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Mycobacterium tuberculosis heat-shock protein 70 impairs maturation of dendritic cells from bone marrow precursors, induces interleukin-10 production and inhibits T-cell proliferation *in vitro*

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Summary

In different inflammatory disease models, heat-shock proteins (hsp) and hsp-derived peptides have been demonstrated to possess anti-inflammatory properties. While some studies have shown that hsp can directly interact with antigen-presenting cells, others report that bacterial hsp can induce specific T cells with regulatory phenotypes. Effective characterization of the immunomodulatory effects of hsp 70, however, has historically been confounded by lipopolysaccharide (LPS) contamination. In this study, we compared the effects of LPS-free *Mycobacterial tuberculosis* hsp 70 (TBhsp70) and its possible contaminants on dendritic cells (DC). We demonstrate herein that LPS-free TBhsp70 inhibits murine DC maturation *in vitro*, while LPS-contaminated TBhsp70 induces DC maturation. Mock recombinant preparations have no effect. In contrast to LPS, TBhsp70 does not induce tumour necrosis factor-α production by DC, but interleukin-10. *In vivo*, only LPS-contaminated TBhsp70 induces up-regulation of CD86 in splenic mature DC. Finally, TBhsp70 inhibited phytohaemagglutinin-induced T-cell proliferation. Our results support the hypothesis that TBhsp70 does not have inflammatory potential, but rather has immunosuppressive properties.

Keywords: cytokines; dendritic cells; heat-shock protein 70; *Mycobacterium tuberculosis*; T-cell proliferation

Introduction

Heat-shock proteins (hsp) are part of a highly evolutionarily conserved response made by all living beings in response to an increase in temperature, and they play a decisive role in thermotolerance and cellular homeostasis.[1,2] They are also immune modulators. The hsp purified from tumours deliver peptides to antigen-presenting cells (APCs) for presentation in the major histocompatibility complex (MHC),[3–5] thus eliciting tumour-specific $CD8^+$ T-cell responses. On the other hand, immunizaton with hsp has also been shown to down-regulate inflammatory pathways.[6] High serum levels of hsp are associated with a low risk for atherosclerosis.[7,8] Different clinical trials are now being conducted using hsp-based immunotherapy for rheumatoid arthritis and type I diabetes.[9]

The most conserved hsp is hsp 70; it is also the most abundantly induced hsp in response to stress. Bacterial and eukaryotic hsp 70 reach levels of 50% identity and exhibit functional similarities.[10] Both *Escherichia coli* and *Mycobacterium tuberculosis* hsp 70 (TBhsp70) are excellent carrier-adjuvants for antibody production.[11,12] TBhsp70 is an extremely powerful antigen, even in lipopolysaccharide (LPS) hyporesponsive mice,[13] leading to early immunoglobulin G production. Immunization with TBhsp70 protects against adjuvant arthritis in rats[14] because of the induction of interleukin-10 (IL-10)-producing T cells that recognize a specific TBhsp70 peptide.[15,16] TBhsp70 induces

Abbreviations: APC, antigen-presenting cells; ATP, adenosine triphosphate; BSA, bovine serum albumin; DEAE, diethylaminoethyl; DEX, dexamethasone; DNA, deoxyribonucleic acid; EgAFFp, *Ecchinococcus granulosus* actin filament fragmenting protein; ELISA, enzyme-linked immunoabsorbent assay; EU, endotoxin units; GST, glutathione-*S*-tranferase; hsp, heat-shock protein; IFN-γ, interferon-γ; IL, interleukin; LAL, *Limulus* amoebocyte lysate; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PHA, phytohaemagglutinin; Pi, inorganic phosphate; TBhsp70, hsp 70 of *Mycobacterium tuberculosis*; TNF-α, tumour necrosis factor-α.

IL-10 production in blood and synovial cells from arthritis patients, leading to a decrease in tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) production.[17] Together, these observations suggest that TBhsp70 has immunosuppressive properties. It is still unclear, however, if the immunomodulatory effects of TBhsp70 are the result of direct interactions with the APCs, or of its action as an antigen, clonally expanding T cells with a regulatory phenotype.

In this study, we analysed the effect of direct TBhsp70 interactions with dendritic cells (DC). Because studies on immune modulation by hsp 70 have been plagued with LPS contamination, we systematically analysed the effect of LPS-free TBhsp70 and its probable contaminants on the maturation of DC. Our results suggested that TBhsp70 has inflammatory properties only when contaminated with LPS. LPS-free TBhsp70 inhibited murine DC maturation as well as T-cell proliferation, inducing IL-10 and not TNF-α production, which is consistent with anti-inflammatory potential.

Materials and methods

Reagents

Dexamethasone (D4902) (DEX) and LPS (L-2630) were purchased from Sigma (St. Louis, MO). DEX was reconstituted in ethanol and used at 10^{-5} to 10^{-7} M. Bovine serum albumin (BSA) was purchased from Gibco-BRL (Gaithesburg, MD) (11018–017). Recombinant TBhsp70 was produced in XL1-blue *Escherichia coli*, and purified according to Mehlert.[18] A mock extract was prepared in the same strain of *E. coli* lacking plasmid, and exactly the same purification procedures as had been used for the transformed bacteria extract [adenosine triphosphate (ATP) column, diethylaminoethyl (DEAE) column, Triton extraction, Centricon concentration) were applied, the purifications being performed side by side with transformed bacterial cultures. Both transformed and mock bacterial extracts were analysed for protein concentration, using the Bradford assay, as well as by densitometry using a BSA standard curve on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels stained with Coomassie blue. Mock preparations were used in cultures and the same microlitre amount of TBhsp70 was used. In some experiments, *Ecchinococcus granulosus* actin filament fragmenting protein (EgAFFP) was used as a recombinant protein control.[19] Recombinant EgAFFP was glutathione-*S*-tranferase (GST) -purified, and provided by Dr Henrique Ferreira (Universidade do Rio Grande do Sul, Porto Alegre, RS, Brazil).

LPS extraction

All reagents, including phosphate-buffered saline (PBS), DNA and mock extract, were screened for LPS contamination, and the preparations used to stimulate the cell cultures were compared before and after LPS extraction. To remove LPS using Triton X-114, the method described by Aida and Pabst[20] was employed. Briefly, 5 μl Triton X-114 (Sigma) was added to 500 μl of 1 μg/ml recombinant protein. After vortexing vigorously, the solution was incubated on ice for 5 min, vortexed again and incubated at 37° for 5 min. The solution was then centrifuged for 7 s at 37° and the supernatant was collected. This procedure was repeated three more times. In some experiments, the Triton extraction protocol was repeated, giving a total of eight extractions. Contaminating Triton was removed by incubating overnight with Biobeads (cat. no. 152–3920, Bio-Rad, Hercules, CA) at 4° with agitation. TBhsp70 was used only when LPS levels were below 0·005 endotoxin units (EU)/ml.

ATPase assay

Integrity of TBhsp70 after Triton extraction was assessed by monitoring its ATPase activity in the presence of Mg^{2+}, using a method described elsewhere.[21] Briefly, 3–5 μg protein was added to the reaction mixture containing Tris–HCl (pH 8·0) and 5 mM $CaCl_2$ or $MgCl_2$ in a final volume of 200 μl. The samples were preincubated for 10 min at 37°. The reaction was initiated by the addition of ATP to a final concentration of 1 mM and stopped by adding 200 μl 10% trichloroacetic acid. The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi). Incubation times and protein concentrations were chosen to ensure linearity of the reactions. Controls with the addition of the enzyme preparation after mixing with the trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol Pi released per minute per mg protein. All enzyme assays were run in triplicate.

Dendritic cell cultures

C57BL/6 mice were purchased from LACEN (Rio Grande do Sul, Brazil). Murine DC were grown from bone marrow with granulocyte–macrophage colony-stimulating factor and IL-4, as described by Inaba *et al.*,[22] and used on day 5 of culture, still immature as assessed by fluorescence-activated cell sorting (FACS) analysis of class II and B7 expression. The cells were then incubated with either DEX, LPS, TBhsp70, EgAFFP, bacterial DNA, mock extract or BSA, for 24 or 48 hr and then analysed for maturation by FACS. The supernatant was collected and used for cytokine analysis.

Flow cytometry and ELISA

Commercially available enzyme-linked immunsorbent assay (ELISA) kits for IL-10 and TNF-α (Quantikine or

Duo-Set, R & D Systems, Minneapolis, MN) were used to measure murine cytokine concentrations in cell culture supernatant. The λ_{450} absorbances were detected using an ELISA plate reader (Biorad, Hercules, CA) and concentrations were extrapolated from a log-transformed curve (GRAPHPAD PRIZM 3·0, San Diego, CA). Data are expressed in pg/ml. Anti-murine antibodies for flow cytometry (anti-IA^b and anti-CD86; catalogue numbers 553551 and 553692) were purchased from Pharmingen (San Diego, CA). The percentage of mature DC was determined by gating on the $CD86^{high}$ $IA^{b\ high}$ population. For the *in vivo* studies, cells were stained with fluorescein iosthiocyanate-conjugated CD11c, phycoerythrin-conjugated CD86 and cychrome-conjugated B220 (Pharmingen). Analysis of CD86 expression was performed on $CD11c^+$ cells.

Cell proliferation/viability assay

T-cell proliferative responses were determined by a modified colorimetric assay.[23] A single cell suspension of splenocytes from C57BL/6 mice was obtained and cells were incubated at 8×10^5 cells/ml with 1% phytohaemagglutinin (PHA), alone or with different amounts of TBhsp70 or DEX. In the last 4 hr of culture, 100 µl of the supernatant was gently discarded and 30 µl freshly prepared MTT [3-(4,5-diamethyl 2-thiazolyl) 2,5 diphenyl-2H-tetrazolium, Sigma] solution (5 mg/ml in RPMI-1640) was added to each well. The dehydrogenase enzymes in metabolically active cells convert this substrate to formazan, producing a dark blue precipitate. The cell cultures were incubated for 4 hr at 37° in a 5% CO_2 atmosphere. After complete removal of the supernatant, 100 µl of dimethyl sulphoxide (Sigma) was added to each well. The optical density (OD) was determined using a Biorad ELISA plate reader at wavelengths of 570 and 630 nm. Data were analysed using the GRAPHPAD PRIZM software, and proliferation/viability was expressed as percentage of PHA-induced proliferation. One-way analysis of variance test was used to determine differences between groups. Multiple comparisons among levels were checked with a Bonferroni post hoc test.

In vivo *assay*

BALB/c $RAG^{-/-}$ mice and regular BALB/c mice were injected intravenously (i.v.) with 100 µl of PBS, 40 µg LPS, 40 µg dirty TBhsp70, or 40 µg clean hsp. Mice were killed 6 or 18 hr after injection. The spleens were removed and treated with collagenase D as described elsewhere.[24] The single cell suspensions obtained were stained with anti-B220, anti-CD11c and anti-CD86 antibodies, and CD86 expression was analysed by flow cytometry on $CD11c^+$ cells.

Results

TBhsp70 arrests the maturation of murine bone-marrow-derived dendritic cells and induces IL-10 production

It is well-established that immature DC express low levels of MHC class II and CD86.[25] When immature DC are triggered to mature with a Toll-like receptor ligand such as LPS, they up-regulate the expression of MHC class II and the costimulatory molecule CD86.[26–28] We previously observed that TBhsp70 blocked *in vitro* differentiation of DC from bone marrow precursors, demonstrated by reduced expression of MHC class II and CD86.[17] We reproduced this finding, this time adding different concentrations of Triton-extracted TBhsp70 to murine bone marrow DC cultures, and analysed the expression of MHC class II (IA^b) and CD86, both 24 and 48 hr after adding stimulus to the cultures. The preparation used in this experiment had 0·005 EU/ml after LPS removal treatment, as determined by the *Limulus* amoebocyte lysate (LAL) assay. To some cultures we added LPS, and to others, DEX, a powerful anti-inflammatory known to inhibit the maturation of DC.[29,30] Controls included BSA as an irrelevant protein control, and PBS.

This *in vitro* system allowed tracking of DC maturation over time in culture, measured by the expression of CD11c, MHC class II and CD86. By day 10, all cells were $CD11c^+$ $CD8^-$ class II^{hi} $CD86^{hi}$ (not shown). Days 6 and 7 were critical time-points in this system, when most of the cells started to acquire the mature phenotype (MHC class II^{hi} $CD86^{hi}$). Accordingly, on the 6th day of culture (Fig. 1, PBS) approximately 15% of cultured cells were already mature DC. As can be seen in Fig. 1, while LPS induced maturation, as shown by 23% of cells being class II^{hi} $CD86^{hi}$ cells, no effect was apparent for DEX or TBhsp70 cultures. On day 7 (48 hr after the addition of stimuli to the cultures), mature DC incubated with PBS or BSA comprised > 30% of the total cells (Fig. 1, 48 hr). Maturation induced by LPS at 48 hr was prominent (almost 50% of the cells were MHC class II^{hi} $CD86^{hi}$). This is the magnitude of stimulation we ordinarily observe in these cultures when we stimulate with LPS (not shown), from very small doses to the high dose used here. Inhibition of maturation by DEX was evident, with cells failing to differentiate to the mature DC phenotype. Similarly to DEX, TBhsp70 inhibition of maturation was observed only at 48 hr. Treatment with both TBhsp70 and DEX also led to a reduction in the population of class II^{lo} $CD86^{lo}$ cells. This suggested that these treatments could either redirect the differentiation of DC precursors or induce apoptosis of differentiated DC, or perhaps both.

TBhsp70 induced IL-10, but no TNF-α, in rat adjuvant arthritis[31] and in synovial cells of arthritis patients.[17] To

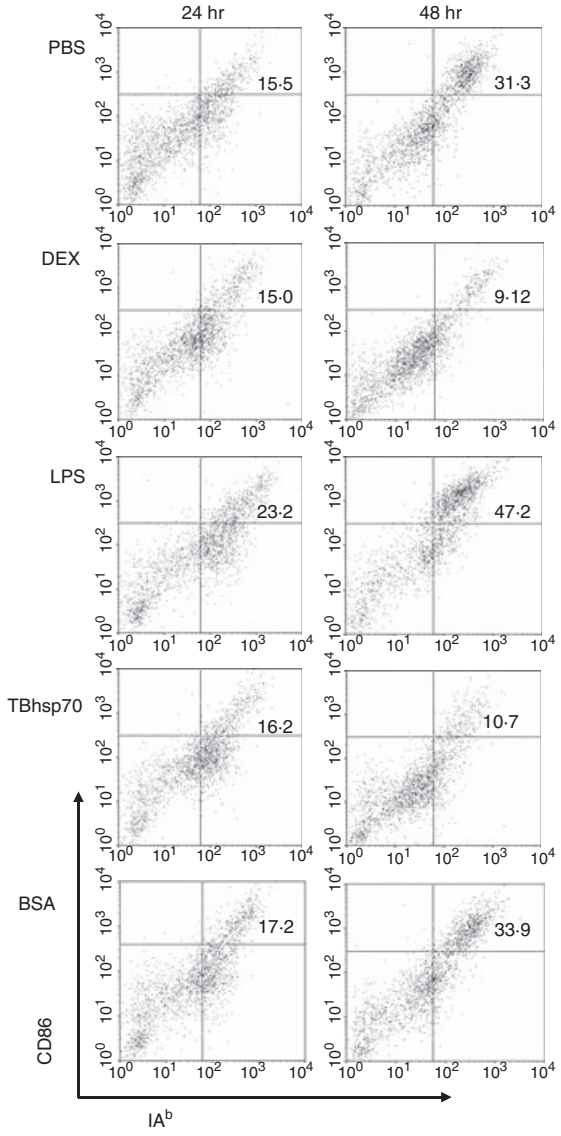

Figure 1. Kinetics of LPS-free TBhsp70 inhibition of dendritic cell maturation. Dendritic cells were grown from bone marrow and incubated on the fifth day of culture with different stimuli: PBS, 10^{-5} M dexamethasone (DEX), 60 μg of either LPS (1 EU/μg), BSA or TBhsp70. Cells were harvested 24 hr and 48 hr later and analysed for CD86 and MHC class II expression. Three populations are typically identified at this time in culture: $CD86^{hi}$ MHC class II^{hi} cells (percentage indicated in the upper right quadrants) are the already mature DC; the $CD86^{lo}$ MHC II^{lo} population are the still immature DC; and the double-negative population has not yet differentiated.

test this in bone marrow-derived DC, we analysed cytokine production in cell culture supernatants within 48 hr of stimulation. While TNF-α (Fig. 2a) was induced in cultures incubated with LPS, it was not observed in cultures with TBhsp70 added. Induction of IL-10 was observed in cultures with TBhsp70 (Fig. 2b), however, in much lower concentrations than previously observed in synovial cells. IL-10 was also induced by LPS, suggesting

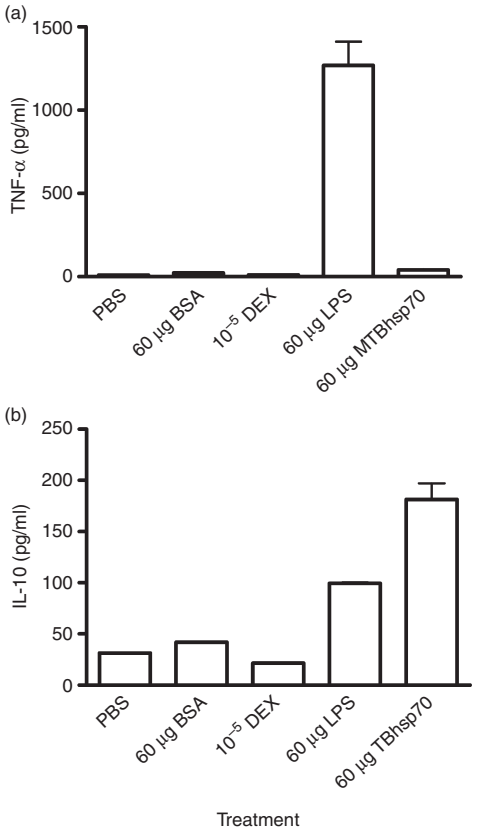

Figure 2. Cytokines detected in the supernatants harvested 48 hr after addition of stimuli to cultures. (a) TNF-α; (b) IL-10. Stimuli included PBS; 10^{-5} M DEX (DEX), 60 μg of either LPS (1 EU/μg), BSA or TBhsp70. This experiment was repeated five times, with comparable results.

that this cytokine was not the sole anti-inflammatory mediator being induced by TBhsp70 leading to DC maturation arrest.

Induction of TNF-α, but not of IL-10 by TBhsp70, is the result of contaminating LPS

In contrast to our observations, other studies reported inflammatory properties for TBhsp70.[32–34] We asked if the effects we observed over DC maturation could be the result of unknown substances present in the TBhsp70 recombinant preparation, and dragged along during the purification process. Alternatively, it was possible that the Triton extraction treatment we used denatured the protein, leading to the abrogation of its ability to induce TNF-α and DC maturation.

To test if the inhibitory effects observed for TBhsp70 preparations were the result of contaminants in the recombinant preparation, we set up a mock preparation. Bacteria not containing the plasmid were grown and lysed, and the lysate was processed side by side (ATP purification, DEAE columns, centricon concentration)

with the lysates from transformed bacteria. Samples from mock preparations were loaded on SDS–PAGE gels and no bands were detected by Coomassie blue staining (not shown). Part of the mock preparation was Triton extracted, and preparations were designated as clean (C) and dirty (D), respectively, for the absence or presence of contaminating LPS. We then analysed the LPS content in TBhsp70 preparations before and after Triton extraction, using the LAL assay, as well as in the clean and dirty mock preparations. We also tested how many endotoxin units were present in our LPS (1 mg/ml) stock solution.

The dirty recombinant preparation of TBhsp70 had 750 EU/μl (0·750 EU/ml), as determined by the LAL assay, almost as much as what was determined for the LPS stock solution (Fig. 3a). After Triton X-114 extractions, this contamination was reduced 1000-fold. Interestingly, the mock preparation showed a much lower LPS content than the protein preparation, suggesting that LPS might be 'carried' in the purification process by TBhsp70. After Triton extraction, the LPS content of the mock preparation was also reduced. As an irrelevant recombinant protein control, a protein from *Ecchinococcus granulosus* (EgAFFP) was used. This protein also seemed to have a higher amount of contaminating LPS in the preparation compared to the mock extract, but around 50% less contaminating LPS compared to TBhsp70. These results suggested that endotoxin tends to be carried along with protein, and not specifically TBhsp70, during the purification process. Also, it suggested that TBhsp70 has a higher ability to carry LPS along the purification process compared to EgAFFP. This was consistently observed every time the experiment was repeated. Endotoxin content was also efficiently removed from EgAFFP by detergent extraction (Fig. 3a).

To determine if LPS extraction by Triton damaged the protein structure, we performed an ATPase assay. The N-terminal domain of hsp 70 acts as an ATPase, allowing the protein to release the peptide being carried in the C-terminal domain (the peptide-binding domain) after hydrolysis of ATP.[35] The concentration of the extracted protein was not significantly affected by the LPS removal process using Triton X-114, as determined by the Bradford assay as well as SDS–PAGE gel (from 0·7 μg/ml to 0·56 μg/ml; not shown); however, it can be altered by Biobead decontamination of residual Triton in the preparation. After concentration determination and adjustments, identical amounts of clean (Triton extracted) and dirty (not extracted) TBhsp70 preparations were tested for ATPase activity (Fig. 3b). As can be observed, ATPase activity of TBhsp70 is not significantly affected by LPS removal treatment ($U = 0{\cdot}0000$, $P = 0{\cdot}1$), suggesting that the structure of the protein is not damaged by the procedure. Together, these results suggested that Triton can efficiently remove endotoxin from all preparations, and does not affect protein structure.

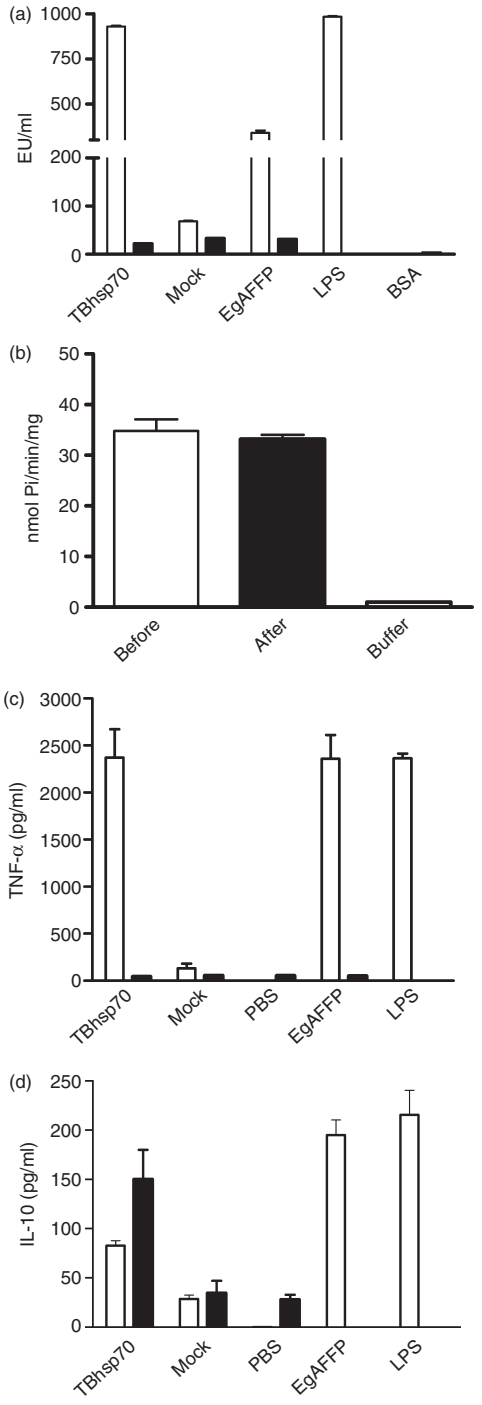

Figure 3. Effect of Triton X-114 treatment on recombinant preparations. (a) Endotoxin content of recombinant protein preparations and stock LPS solution (1 μg/ml) measured by the LAL assay, before and after Triton extraction. (b) ATPase activity of TBhsp70 before and after Triton extraction. (c, d) Cytokine content in DC culture supernatant after 48-hr incubation with different doses (10, 20 or 40 μg/ml) of either LPS, TBhsp70, EgAFFP or (microliztre equivalents of) mock preparation, before or after Triton extraction. Open bars, before Triton extraction; black bars, after Triton extraction. This experiment was repeated six times, with similar results.

We next tested the effect of the dirty and clean preparations on the induction of TNF-α and IL-10 production by DC. To do that, we added 40 μg/ml of each of the different preparations to cultures. Equivalent amounts in microlitres of the clean and dirty mock preparations were added for comparison. Each of the preparations was added to DC cultures on day 5, and TNF-α was assayed in the supernatant 48 hr later. As shown in Fig. 3(c), LPS induced high levels of TNF-α. A dirty TBhsp70 preparation, that had amounts of EU/μl similar to the LPS stock solution, was added to immature DC and induced TNF-α levels comparable to those induced by LPS. No TNF-α was observed in cultures incubated with clean TBhsp70. The same was observed for dirty and clean EgAFFP, again in amounts compatible with its level of endotoxin contamination. To compare TNF induction by TBhsp70 and mock preparations, we matched the microlitre amounts of TBhsp70 added to cultures in Fig. 3(c). The clean mock preparation did not induce TNF-α production by DC. The dirty mock preparation induced modest amounts of TNF-α but dose-dependently and consistently with its much lower level of contaminating LPS. Taken together, these results suggest that any recombinant preparation that has not had its contaminating LPS removed can appear to have an inflammatory effect on dendritic cells. That was not the case for IL-10 induction (Fig. 3d). Incubation with LPS and all dirty preparations induced IL-10 production. However, only clean TBhsp70, but neither clean EgAFFP nor clean mock preparation induced IL-10 in DC cultures (Fig. 3d). Interestingly, dirty TBhsp70 induced lower levels of IL-10 than dirty EgAFFP and LPS preparations.

Inhibition of DC maturation from bone marrow precursors by TBhsp70 is not caused by contaminants

Neither LPS-free TBhsp70 nor clean mock preparations induced TNF-α production by DC. This suggested that TBhsp70 was not inherently inflammatory, and that induction of TNF-α in DC cultures could be explained by LPS contamination. We next investigated if the inhibitory effects of TBhsp70 on DC maturation could be explained by unknown contaminants present in the mock preparations. The same microgram amounts of either clean or dirty TBhsp70 were added to cultures on day 5. In other cultures, the equivalent microlitre amount of either dirty or clean mock preparations was added. The percentage of mature DC was assessed by FACS 48 hr later, measured by CD86 and MHC class II expression.

Figure 4(a) shows that while dirty TBhsp70 can induce DC maturation, clean TBhsp70 has either no effect on maturation (10 and 20 μg/ml) or inhibits it (40 μg/ml). The dirty mock preparation induced a slight increase in maturation, only at the highest dose, compatible with its previously determined LPS contam-

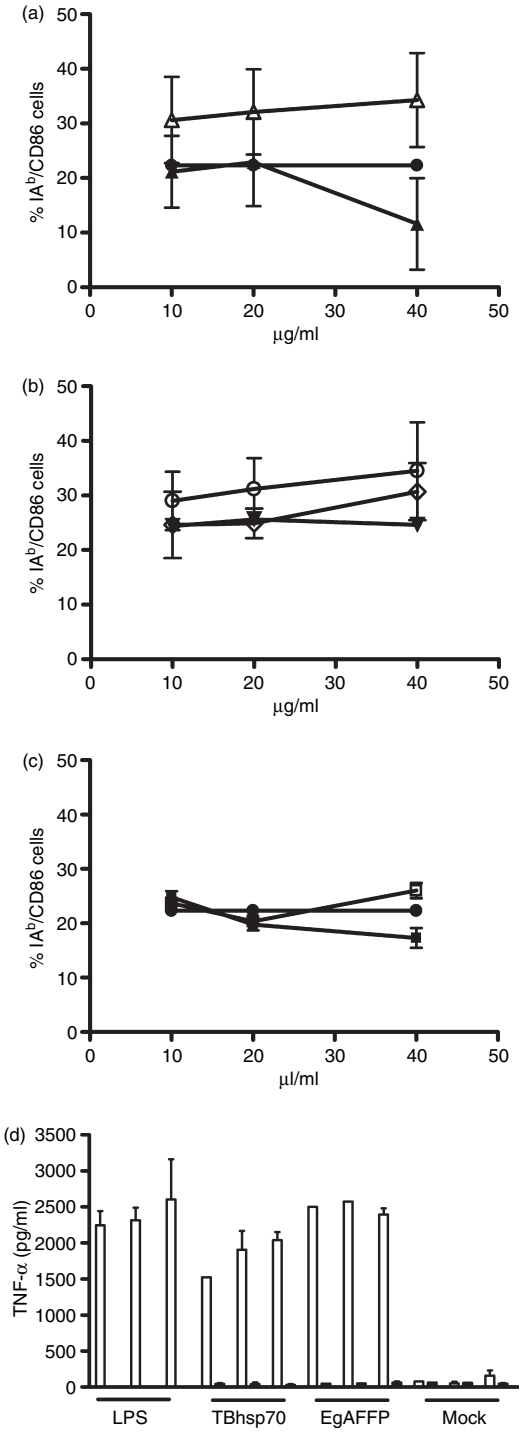

Figure 4. LPS-free TBhsp70, but not its contaminants, inhibits DC maturation and does not induce TNF-α or NO. Murine DC were incubated with 10, 20 or 40 μg/ml of LPS-contaminated (open shapes) or Triton-extracted (black shapes) preparations. For mock preparations, equivalent μl amounts were used. (a) ●, PBS; ▲, clean TBhsp70; △, dirty TBhsp70; (b) ○, LPS; ▼, clean EgAFFP; ◇, dirty EgAFFP; (c) ●, PBS, ■, clean mock prep; □, dirty mock preparation; (d), TNF-α production by the cultured DC, incubated with stimuli described above, with LPS contaminated (open bars) or Triton-extracted (black bars) preparations.

ination content (Fig. 4b), and no inhibition of maturation was observed for the clean mock preparation, even at the highest dose. LPS alone induced DC maturation, and so did the dirty TBhsp70 preparation that had comparable amounts of contaminating endotoxin (Fig. 4c). The same was true for dirty EgAFFP. Together, these results suggest that the inhibitory effect observed for TBhsp70 is not from an unknown contaminant in the purification procedure. The cytokine production profile of the supernatants of the same cultures showed concurrent results. TNF-α production was observed only in cultures incubated with the dirty preparations (Fig. 4d).

LPS-free TBhsp70 does not activate DC *in vivo*

In vivo, TBhsp70 has carrier-adjuvant effects, and is also an immunodominant antigen. It was therefore possible that we were unable to observe DC-maturation induction by TBhsp70 because we were working on *in vitro* systems, and some unknown *in vivo* component of the immune response was missing that was needed to fully accomplish APC activation by TBhsp70. *In vivo*, APCs up-regulate CD86 as early as 6 hr after i.v. injection of LPS[24] and at this time-point after LPS injection DC have rapidly migrated to the T-cell zones in the spleen.[36] It was possible that TBhsp70 could induce the activation of DC *in vivo* by directly binding to mature APCs, as described in many *in vitro* studies. Alternatively, up-regulation of activation markers on APCs could be a by-product of APC presentation of TBhsp70 peptides to TBhsp70-specific T cells, which could exist in a high precursor frequency in normal animals. A high precursor frequency for TBhsp60-specific T cells has been described in cord blood[37] and the unusual secondary-like humoral response observed upon immunization with TBhsp70[13] could reflect a similarly high precursor frequency of T cells with specificity for this protein.

To test if TBhsp70 would activate APCs *in vivo*, we injected 40 μg of either clean or dirty TBhsp70, 40 μg of LPS, or the same volume of PBS, i.v. into BALB/c mice. To test the possibility that an activation effect, if observed, would be the result of precursor T-cell interactions with the APC, we simultaneously injected BALB/c RAG$^{-/-}$ mice with the same preparations. We performed the experiment at two time-points, 6 and 18 hr, to ensure that an absence of APC activation, if observed, was not because hsp 70 needed more time than LPS to induce up-regulation of CD86 in DC. The results were identical at the two time-points, and are shown in Fig. 5 (for the 18 hr time-point). Spleens were removed and treated with collagenase D; CD86 expression was analysed in CD11c$^+$ cells. Only LPS and dirty TBhsp70 induced up-regulation of CD86 in DC, both in normal and RAG$^{-/-}$ mice. No effect was observed for clean hsp 70. This suggested that clean TBhsp70 does not induce APC activation *in vivo*.

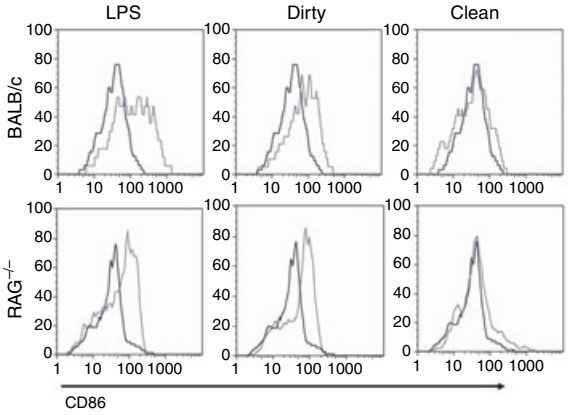

Figure 5. Clean TBhsp70 does not induce up-regulation of CD86 in DC *in vivo*. Mice (regular BALB/c or Rag$^{-/-}$ BALB/c) were injected intravenously with either PBS, 40 μg LPS, dirty TBhsp70, or clean TBHSp70. Spleens were removed 18 hr later, treated with collagenase D and the single-cell suspension was analysed by flow cytometry. Expression of CD86 was analysed in CD11c$^+$ B220$^-$ cells. This experiment was repeated twice, with identical results, and is currently the standard assay in our laboratory for LPS contamination of recombinant proteins because of its sensitivity. A variant of this experiment, with spleen removal after 6 hr of injection, was performed three times. Dark line represents PBS control; grey line represents experimental data.

LPS-free TBhsp70 inhibits PHA-induced-cell proliferation *in vitro*

The i.v. injection experiment suggested that TBhsp70 had no inherent inflammatory properties. *In vitro*, clean TBhsp70 delayed maturation and induced IL-10, but not TNF-α, in bone-marrow-derived DC cultures, suggesting an immunosuppressive potential. It was still unclear if the observed effects could indeed modulate T-cell function. We had previously observed that LPS-free TBhsp70 could induce IL-10 in human peripheral blood mononuclear cells from blood and synovial fluid.[17] To determine if clean TBhsp70 could affect PHA-induced T-cell proliferation *in vitro*, we cultured mouse splenocytes with different amounts of either DEX, clean or dirty TBhsp70 for 96 hr, and measured proliferation using an MTT assay. The results, shown in Fig. 6, showed that DEX (Fig. 6a) significantly inhibited T-cell proliferation, in all three concentrations used ($F_{1,9} = 46{\cdot}69$, $P < 0{\cdot}0001$). Inhibition of T-cell proliferation by LPS-free TBhsp70 was observed (Fig. 6b) ($F_{1,9} = 4{\cdot}63$, $P < 0{\cdot}01$). A dose-dependent tendency of reduced proliferation could be detected at concentrations of 10 and 20 μg/ml, with Bonferroni tests showing statistical significance only at the concentration of 40 μg/ml. LPS-contaminated TBhsp70 induced an

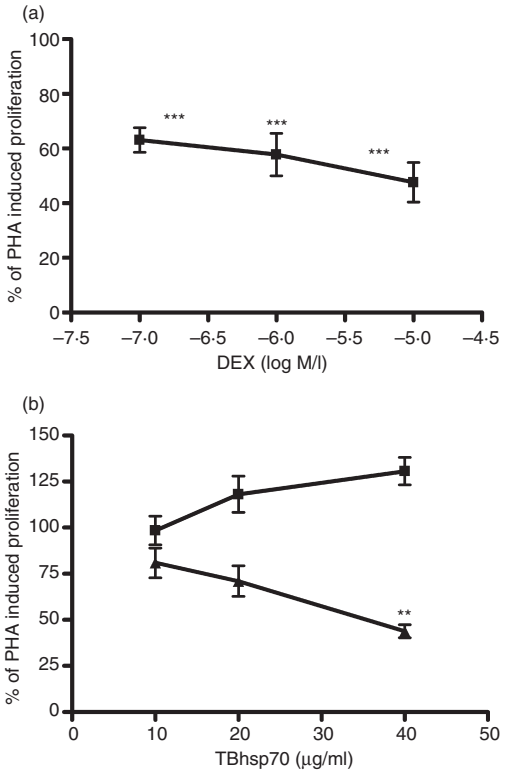

Figure 6. Clean TBhsp70 inhibits PHA-induced T-cell proliferation *in vitro*. In this experiment, splenocytes from four mice were incubated with 1% PHA plus either (a) DEX (10^{-7} to 10^{-5} M); or (b) clean (▲) TBhsp70; or (c) dirty (■) TBhsp70, for 96 hr. Proliferation/viability is expressed as percentage of PHA-induced proliferation. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. This experiment was performed five times, with comparable results.

increase in proliferation (Fig. 6c); however, that was not statistically significant.

Discussion

In this study, we asked if TBhsp70 could influence murine DC maturation and activation. We also asked if inconsistencies observed in different reports in the literature could be explained by contaminants in the hsp 70 preparation. Our results show that TBhsp70 inhibits DC differentiation from bone marrow precursors, inducing IL-10 but not TNF-α. Also, we demonstrated that clean TBhsp70 does not induce APC activation *in vivo*, but can suppress PHA-induced T-cell proliferation *in vitro*.

While the chaperoning activity of eukaryotic hsp 70 is confirmed by independent studies, its inflammatory potential is controversial. The ability to induce inflammatory cytokines that has been described for this protein[38] disappears when contaminant LPS is removed from the hsp 70 preparation.[39,40] This observation is crucial to the understanding of the true immunomodulatory properties of this protein.[40,41]

The issue of contaminating bacterial substances in recombinant hsp preparations has been intensively debated. It has undermined all the studies that have reported immunomodulatory roles for these proteins. LPS is difficult to remove, particularly from hsp 70. The results of our endotoxin removal experiments show that LPS seems to be carried along with the protein in the purification process, something that does not happen in a mock preparation. EgAFFP also presented somewhat high levels of contaminating endotoxin, which could be the result of this protein's known affinity for hydrophobic moieties.[19] The observation that LPS-contaminated hsp activates DC maturation and enhances T-cell proliferation could suggest that the immunosuppressive effect of the clean protein is not sufficient to interfere with LPS effects. However, it is possible that LPS binds to the protein, as suggested by Triantafilou and collaborators,[42] which could explain why the immunodulatory effects of TBhsp70 would be different in the absence versus presence of LPS, especially in a recombinant preparation, which contains such high amounts of LPS. Finally, even when LPS could be clearly removed from hsp preparations, immunomodulation by hsp is not fully acknowledged, because unknown bacterial contaminants could be responsible for the observed effects. We tried to address these issues in this study.

In our hands, Triton extraction was the most satisfactory method for efficiently removing endotoxin from our protein preparation, compared to polymyxin B purification, or boiling (not shown here). Unaltered ATPase activity of the protein after detergent treatment ensured that protein structure had not been damaged. This result is consistent with others that have removed endotoxin using Triton and reported different proteins to retain biological activity and physical integrity.[43–45] After we were confident that we had an endotoxin-free, active form of TBhsp70, we extended the treatment to control proteins and preparations, and then compared them for possible effects on DC.

The induction of an anti-inflammatory/immunosuppressive environment by TBhsp70 observed in this study agrees with many independent findings described in the literature. Reports of immunological activity of TBhsp70 invariably point to the generation of strong antibody responses,[11–13] which are driven by T helper type 2 (Th2) cytokines, and are associated with anti-inflammatory environments. Moreover, different groups have reported that TBhsp70, or peptides of this protein, could be used to successfully treat rats in an animal model of arthritis[14] or listeriosis.[31,46] The protection was explained by the generation of IL-10-producing T cells that were specific for a TBhsp70 peptide. Pre-immunization with BiP, an endoplasmic reticulum homologue of hsp 70, suppresses the development of adjuvant-induced arthritis[47,48] and stimulates IL-10-producing $CD8^+$ T cells in arthritis

patients.[49] The generation of T-cell responses, as well as their skewing towards Th1, Th2 and even Th3 (tolerogenic) responses, is known to be a function of the interactions of T cells and DC.[50] In particular, interaction of T cells with immature DC, or IL-10-producing DC, leads to the development of tolerogenic and/or TH2 responses.[51–54] The results of our experiments suggest that TBhsp70 can influence the DC phenotype in a way that would favour a Th2 or a tolerogenic response in the T cells that interact with these APCs. Tolerogenic effects have also been extensively reported for TBhsp60, reviewed in ref. 9. Also, immunizations with high doses of gp96, a eukaryotic hsp,[55,56] lead to tolerance, suggesting that this may not be a unique property of TBhsp70.

Taken together, our results demonstrate that endotoxin-free TBhsp70 has anti-inflammatory potential, which cannot be explained by contamination, and that it can affect DC differentiation with a magnitude comparable to DEX. The mechanisms by which DEX affects DC maturation are still not clear. A previous study[57] reported that DEX did not induce apoptosis in differentiating DC but rather redirected DC differentiation, leading to DC with suboptimal T-cell-stimulating potential. A study in human cord-blood-derived DC[58] reported that DEX treatment during DC differentiation from $C34^+$ precursors selectively inhibited the differentiation of dermal DC, but not of Langerhans' cells, by blocking their differentiation from $CD14^+$ precursors as well as by inducing apoptosis in the $CD14^+$ DC precursors. $CD4^+$ macrophages were not killed by treatment with DEX. A more recent study,[59] also on cord-blood-derived DCs, showed that DEX added to differentiating DC enhanced DC apoptosis, suppressed differentiation to $CD1a^+$ cells, inhibited expression of CD86 and enhanced IL-10 secretion. If DEX was added during the maturation stage, it caused less dramatic effects.

The exact mechanisms by which TBhsp70 lead to IL-10 induction, DC maturation arrest and inhibition of T-cell proliferation remain to be elucidated. We have tested if TBhsp70 could be toxic – if it would kill splenocytes in culture. The results were negative (not shown). However, it is possible that, similarly to DEX, TBhsp70 could lead to apoptosis of different subpopulations of the differentiating DC. We are currently investigating this hypothesis.

We have previously observed that in human peripheral blood mononuclear cells TBhsp70 induces IL-10 in adherent cells but not in purified T cells.[17] We believe that the proliferation inhibition observed in the present study is not a direct effect on T cells, but rather is a consequence of modulation of the microenvironment by TBhsp70. Further studies are necessary to determine if the effect observed *in vitro* can actually influence *in vivo* immune responses such as graft rejection. Also, it remains to be determined if hsp 70 immunomodulatory activity is solely on the APC or, as was originally demonstrated for hsp 60, also involves the generation of hsp-specific T cells with regulatory potential[60] or a direct effect over T cells.[61,62] We are currently pursuing these studies in our laboratory. Recently, others have shown the ability of TBhsp70 to chaperone peptides to MHC routes[63,64] generating peptide-specific T-cell responses. If this protein proves to be of use as a chaperone to deliver peptides into the MHC, TBhsp70 may constitute a unique tool for autoimmunity therapy, with the advantage of antigen specificity.

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