a single-cell suspension. Cells were washed twice and resuspended in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Paisley, Scotland, UK) comprising 10% heat-inactivated fetal calf serum (FCS) (Irvine Scientific Co. Ltd.), 10 mM HEPES buffer, (GIBCO^{TM} Ltd.), 2 mM L-glutamine (GIBCO^{TM} Ltd.), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Corporation), 50 μM 2-mercaptoethanol. Bone marrow cells (1×10^6 cells ml^{-1}) were cultivated in 96-well cell culture plate (Costar, Corning Incorporated, Corning, NY, USA) for 7 days at 37 °C under 5% CO_2 in the presence of 20% conditioned medium derived from the cell line L929 as a source of macrophage colony stimulating factor (M-CSF). The culture medium was changed every 2 days.

2.4. IL-12 assay

On day 7, BMMs were washed three times with PBS and were stimulated with 1 μg/ml LPS, 10 μg/ml Zym, 0.1 mM MTB70, Tc70, Lb70, Pf70C, MTB70C for 72 h at 37 °C in the presence of 5% CO_2. Supernatants were harvested and assessed for cytokine release using murine IL-12 p70 enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Mabtech AB, Stockholm, Sweden).

2.5. Determination of serum antibody levels by ELISA

To determine OVA specific antibody responses, microtitre plates (Costar, Corning Incorporated) were coated with 5 μg/ml of OVA. Plates were incubated overnight (ON) at room temperature (RT) followed by incubation ON with serial dilutions of sera at RT. Sera were initially diluted to 1:100 to determine specific antibodies. Isotypes were determined by incubating the plates for 2 h at RT with alkaline phosphatase-conjugated goat immunoglobulin specific for mouse total IgG, IgG1 and IgG2a (Southern Biotechnology Inc., Birmingham, AL, USA). Colour was developed at RT with *p*-nitrophenyl phosphate disodium (Sigma Diagnostics, St. Louis, MO, USA), and the absorbance was measured at 405 nm at different time points.

2.6. Statistical analyses

Three mice were included in each experimental group and the results were expressed as mean ± S.D. of triplicate samples (sera) from individual mice from each group. Two-tailed

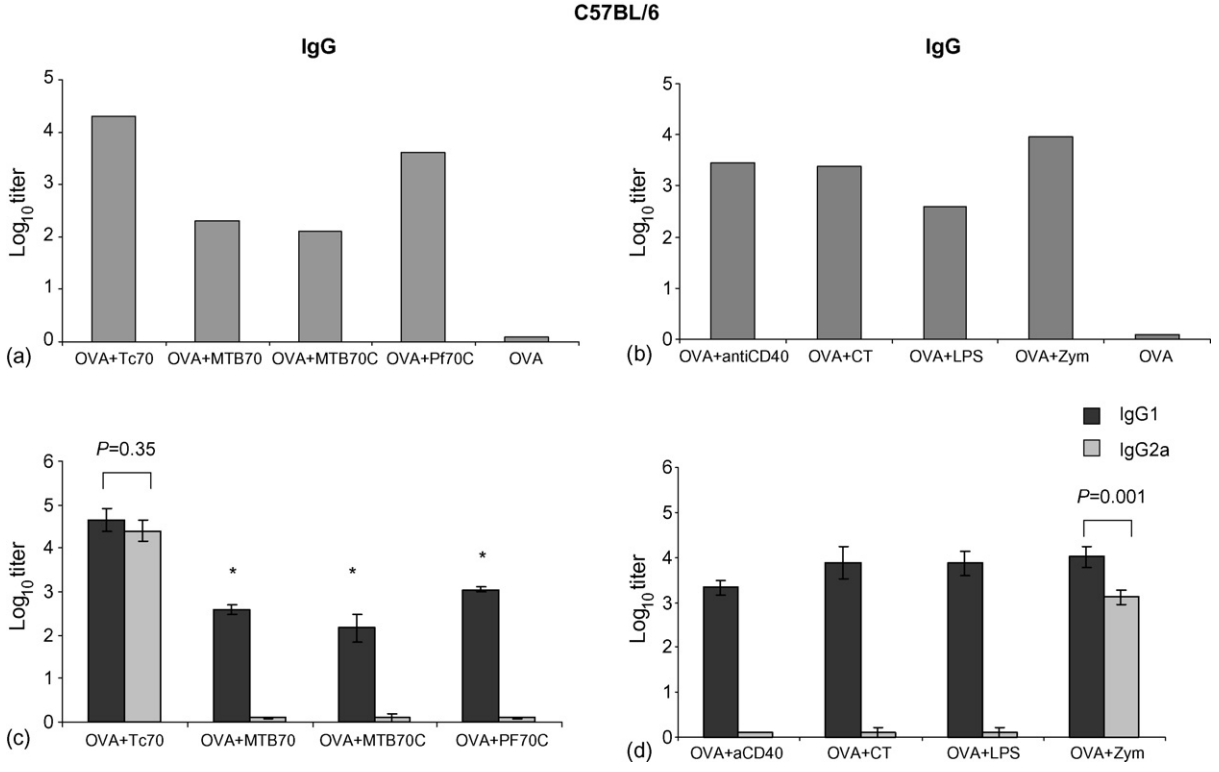

Fig. 1. Comparative analysis of antibody responses to OVA induced in C57BL/6 (wild type) mice. Mice were immunized i.p. twice in a 4 weeks interval with 50 μg of OVA per mouse formulated individually with (a, c) Tc70, MTB70, MTB70C or Pf70C and (b, d) anti-CD40, CT, LPS or Zym. ELISA using serial dilutions of the sera detected the presence of OVA-specific antibodies. Titers were expressed as the $\log_{10}$ of the reciprocal of the highest dilution giving OD = 1.0. Pooled sera of three mice were used for detection of IgG. For the assessment of IgG1 and IgG2a profile, analysis was done with sera from individual mouse and the results were presented as mean $\log_{10}$ titer ± S.D. of three mice. Regarding IgG1 and IgG2a isotypes, significant differences between OVA plus Tc70 groups with other HSP70 treated groups following a Student's *t*-test are depicted with asterisks, $^{*}p < 0.005$.

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Student's *t*-test was performed to identify significant differences between experimental groups. Responses in wild type group were considered as the standard and all the comparisons were done individually with the wild type group.

3. Results

3.1. Analysis of the adjuvant effect of HSP70 molecules in C57BL/6 mice

We have previously shown that both Tc70 and Pf70C are efficient adjuvants when coadministered with TD antigens [32]. The objective of the present study was to evaluate the adjuvant effect of other HSP70s. C57BL/6 mice were immunized twice i.p. in a 4 weeks interval with 50 μg of OVA administered either alone (negative control) or together with Tc70, Pf70C, MTB70 or MTB70C. We compared the antibody response induced in the presence of HSPs with the response induced in the presence of some common adjuvants such as CT, LPS, anti-CD40 and zymosan. The anti-OVA antibody titres were determined 7 days after the second immunization. Fig. 1a shows that both the entire HSP70s and their C-terminal parts promoted the generation of secondary IgG antibodies against OVA. This response was comparable with the response induced in the presence of CT, LPS, anti-CD40 or zymosan (Fig. 1b). Nearly undetectable amounts of IgG antibodies were produced in mice immunized with OVA alone. The IgG1 and IgG2a subclasses were also determined as an indication of Th2 and Th1 type of responses, respectively. As illustrated in Fig. 1c and 1d, only Tc70 and zymosan induced OVA specific IgG1 and IgG2a antibodies whereas MTB70, MTB70C, Pf70C, CT, LPS and anti-CD40 only stimulated the production of antibodies of the IgG1 isotype. Statistical analysis indicated that the IgG1 and IgG2a titres were not significantly different ($p = 0.35$) in the OVA plus Tc70 immunized group, while the OVA specific IgG1 was the predominant isotype ($p = 0.001$) in the OVA plus zymosan immunized group. Tc70 displayed the strongest adjuvant effect of all HSPs tested.

3.2. Dependency on TLR receptor signaling in the adjuvant effect promoted by HSP70s

Since both TLR-2 and TLR-4 have been suggested to be involved in HSP70 mediated signaling [20,21], we decided here to investigate whether TLR-2 and TLR-4 are involved in the adjuvant effect caused by HSP70 molecules. TLR-$2^{-/-}$ and TLR-$4^{-/-}$ mice were immunized twice with OVA, administered together with Tc70, Pf70C, MTB70 or MTB70C or given alone. As control adjuvants, we used LPS and zymosan, ligands for TLR-4 and TLR-2, respectively;

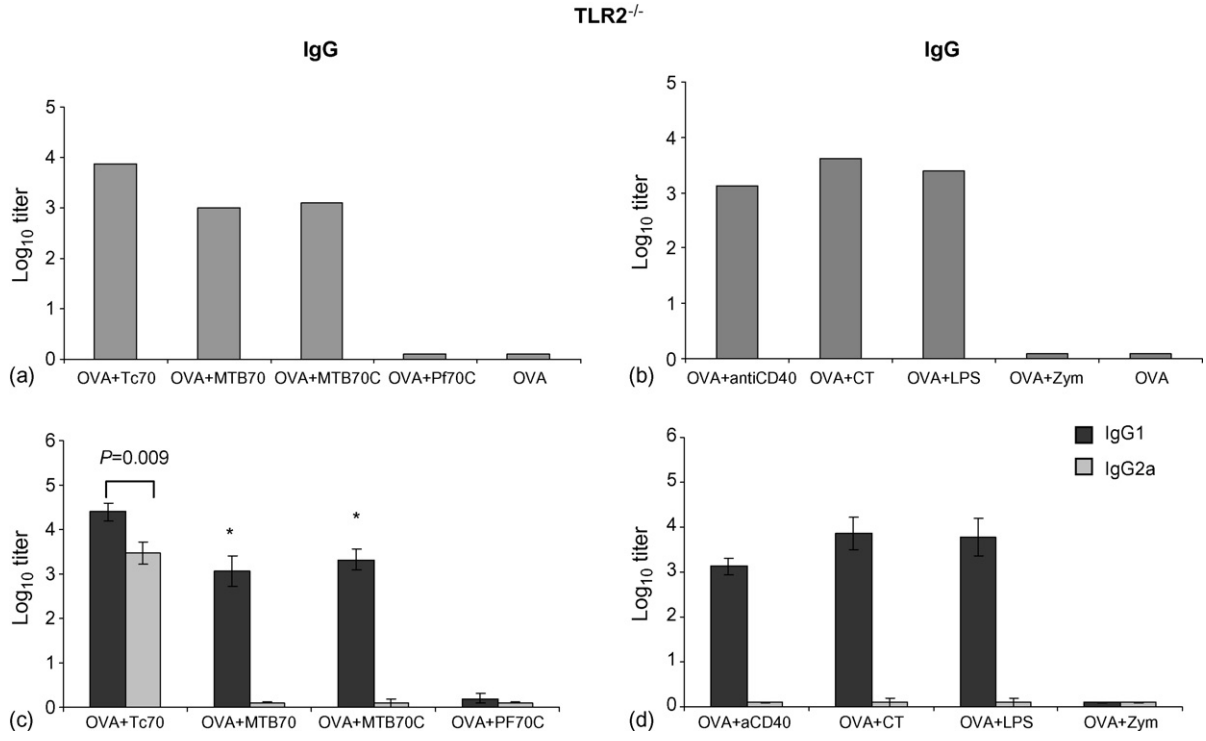

Fig. 2. Comparative analysis of antibody responses to OVA induced in TLR-$2^{-/-}$ mice. Mice were immunized i.p. twice in a 4 weeks interval with 50 μg of OVA per mouse formulated individually with (a, c) Tc70, MTB70, MTB70C or Pf70C and (b, d) anti-CD40, CT, LPS or Zym. The presence of OVA-specific antibodies was detected by ELISA using serial dilutions of the sera. Titers were expressed as the log10 of the reciprocal of the highest dilution giving OD = 1.0. Pooled sera of three mice were used for detection of IgG. For the assessment of IgG1 and IgG2a profile, analysis was done with sera from individual mouse and the results were presented as mean log10 titer ± S.D. of three mice. Regarding IgG1 and IgG2a isotypes, significant differences between OVA plus Tc70 groups with other HSP70 treated groups following a Student's *t*-test are depicted with asterisks, $^{*}p < 0.005$.

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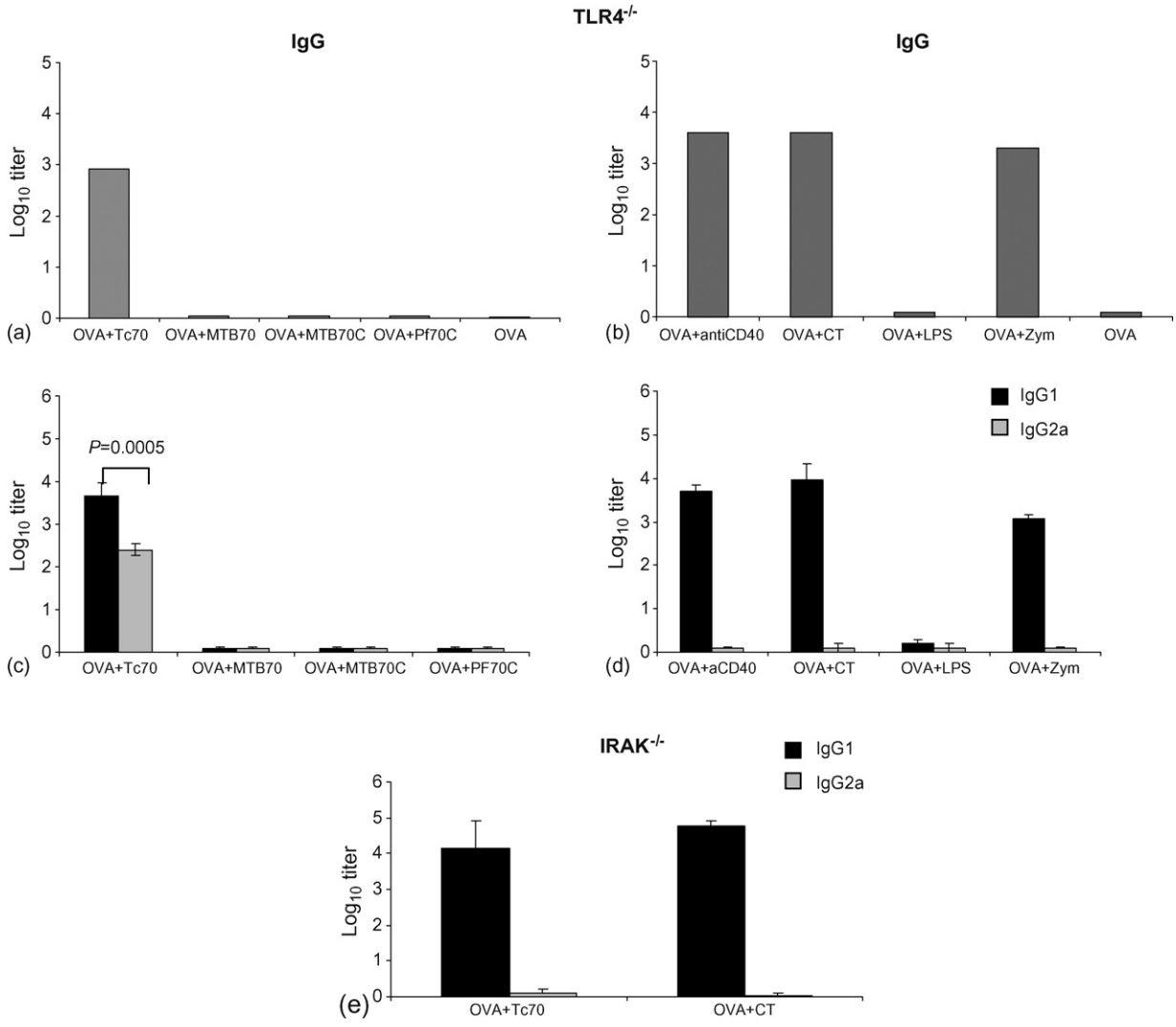

Fig. 3. Comparative analysis of the anti-OVA IgG (a, b) and IgG (c, d) isotypes profile of serum samples obtained from TLR-4$^{-/-}$ and IRAK4$^{-/-}$ (e) mice immunized with OVA delivered together with (a, c) Tc70, MTB70, MTB70C or Pf70C and (b, d) anti-CD40, CT, LPS or Zym. Antibody titers were determined as described in Fig. 2.

other groups of mice received OVA together with CT or anti-CD40 known to exert their effects independently of TLR-2 and TLR-4 signaling pathways. Anti-OVA response was not observed in mice immunized with OVA given alone but TLR2$^{-/-}$ mice responded to the adjuvant effect of all HSPs tested with the exception of Pf70C (Fig. 2a). The control adjuvants worked as expected: LPS, anti-CD40 and CT elicited anti-OVA IgG antibodies while the TLR-2 ligand zymosan stimulated negligible amounts of anti-OVA IgG specific antibodies (Fig. 2b). The isotype profile of anti-OVA IgG was also assessed. Coadministration of Tc70 with OVA resulted in the generation of mixture of anti-OVA IgG1 and IgG2a isotypes, where the level of IgG1 was significantly higher than IgG2a ($p = 0.009$) (Fig. 2c). However, MTB70 and MTB70C promoted only anti-OVA IgG1 isotype (Fig. 2c). The IgG2a isotype was not detected in mice that received OVA with CT, anti-CD40 or LPS (Fig. 2d).

The adjuvant effect of HSP70 was also evaluated in TLR-4$^{-/-}$ mice. Only coadministration of Tc70 with OVA elicited enhanced anti-OVA IgG antibody responses, whereas nearly undetectable amounts of anti-OVA IgG antibodies were induced in mice receiving OVA together either with MTB70, MTB70C or Pf70C (Fig. 3a). As expected, with the exception of LPS, the CT, anti-CD40 and zymosan also induced OVA specific secondary responses in TLR-4$^{-/-}$ mice (Fig. 3b). As in the experiments described before, coadministration of Tc70 with OVA resulted in the generation of mixture of anti-OVA IgG1 and IgG2a isotypes (Fig. 3c). The ratio IgG2a:IgG1 was 0.65 and the difference was statistically significant ($p < 0.005$). It should be mentioned that in this Tc70 group the magnitude of the anti-OVA response was lower in TLR-4$^{-/-}$ mice compared to the wild type C57BL/6 mice. Anti-OVA IgG2a response was absent in mice treated with CT, anti-CD40 and zymosan (Fig. 3d).

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Table 1
IL-12 production by BMM in response to various HSP70s

Mouse strain	Stimuli						
	MTB70	MTB70C	Tc70	Lb70	Pf70C	LPS	Zym
WT	1.0 ± 0.3	3.9 ± 1.0	5.3 ± 1.3	7.0 ± 0.7	6.3 ± 2.2	5.4 ± 2.3	2.5 ± 1.3
TLR2$^{-/-}$	1.7 ± 0.9	6.2 ± 1.0	4.3 ± 2.0	5.5 ± 1.4	6.3 ± 1.3	7.5 ± 2.0	**1.7 ± 0.4**
TLR4$^{-/-}$	**0.4 ± 0.4**	**0.7 ± 0.5**	4.7 ± 1.5	4.4 ± 1.3	**0.2 ± 0.3**	**0.1 ± 0.1**	4.0 ± 2.1
MyD88$^{-/-}$	**0.4 ± 0.5**	**1.1 ± 1.3**	**0.6 ± 0.5**	**1.0 ± 1.0**	**0.6 ± 0.8**	**1.0 ± 0.7**	**0.5 ± 0.4**

Data represent the mean IL-12 concentration ± S.D. in ng/ml for each stimulus. The data is the average of two experiments performed with three individual mice per experiment. Reduced responses are marked in bold.

That Tc70 was working as adjuvant in both TLR-2 and -4 knockout mice, could be interpreted in two ways. Either that signaling occurs via other TLRs or that the receptor for Tc70 is a heterodimer composed by both TLR-2 and -4. In this second alternative, the two receptors could function independently. To assess this issue, we immunized IRAK4 knockout mice with OVA formulated together with Tc70. IRAK is a serine/threonine kinase associated with the IL-1 receptor and functions downstream to the TLR-2, TLR-3, TLR-4 and TLR-9 -MyD88 mediated signaling pathways [34]. We found that IRAK knockout mice also responded to Tc70, generating OVA specific IgG antibodies (not shown). However, analysis of the IgG1 and IgG2a subclasses revealed only the production of anti-OVA IgG1 antibodies with no or negligible amount of IgG2a (Fig. 3e).

3.3. Dependency on TLR receptor signaling in the stimulation of BMMs by HSP70s

The ability of HSP70 to induce IL-12 production by macrophages in culture is well documented [35]. IL-12 is a key cytokine responsible for polarizing T cells toward the Th1 phenotype [36] and DC maturation. In order to examine the contribution of TLRs in HSP70-induced inflammatory responses, the production of IL-12 p70 was analyzed in supernatants of cultured BMM from TLR-2, TLR-4 and MyD88 deficient mice and compared with WT mice. We studied the stimulation promoted by different HSP70 from several organisms and compared with the stimulation induced by zymosan and LPS, ligands for TLR-2 and TLR-4, respectively.

Similar to what was observed regarding antibody production, HSP70s had also different TLR signaling requirements for the induction of IL-12. BMM from TLR-2 deficient mice responded normally to all HSP70 tested but macrophages from TLR-4$^{-/-}$ mice induced IL-12 secretion only in response to Tc70 and Lb70 and not to MTB70, MTB70C or Pf70C (Table 1). However, the IL-12 secretion in response to all the HSP70 was largely reduced in macrophages deficient in MyD88 when compared with WT macrophages ($p < 0.05$) suggesting that IL-12 induction promoted by Tc70 and Lb70 may be dependent on signals mediated by other TLRs. The unstimulated cells secreted negligible amounts of IL-12 in the culture. The level of IL-12 produced by resting cells was always less than 0.04 ng/ml.

4. Discussion

The immunostimulatory properties of microbial HSPs, preferentially the HSP70 family, have been extensively investigated [37]. Differences between human and microbial HSPs are documented but, due to high sequence homology, it has been presumed that members of the same family would possess similar properties. Therefore, a systematic comparison of different microbial HSPs has not been done. In this work we have studied four members of the HSP70 family derived from *M. tuberculosis, P. falciparum, T. cruzi and L. brasiliensis.* We have analyzed their ability to act as adjuvants *in vivo* and to stimulate the induction of the proinflammatory cytokine IL-12 by macrophages *in vitro,* as well as their dependency on TLR signaling for optimal effects in both readout systems. HSP70 is known to interact and activate professional APCs by binding to TLRs and other receptors expressed on APCs. This event is important for efficient priming of T cells by HSPs, which in turn influences the antigen specific adaptive responses. It has been demonstrated that stimulation of TLR-4/TLR-2 transfected HEK293T fibroblast cell lines with human HSP70 preparations results in signaling events and the induction of proinflammatory cytokine production [21]. This response is mediated via the MyD88/NF-κB signal transduction pathway. HSP70 utilizes both TLR-2 and TLR-4 to transduce its proinflammatory signals [20,21,38].

In vivo, the HSP70 molecules tested were found to be very strong immune stimulators, although we found that they displayed different degrees of adjuvant potential regarding both the strength and the profile of the induced immune response. Furthermore, they possessed different requirements for signaling through TLRs. Tc70 from *T. cruzi* induced OVA specific humoral responses in both TLR-2 and TLR-4 knockout mice. In contrast, both MTB70 and its C-terminal fragment induced a response in TLR-2$^{-/-}$ but not in TLR-4$^{-/-}$ mice. We also observed that only Tc70 potentate the induction of a mixture of Th1 and Th2 type of antibodies in WT, TLR-2$^{-/-}$ and TLR-4$^{-/-}$ mice. Regarding the IL-12 production from BMMs *in vitro*, we observed that activation is apparently dependent on TLR signaling since the response was abrogated in MyD88$^{-/-}$ mice. We observed, however, that the dependency on TLR-2 and TLR-4 signaling was also different. The same tendency as in the humoral response was found in TLR-2 and TLR-4 deficient mice for all the HSP70 except for Pf70C, which induced IL-12 release from BMM derived

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Table 2
Summary of the different stimulatory properties of HSP70

	TLR2$^{-/-}$	TLR4$^{-/-}$	MyD88$^{-/-}$	
MTB70	Yes	No	–	Ab responses
	Yes	No	No	IL-12 production
MTBC70	Yes	No	–	Ab responses
	Yes	No	No	IL-12 production
Pf70C	No	No	–	Ab responses
	Yes	No	No	IL-12 production
Tc70	Yes	Yes	–	Ab responses
	Yes	Yes	No	IL-12 production
Lb70	Yes	Yes	–	Ab responses
	Yes	Yes	No	IL-12 production

from TLR-2$^{-/-}$ mice but was dependent on TLR-2 signaling as adjuvant *in vivo* (Tables 1 and 2). This may indicate that the two aspects, studied here, have different signaling requirements. Alternatively, it may occur that there are differences between *in vitro* and *in vivo* conditions since Pf70C is a truncated molecule and some epitopes could be missing or modified causing reduction in affinity. In fact, stimulatory and inhibitory epitopes for the chemokine production and DC maturation were identified in the HSP70 from *M. tuberculosis* (MTB70). Wang Y et al. [39] observed that the whole HSP70 molecule was less effective in cytokine induction *in vitro* than the truncated MTB70C (359–610 aa), which is in agreement with the results presented here (Table 1). However, we found no differences in adjuvanticity *in vivo* since both forms induced similar strong responses and triggered a similar isotype profile reinforcing the argument of the existence of differences in *in vivo* and *in vitro* conditions.

As Tc70 and Lb70 are efficient adjuvants in both TLR-2$^{-/-}$ and TLR-4$^{-/-}$ but not in MyD88$^{-/-}$ mice, it is possible that both TLR-2 and TLR-4 are redundant and function independently. This has been shown for *Chlamydia* derived HSP60 [40]. TLR-2/-4 double knockout mice were completely unable to respond in terms of CC chemokine production while the single knockout strain responded normally [40]. It may also be possible that other TLRs, different from TLR-2 and TLR-4, are involved in this process. This will confer a broader and therefore, more interesting role for HSPs as sensors for danger. In this scenario, HSPs could be able to recognize not only LPS, but also other PAMP (pathogen associated molecular patterns) on microorganisms. Finally, other receptors different from TLRs might be involved in the HSP promoted adjuvant potential. Since CD40 was reported to be the receptor for HSP70 expressed on macrophages and dendritic cells [18,19,41,42], it is also possible that the adjuvant effect of Tc70 may be mediated through direct binding of HSP70 to CD40. Ligation of CD40 may then activate APCs by increasing expression of costimulatory molecules [43,44] and the production of inflammatory cytokines [45], which ultimately instructs the adaptive immune response to generate antigen specific T and B cells. In conclusion, we here demonstrate that HSP70 from various sources possess different requirements for signaling through TLRs. This finding sheds new light towards the adjuvanticity of HSP70, and paves the way for the development of effective vaccines against infections.

Acknowledgments

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