THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 280, No. 6, Issue of February 11, pp. 4037–4047, 2005

Printed in U.S.A.

Heat Shock Protein 10 Inhibits Lipopolysaccharide-induced Inflammatory Mediator Production*

Received for publication, October 12, 2004
Published, JBC Papers in Press, November 16, 2004, DOI 10.1074/jbc.M411569200

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Heat shock protein 10 (Hsp10) and heat shock protein 60 (Hsp60) were originally described as essential mitochondrial proteins involved in protein folding. However, both proteins have also been shown to have a number of extracellular immunomodulatory activities. Here we show that purified recombinant human Hsp10 incubated with cells *in vitro* reduced lipopolysaccharide (LPS)-induced nuclear factor-κB activation and secretion of several inflammatory mediators from RAW264.7 cells, murine macrophages, and human peripheral blood mononuclear cells. Induction of tolerance by contaminating LPS was formally excluded as being responsible for Hsp10 activity. Treatment of mice with Hsp10 before endotoxin challenge resulted in the reduction of serum tumor necrosis factor-α and RANTES (regulated upon activation, normal T cell expressed and secreted) levels and an elevation of serum interleukin-10 levels. Hsp10 treatment also delayed mortality in a murine graft-*versus*-host disease model, where gut-derived LPS contributes to pathology. We were unable to confirm previous reports that Hsp10 has tumor growth factor properties and suggest that Hsp10 exerts anti-inflammatory activity by inhibiting Toll-like receptor signaling possibly by interacting with extracellular Hsp60.

Mammalian heat shock protein 10 (Hsp10)[1] and heat shock protein 60 (Hsp60), also known as chaperonins 10 and 60, are mitochondrial proteins involved in protein folding. In the mitochondria Hsp10 forms a heptameric lid, which binds to a double-ring toroidal structure comprising seven Hsp60 subunits per ring (1–3). In addition to its mitochondrial role, Hsp60 has also been found at the cell surface (4–10) and in the extracellular fluid (11, 12) and has been associated with a variety of immunomodulatory activities (13, 14). Heat shock proteins (Hsps) exported to the plasma membrane or released from dying cells are believed to be a source of "danger" signals, informing the innate and adaptive immune systems of tissue damage induced by various insults including infection, injury, toxins, heat, and/or cellular stress (13, 15, 16). Elevated expression of Hsp60 has been associated with a number of inflammatory disorders (11, 17, 18), and a correlation has been found between elevated serum Hsp60 and increased levels of the pro-inflammatory cytokine, TNF-α, psychological distress, low socioeconomic status, and social isolation (12).

Toll-like receptors (TLRs) have emerged as central players in inflammation and innate immunity and are triggered by a variety of pathogen- and host-derived factors, thereby detecting both infection and tissue damage. Host Hsp60, Hsp70, and Gp96 have been reported to be agonists for TLR2 and TLR4 (14, 16, 19), and these receptors are also triggered by lipoproteins and lipopolysaccharides (LPS), respectively. How cell surface or serum Hsp60 might interact with TLR4 signaling remains unclear. Hsps have been localized to the multicomponent TLR4 receptor complex and may influence signal transduction (20). Alternatively, Hsp60 may interact with other components of the multifaceted mammalian LPS recognition system leading to modulation of LPS-mediated signaling (7, 21). Recent evidence has, however, cast doubt on the ability of Hsp60 and Hsp70 to induce pro-inflammatory responses by themselves, since Hsp preparations substantially free of LPS and lipoprotein contaminations failed to stimulate macrophages (22, 23). Nevertheless, anti-Hsp60 antibodies can inhibit Hsp60-induced pro-inflammatory cytokine production by macrophages (24, 25), perhaps suggesting that Hsp60 may potentiate TLR4 signaling by low levels of LPS rather than being responsible for TLR-mediated cellular activation in isolation (13).

TLR engagement primarily results in activation of the nuclear factor-κB (NF-κB) (16), although a series of other pathways are also triggered, including interferon regulatory factor 3, p38 mitogen-activated kinase, c-Jun-N-terminal kinase, and extracellular signal related kinase pathways (14, 21, 26). The patterns of gene expression induced by ligation of the different TLRs are distinct but often overlap. For instance a large proportion of the genes up-regulated by double-stranded RNA via TLR3 ligation are also up-regulated by LPS via TLR4 ligation (26). TLRs are expressed on cells of the monocyte lineage including macrophages and dendritic cells, and their ligation usually leads to secretion of a number of pro-inflammatory cytokines and chemokines; for instance LPS binding to macrophage TLR4 results in TNF-α, IL-6, and RANTES secretion (14, 27). Excessive inflammation or uncurtailed immune responses can be detrimental to the host; hence, a number of negative feedback systems have evolved to dampen secretion of pro-inflammatory mediators (28, 29). These include IL-10, an

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[1] The abbreviations used are: Hsp10, heat shock protein 10; Hsp60, heat shock protein 60; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; GVHD, graft-versus-host disease; IFN-γ, interferon γ; IL, interleukin; LPS, lipopolysaccharide; PBMC, human peripheral blood mononuclear cells; RANTES, regulated upon activation, normal T cell expressed and secreted; RLU, relative light units; TLR, Toll-like receptor; TLR2/4, Toll-like receptor 2/4; TNF-α, tumor necrosis factor-α; HIV, human immunodeficiency virus; LTR, long terminal repeat.

important immunoregulatory cytokine that is involved in limitation of inflammatory responses and induction of immune tolerance (29–31). Many diseases are also associated with excessive or chronic inflammation; hence, pro- and anti-inflammatory pathways have become the subject of extensive research with a view to developing novel therapeutic anti-inflammatory and immunomodulatory interventions (28, 29, 31–34).

Hsp10 was originally identified as a factor present in sera during early pregnancy and has shown immunosuppressive activity in experimental autoimmune encephalomyelitis, delayed type hypersensitivity, and allograft rejection models (35, 36). We demonstrate here that recombinant Hsp10 substantially free of endotoxin inhibited LPS-induced activation of NF-κB, reduced LPS-induced TNF-α, RANTES, and IL-6 secretion, and enhanced IL-10 production. Anti-Hsp60 antibodies prevented and mimicked Hsp10 inhibitory activity, suggesting that Hsp10 may inhibit pro-inflammatory responses by interacting with extracellular Hsp60. We were unable to confirm earlier reports that Hsp10 has tumor growth factor properties (37–41).

EXPERIMENTAL PROCEDURES

RAW264-HIV-LTR-LUC Bioassay—RAW264-HIV-LTR-LUC cells (42) (a kind gift from Prof. D. Hume, University of Queensland) were cultured (43) in the presence of G418 (200 μg/ml) for 1 week after recovery from liquid nitrogen and grown as suspension cultures in 25-cm^3 flasks (Greiner Labortechnik, Frickenhausen, Germany). RAW264-HIV-LTR-LUC cells were disaggregated by repeated pipetting and plated at 2.5×10^5 cells/well in 24-well plates and incubated overnight (37 °C and 5% CO_2). LPS from *Escherichia coli* (Sigma L-6529; strain 055:B5, Sigma) was dissolved in sterile distilled water and stored at 4 °C at 1 mg/ml in glass vials. Immediately before use the solution was vigorously vortexed before aliquots were taken. The cells were incubated with LPS, Hsp10, or control buffers for 2 h followed by the addition of stimulating LPS at the indicated concentrations. After a further 2-h incubation the adherent cells were processed for the luciferase assay (Luciferase Assay System, Promega, Madison, WI). Luciferase activity was read for 15 s on a Turner Designs Luminometer TD 20/20.

Production and Purification of Hsp10—Recombinant human Hsp10 (GenBank^{TM} accession number X75821) was produced in *E. coli* essentially as described by Ryan *et al.* (44). In addition, the material that did not bind Macro-Prep High Q (Bio-Rad) was further purified by S-Sepharose and then gel filtration (Superdex 200, Amersham Biosciences). Purified Hsp10 in a 50 mM Tris-HCl (pH 7.6) and 150 mM NaCl buffer was filtered through an Acrodisc filter system with a 0.2-mm Mustang E membrane according to the manufacturer's instructions (Pall Corp., Ann Arbor, MI; catalog number MSTG5E3) to remove residual endotoxins and was stored at −70 °C. The purity of Hsp10 was determined to be >97% by SDS-PAGE. Aliquots were thawed once before use. All batches of Hsp10 showed the same molar activity as GroES in GroEL-mediated rhodanese refolding assays (45) (data not shown). LPS contamination of Hsp10 was determined by the Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, MD) to be <1 endotoxin units/mg purified Hsp10 protein.

Trypsin Treatment of Hsp10—2.5% trypsin (Gibco) was filtered through an Acrodisc filter as above twice and added at 40 μg/ml to Hsp10 (at 2–3 mg/ml). After incubation at 37 °C overnight the trypsin/Hsp10 solution was heated to 90 °C for 15 min to destroy trypsin activity and cooled before addition to the bioassays. After trypsin treatment no Hsp10 could be detected by SDS-PAGE and Western blotting, and the material was inactive in the rhodanese refolding assay (data not shown).

Tumor Cell Lines—K562 (human erythroleukemia), Mono Mac 6 (human monocytic line), U937 (human histiocytic lymphoma), P815 (mouse mastocytoma), EL4 (mouse T cell lymphoma), Jurkat (human T cell leukemia), RAW 264.7 (ATCC TIB 71, mouse macrophage), L929 (mouse fibrosarcoma), B16 (mouse melanoma), HeLa (human cervical carcinoma), and MCA-2 (mouse fibrosarcoma) cell lines were shown to be mycoplasma negative (46). Cells were grown in medium including RPMI 1640 (or Dulbecco's modified Eagle's medium for MCA-2 cells) (Gibco), 10% fetal bovine serum (Gibco), 2 mM glutamine (Sigma), 10 mM HEPES (Sigma), 100 μg/ml streptomycin, and 100 IU/ml penicillin (CSL Ltd, Melbourne, Australia).

In Vitro Growth Assay—Cells were seeded into 96-well flat bottom plates at 4×10^3 cells/well at the indicated concentration of Hsp10. After 2–4 days in culture and before cells reached confluence, adherent cells were fixed in 100% methanol, and protein was determined using the sulforhodamine B assay (47). Non-adherent cells were counted under microscope hemocytometer in the presence of trypan blue to indicate non-viable cells. Anti-Hsp10 antibody was raised in rabbits by immunizing four times with Hsp10 (100 μg) emulsified in complete Freund's adjuvant. IgG was purified from serum using protein A-Sepharose (Sigma) followed by affinity column chromatography using Hsp10 coupled to Affi-Gel 10 (Bio-Rad). Purified anti-Hsp10 IgG at 2.74 mg/ml had an end point titer of 1 in 2 million by enzyme-linked immunosorbent assay (ELISA). IgG purified from naive rabbit serum was used as a control and had no detectable reactivity to Hsp10 by ELISA.

In Vivo Tumor Growth—B16 cells, Lewis lung, or MCA-2 tumors were inoculated subcutaneously (10^5 cells per site, 2 sites per mouse, 5 mice per group) into the shaved backs of C57BL/6 or CBA mice (for MCA-2). Doses of 10 or 100 μg/mouse of Hsp10 were given subcutaneously daily for 10 days beginning on the day of tumor inoculation. Control groups received the same volume of 50 mM Tris/saline buffer. Tumor growth was monitored as described previously (48). Mice bearing tumors greater than 100 mm^2 were euthanized.

RAW264.7 IL-6 and RANTES Assays—RAW264.7 cells were seeded at 2.5×10^5 cells/well in 24-well plates and cultured overnight (37 °C and 5% CO_2). Hsp10 or buffer was added to cells in triplicate for 2 h followed by the addition of LPS (1 ng/ml). After 6 h, supernatants were collected and analyzed for RANTES and IL-6 using specific ELISA-paired antibody kits (R & D Systems, Minneapolis, MN). ELISA standards and samples were run in duplicate, and the sensitivity of the assays was 31 and 15.6 pg/ml for RANTES and IL-6, respectively. The optical density (450 nm) of each sample was determined using a microplate reader (Tecan Sunrise, Austria, with Magellan V3.11 software).

Cytokine Production from Splenocytes and Macrophages Derived from Hsp10-treated Animals—C57BL/6 (H-2^b, Ly-5.2$^+$) mice were purchased from the Australian Research Centre (Perth, Western Australia, Australia) and C57BL/6 IL-10$^{-/-}$ mice (H-2^b, Ly-5.2$^+$) were supplied by the Australian National University (Canberra, Australia). The culture medium used throughout was 10% fetal bovine serum/Iscove's modified Dulbecco's medium (JRH Biosciences, Lenexa, KS) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acid, 0.02 mM β-mercaptoethanol, and 10 mM HEPES, and cells were cultured at pH 7.75, 37 °C, and 5% CO_2. C57BL/6 mice ($n = 3$ per group) were treated with subcutaneous injections of Hsp10 (100 μg) or diluent buffer daily for 5 days, and peritoneal macrophages were harvested on the sixth day by peritoneal lavage and pooled from individual animals within the treatment group. Cells were plated in triplicate at 2×10^5/well in the presence of LPS (1 μg/ml). Culture supernatants were collected at 5 h, and levels of TNF-α were assessed by ELISA. Results were normalized to production per 10^5 macrophages based on CD11b staining by fluorescence-activated cell sorter analysis of input cells.

For IL-10 determination, splenocytes were harvested from the same animals and pooled as above and cultured in triplicate at 5×10^5 cells/well in the absence (not shown) or presence of LPS (10 μg/ml). Culture supernatants were collected at 48 h, and levels of IL-10 were determined by ELISA.

For T cell-derived cytokine production, spleen-derived nylon wool-purified T cells (49) (2×10^6 CD3-positive cells/24 well) from Hsp10-treated or control-treated C57BL/6 mice were stimulated *in vitro* with irradiated (2000 rads) allogeneic B6D2F1 splenocytes (3×10^6/24 wells). After 7 days cells were collected and plated in triplicate (5×10^5 CD3-positive cells/96-well) and re-stimulated with plate-bound antibodies to CD3 and CD28 (from hybridomas 2C11 and N37.51), which were prepared by incubating the antibodies (10 μg/ml) overnight at 4 °C in 96-well flat bottom plates followed by washing twice with phosphate-buffered saline (49). Culture supernatants were harvested at 24 h, and concentrations of cytokines were determined by ELISA.

The monoclonal antibody pairs used in the TNF-α, interferon-γ (IFN-γ), IL-10, and IL-4 ELISA assays were used at concentrations recommended by the supplier (Pharmingen). Supernatants were diluted in culture medium 1:1 for IL-4, IL-10, and TNF-α and 1:50 for IFN-γ. Cytokines were captured by the capture antibody and detected by the direct biotin-labeled detection antibody. Streptavidin-labeled horseradish peroxidase (Kirkegaard and Perry laboratories, Gaithersburg, MD) and substrate (Sigmafast OPD, Sigma) was then used to measure immobilized biotin. Plates were read at 492 nm using the Spectraflour Plus microplate reader (Tecan). Recombinant cytokines (Pharmingen) were used as standards for ELISA assays. Standards were run in

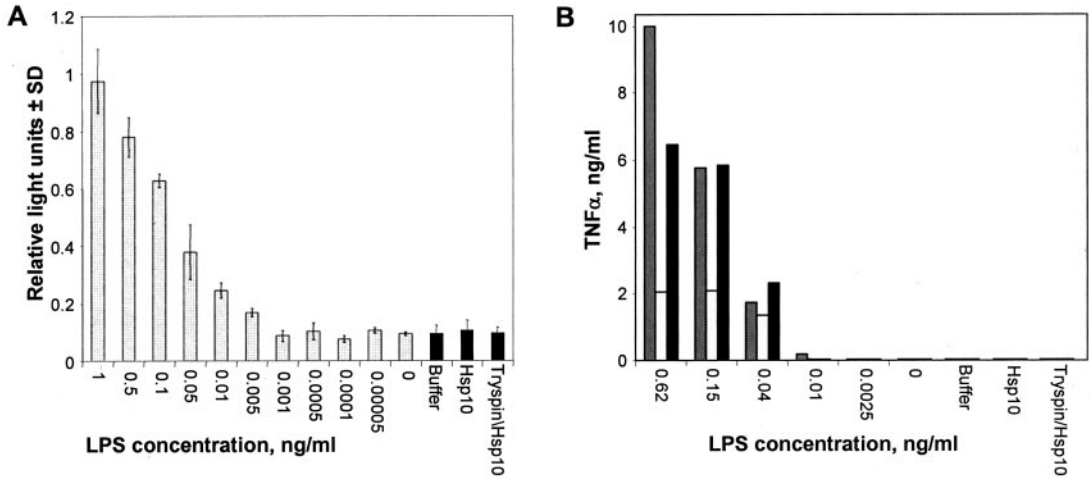

FIG. 1. **Lack of detectable endotoxin contamination in preparations of recombinant Hsp10.** *A*, RAW264-HIV-LTR-LUC cells were stimulated for 2 h with a range of LPS concentrations ($n = 3$ replicates per concentration, *light bars*), the Tris/NaCl buffer used to dissolve the Hsp10 (Buffer, $n = 5$), Hsp10 (Hsp10, $n = 5$), and trypsin/heat-treated Hsp10 (Trypsin/Hsp10, $n = 4$). Results are expressed as relative light units of luciferase activity. *B*, human PBMC from three different donors (*dark gray*, donor 1; *light gray*, donor 2; *black bars*, donor 3) were stimulated with a range of LPS concentrations, diluent buffer (*Buffer*), Hsp10 (*Hsp10*), and trypsin/heat-treated Hsp10 (*Trypsin/Hsp10*). Supernatants were removed after 20 h and analyzed for levels of TNF-α by ELISA. The limit of detection of this assay was 31 pg/ml TNF-α.

duplicate, and the sensitivity of the assays was 0.063 units/ml for IFN-γ and 15 pg/ml for IL-10, IL-4, and TNF-α.

Human Peripheral Blood Mononuclear Cell (PBMC) TNF-α and IL-6 Assays—Human PBMC were isolated from heparinized blood of healthy volunteers by buoyant density gradient centrifugation on Ficoll-Paque Plus (Amersham Biosciences). PBMC were dispensed at 10^6 viable cells/ml in 200 μl in 96-well tissue culture plates (Greiner Bio-One, Kremsmuenster, Austria). Hsp10 was then added, and plates were incubated for 1 h followed by LPS addition and a further 20-h incubation at 37 °C and 5% CO_2, after which supernatants were collected, and duplicate samples were analyzed for TNF-α and IL-6 levels (Duoset ELISA kits, R & D Systems). The sensitivity of these assays was 31 pg/ml TNF-α and 9 pg/ml IL-6.

Mouse Serum TNF-α, RANTES, and IL-10 Assays after LPS Injection—Female BALB/c mice aged 6–8 weeks were given 100 μg of Hsp10/mouse with or without LPS at 10 μg/mouse. Hsp10 was administered intravenously ~30 min before the intravenous injection of LPS diluted in phosphate-buffered saline (the final volume was 100 μl; see above for preparation and storage of LPS stock solution). The mice were sacrificed, and blood was collected via cardiac puncture 1.5 h after LPS challenge. Blood was collected into 0.8-ml clotting accelerator tubes (MiniCollect, Interpath) and serum-stored at 4 °C for analysis of TNF-α, RANTES, and IL-10 using commercially available ELISA kits (R & D Systems, BD Biosciences Pharmingen).

Bone Marrow Transplantation and Graft Verses Host Disease—Female 8–14-week-old C57BL/6 (B6, H-2^b, Ly-5.2^+), B6 Ptprc^a Ly-5^a (H-2^b, Ly-5.1^+), and B6D2F1 (H-$2^{b/d}$, Ly-5.2^+) (50) mice were purchased from the Australian Research Centre (Perth, Western Australia). Hsp10 (100 μg per animal) or control diluent buffer was injected subcutaneously daily for 5 days into donor and recipient animals before transplant. Mice were housed in sterilized microisolator cages and received acidified autoclaved water (pH 2.5) and normal food for the first 2 weeks post-transplantation. Mice were transplanted according to a standard protocol described previously (51–53). Briefly, on day 1 B6D2F1 mice received 1300 centigray total body irradiation (^{137}Cs source at 108 centigray/min) split into two doses separated by 3 h to minimize gastrointestinal toxicity. Donor bone marrow (5×10^6) and donor nylon wool purified splenic T cells (2×10^6) were resuspended in 0.25 ml of Leibovitz's L-15 media (Invitrogen) and were injected intravenously into each recipient. Survival was monitored daily, and graft-versus-host disease (GVHD) clinical scores were measured weekly. The degree of systemic GVHD was assessed by a scoring system, which sums changes in five clinical parameters, which are weight loss, posture (hunching), activity, fur texture, and skin integrity (maximum index = 10) (51–54). Individual mice were ear-tagged and graded from 0 to 2 for each criterion without knowledge of treatment group. Animals with severe clinical GVHD (scores >6) were sacrificed according to ethical guidelines, and the day of death was deemed to be the following day.

Inhibition of Hsp10 Activity with Anti-Hsp60 Serum—RAW264-HIV-LTR-LUC cells were plated and incubated overnight as above. The medium was replaced, and goat polyclonal anti-Hsp60 serum (1/1000 dilution) (Stressgen, Victoria, Canada) or the same dilution of normal goat serum was added for 1 h. Hsp10 or buffer was then added for a further 2 h followed by buffer or LPS at the indicated concentrations, and luciferase activity was measured 2 h later.

Statistical Analysis—Statistical analysis (univariate analysis of variance: ANOVA, Student's t test, or log rank statistic) was performed using SPSS for Windows 11.5.0 (SPSS Inc.).

RESULTS

The Endotoxin Content of Purified Recombinant Hsp10 Was Below Detection—The Hsp literature has been complicated by the potential presence of contaminating endotoxin in recombinant Hsp preparations (22, 23). To illustrate that recombinant Hsp10 preparations were substantially free of endotoxin, two highly sensitive bioassays were used. The RAW264-HIV-LTR-LUC cell line is a mouse macrophage cell line (RAW264.7) stably expressing a luciferase reporter gene under the control of an HIV long terminal repeat promoter, which is highly and rapidly responsive to NF-κB stimulation. These cells provide a sensitive bioassay for the presence of LPS (42, 55), and in our hands the assay was able to detect 5–10 pg/ml *E. coli* LPS (Fig. 1*A*, *light bars*). Importantly, Hsp10 (100 μg/ml), and the diluent Tris/NaCl buffer failed to generate luciferase activity above background (Fig. 1*A*, *Hsp10* and *Buffer* (*dark bars*)).

The second endotoxin bioassay utilized TNF-α production by human PBMC and was able to detect 40 pg/ml LPS (Fig. 1*B*). Using this assay neither Hsp10 (100 μg/ml) nor the diluent buffer induced detectable TNF-α secretion in PBMC from three donors (Fig. 1*B*). Thus, the Hsp10 material used in this study contained levels of endotoxin below the level of detection of two highly sensitive bioassays and failed to stimulate either RAW264-HIV-LTR-LUC cells or PBMC when used at 100 μg/ml.

Because Figs. 3 and 5 will illustrate that Hsp10 inhibits LPS stimulation of RAW264-HIV-LTR-LUC cells and PBMC, respectively, it was important to establish that Hsp10 did not inhibit the ability to detect potential LPS contamination in these bioassays. Trypsin treatment of Hsp10 resulted in the degradation of Hsp10 to levels undetectable by SDS-PAGE and Western blotting and the rhodanese refolding assay (data not shown). Trypsin treatment included a final heating to 90 °C for 15 min to destroy trypsin activity before the use of the material in the bioassays (see "Materials and Methods"). This heat treatment did not affect LPS activity (*Trypsin LPS*, Fig. 3*B*). Im-

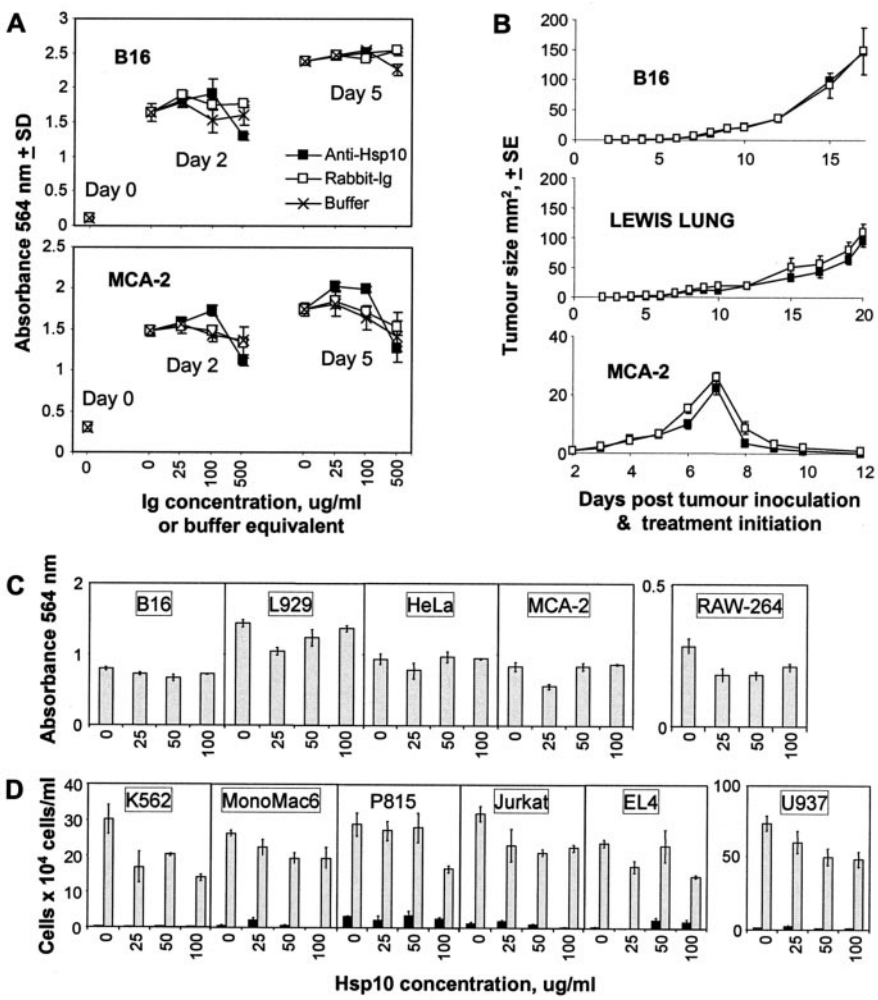

FIG. 2. **Anti-Hsp10 antibodies did not inhibit and Hsp10 did not enhance growth of tumor lines *in vitro* and *in vivo*.** *A*, anti-Hsp antibodies did not influence tumor cell growth *in vitro*. B16 and MCA-2 cells were seeded into 96-well plates in triplicate in the presence of the indicated concentrations of affinity purified rabbit anti-Hsp10 immunoglobulin (*Anti-Hsp10*), control rabbit immunoglobulin (*Rabbit Ig*), or buffer. Parallel plates were fixed 4 h after seeding (Day 0) and on days 2 and 5, and cell numbers were assessed using the sulforhodamine B protein stain. *B*, Hsp10 did not influence tumor growth *in vivo*. Mice (n = 5 per group) were inoculated with B16, Lewis Lung, or MCA-2 tumors subcutaneously (2 tumors per mouse) and given 100 μg of Hsp10 (*black squares*) or the same volume of buffer (*white squares*) daily for 10 days starting on the day of tumor inoculation. Tumor size was determined at the indicated times. *C* and *D*, Hsp10 did not enhance tumor growth *in vitro*. Tumor cell lines were seeded into 96-well plates and cultured for 2–3 days (before reaching confluence) in the presence of the indicated concentration of Hsp10. Cell numbers were then assessed using the sulforhodamine B protein stain for adherent cells (*C*) or trypan blue exclusion (*D*) for non-adherent cells (*dark bars* represent non-viable cell counts, and *light bars* represent viable cell counts, which exclude the dye). Data represent the mean of triplicate wells ± S.D.

portantly, trypsin-treated Hsp10 failed to stimulate RAW264-HIV-LTR-LUC cells or PBMC (Fig. 1, *A* and *B*, *Trypsin/Hsp10*), illustrating that Hsp10 did not mask potential LPS contamination.

Hsp10 and Anti-Hsp10 Antibodies and the Growth of Tumor Cells in Vitro and in Vivo—Monoclonal antibodies to Hsp10 have been reported to inhibit the growth of mouse B16 melanoma and MCA-2 fibrosarcoma cells *in vitro* (40) and *in vivo* (41), leading to the conclusion that Hsp10 has growth factor properties. Using a high titer, affinity-purified polyclonal anti-Hsp10 antibody at concentrations up to 500 μg/ml, we were unable to influence the growth of B16 or MCA-2 *in vitro* when compared with purified control antibodies used at the same dose (Fig. 2*A*). The results from the previous studies may be explained by the use of ascites-derived anti-Hsp10 monoclonal antibodies, whereas the control antibodies were derived from tissue culture supernatants (40, 41). Ascites fluid-derived material frequently contains growth-suppressing inflammatory cytokines, which are not usually present in tissue culture-derived antibody preparations (56).

To further investigate potential growth factor activity of Hsp10, the growth of a panel of tumor cells was tested *in vivo* and *in vitro* in the presence of purified recombinant Hsp10. Hsp10 administered at 10 μg (data not shown) or 100 μg per day for 10 days did not influence the growth of B16, MCA-2, or Lewis Lung tumors *in vivo* (Fig. 1*C*) (in our hands the MCA-2 tumor spontaneously regressed and was no longer visible on day 12). Furthermore, Hsp10 did not enhance the growth of a large panel of tumor cell lines including B16 and MCA-2 *in vitro* (Fig. 1, *C* and *D*). Taken together with the antibody data, these experiments do not support a role for Hsp10 as a tumor growth factor.

Hsp10 Inhibited LPS Signaling in RAW264-HIV-LTR-LUC Indicator Cells—During the endotoxin testing experiments (Fig. 1), it emerged that Hsp10 was able to inhibit LPS-mediated activation of the RAW264-HIV-LTR-LUC cell line. The HIV long terminal repeat promoter present in these cells is highly and rapidly responsive to NF-κB stimulation, and these cells provide a sensitive bioassay for analysis of TLR4 signaling pathways in macrophages stimulated with

bacterial LPS (42, 55). To avoid the use of supra-physiological levels of LPS (23), a titration range for LPS concentration was established that represented ~80, 50, and 20% of maximal LPS-stimulated luciferase activity; these were 5, 1, and 0.2 ng/ml, respectively (data not shown). At these doses of LPS, preincubation of RAW264-HIV-LTR-LUC cells with 100 μg/ml Hsp10 for 2 h significantly inhibited LPS-stimulated luciferase activity by ≈30–50% (Fig. 3*A*). Preincubation periods below 30 min resulted in less reproducible inhibition (data not shown).

To illustrate that the inhibitory activity of Hsp10 (Fig. 3*A*) was lost after proteolytic degradation, Hsp10 was digested with trypsin, and the trypsin activity was destroyed by heating (90 °C for 15 min) before the addition to the bioassays (see "Materials and Methods"). Destruction of Hsp10 by trypsin was confirmed by SDS-PAGE/Western blotting and the rhodanese refolding assay (data not shown). (Heating alone failed to affect significant Hsp10 activity in the LUC or the rhodanese refolding assays; data not shown). Hsp10 pretreatment again significantly inhibited LPS-mediated luciferase activity (Fig. 3*B*, *Control versus Hsp10*, $p < 0.001$, see the figure legend). However, trypsin/heat-treated Hsp10 failed to inhibit LPS signaling (Fig. 3*B*, *Trypsin Hsp10*), giving values similar to trypsin/heat-treated buffer controls (Fig. 3*B*, *Trypsin Buffer*). As expected, trypsin treatment (followed by heating at 90 °C for 15 min) failed to affect significantly the activity of *E. coli* LPS (Fig. 3*B*, *Trypsin LPS*). Thus, proteolytic degradation of Hsp10 resulted in the loss of inhibitory activity by Hsp10 preparations, illustrating that trypsin-resistant contaminants (*e.g.* LPS) of the Hsp10 preparations were not responsible for the inhibitory activity.

Tolerance Induction Was Not Responsible for Inhibition of LPS Signaling in RAW264-HIV-LTR-LUC Cells—LPS tolerance is a well recognized phenomenon whereby the response to a second stimulus with LPS is reduced. LPS tolerance is normally induced if the time interval between the two LPS exposures exceeds 3 h and the concentration of the LPS during the initial exposure is sufficiently high to stimulate the macrophages (57, 58). To formally discount tolerance induction as responsible for the observations in Fig. 3*A*, RAW264-HIV-LTR-LUC cells were pretreated with a range of LPS concentrations for 2 h followed by stimulation with 5, 1, and 0.2 ng/ml LPS. Pretreatment with LPS concentrations ranging from 1 to 0.0005 ng/ml did not inhibit the induction of LUC activity by the second LPS exposure (Fig. 3*C*). Thus, for the RAW264-HIV-LTR-LUC system the 2-h pretreatment period appeared insufficient for tolerance induction. Furthermore, sub-stimulating doses of LPS (0.005–0.0005 ng/ml), potentially similar to those found in Hsp10 preparations, also failed to inhibit LPS-mediated LUC activity. LPS tolerance was, therefore, not responsible for the observations in Fig. 3*A* since (i) the 2-h interval between LPS exposures was insufficient for LPS tolerance induction in the RAW264-HIV-LTR-LUC system, and (ii) undetectable (or sub-stimulating) levels of LPS, which are potentially contaminating the Hsp10 preparations, were unable to mediate LPS tolerance.

Hsp10-mediated Reduction of LPS-stimulated LUC Activity in RAW264-HIV-LTR-LUC Cells Was Dose-responsive—The reductions in LUC activity shown in Fig. 3*A* were obtained using 100 μg/ml Hsp10. To determine whether the activity of Hsp10 was dose-related, RAW264-HIV-LTR-LUC cells were treated with a range of Hsp10 concentrations before the addition of LPS (treatment with 100 μg/ml, thus, represents a repeat of the experiments shown in Fig. 3*A*). A clear dose response emerged with increasing levels of inhibition apparent from 2 to 100 μg of Hsp10, with the inhibition appearing to level off after 100 μg/ml (Fig. 3*D*).

Hsp10 Reduced RANTES and IL-6 Production in LPS-stimulated RAW264.7 Cells—To determine whether the Hsp10-mediated reduction in NF-κB activation seen in Fig. 3*A* also affected LPS-induced pro-inflammatory mediator secretion, the affect of Hsp10 on RANTES and IL-6 secretion levels by RAW264.7 cells was investigated. An LPS dose of 1 ng was used that induced ~50% of maximal RANTES production in this 6-h assay (data not shown). The 2-h preincubation period (similar to that used above) was unable to induce tolerance in these assays irrespective of the dose of LPS used (data not shown). Hsp10 mediated a dose-related reduction in both LPS-induced RANTES (Fig. 3*E*) and IL-6 (Fig. 3*F*) secretion, illustrating that inhibition of NF-κB activation in this system leads to reduction in pro-inflammatory mediator secretion.

Hsp10 Activity Was Independent of IL-10—To determine the effect of Hsp10 on cells *in vivo*, mice were treated with Hsp10 followed by isolation of their peritoneal macrophages for stimulation with LPS *in vitro*. LPS-induced secretion of TNF-α from the peritoneal macrophages was significantly lower in animals that had been treated with Hsp10 (Fig. 4*A*), illustrating that Hsp10 mediated similar effects on primary macrophages treated *in vivo* as seen with RAW264.7 cells treated *in vitro*.

IL-10 is a potent immunosuppressive cytokine able to inhibit TLR4 signaling (59, 60). When splenocytes from Hsp10-treated animals were stimulated with 10 μg/ml LPS (the minimum dose that reliably induces detectable IL-10 in this system), a significant increase in IL-10 production was observed compared with buffer-treated animals (Fig. 4*B*). However, the Hsp10-mediated reduction in LPS-induced TNF-α production (Fig. 4*A*) did not require IL-10, since similar reductions in TNF-α secretion were observed when peritoneal macrophages from Hsp10-treated IL-$10^{-/-}$ mice were stimulated with LPS *in vitro* (Fig. 4*C*). Thus, reduced TNF-α secretion and increased IL-10 production appear to be independent consequences of Hsp10 treatment.

Hsp10 Treatment and T Cell IFN-γ and IL-4 Secretion—Th1 immune responses are usually characterized by the production of pro-inflammatory cytokines such as TNF-α and IFN-γ, whereas Th2 responses involve IL-4 and IL-10 secretion. Because Th2 responses are often able to suppress Th1 cytokine production, the influence of Hsp10 on Th1/Th2 balance was investigated. Mice were treated with Hsp10 as above, and the splenocyte-derived T cell populations were stimulated *in vitro* with allogeneic splenocytes for 7 days in a mixed lymphocyte culture. After this Hsp10 treatment protocol, the proliferation of T cells within the mixed lymphocyte cultures was not significantly different between Hsp10-treated and control animals (data not shown). The resulting cultures were then stimulated with plate-immobilized CD3/CD28 antibodies (to stimulate T cells), and the culture supernatants were examined for cytokines. Neither T cell IL-4 secretion (Fig. 4*D*) nor IFN-γ secretion (Fig. 4*E*) differed significantly between Hsp10-treated and control animals. Thus, at the Hsp10 dose and treatment regimens used here, Hsp10 did not significantly influence the Th1/Th2 profile. T cell IL-10 secretion was slightly but significantly elevated (Fig. 4*F*), and a similar elevation in IL-10 secretion could be observed when Hsp10 was added *in vitro* to a mixed lymphocyte culture with the cells derived from untreated animals (data not shown). These data indicate that Hsp10 can enhance IL-10 production in response to mixed lymphocyte culture stimulation and suggest that the Hsp10-enhanced IL-10 production seen above may arise in part from T cell-derived IL-10.

Hsp10 Reduced LPS-induced TNF-α and IL-6 Production in Human Peripheral Blood Mononuclear Cells—To determine whether Hsp10 is also active on primary human cells, PBMC

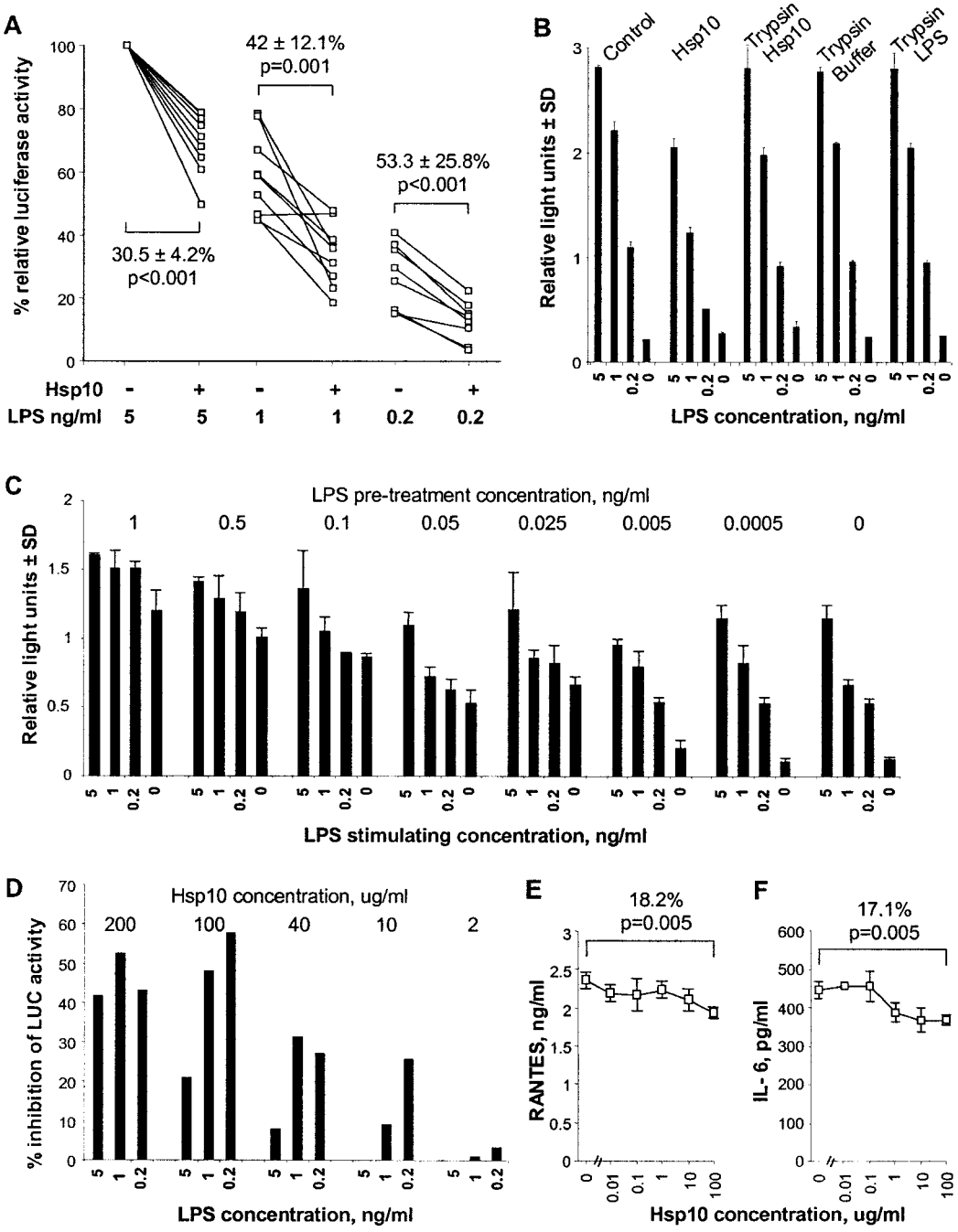

FIG. 3. **Hsp10 inhibited LPS-induced activation of NF-κB activity in RAW264.7 cells.** *A*, Hsp10-mediated inhibition of LPS-induced NF-κB activity. In nine separate experiments 100 μg/ml Hsp10 (*Hsp10+*) or buffer (*Hsp10−*) was preincubated with RAW264-HIV-LTR-LUC cells for 2 h. LPS was then added at 5, 1, or 0.2 ng/ml, and luciferase activity was measured after another 2-h incubation. The relative light units (RLU) of luciferase obtained with 5 ng/ml LPS (in the absence of Hsp10) was set at 100% relative luciferase activity, and 0% represents the RLU obtained in the absence of LPS. Hsp10, in the absence of LPS, did not stimulate significant luciferase activity (data not shown). The mean percentage reduction (±S.D.) in RLU mediated by Hsp10 is indicated for each concentration of LPS, and the significance was calculated using a paired *t* test. *B*, trypsin-treated Hsp10 failed to inhibit LPS-induced NF-κB activity. Treatment of RAW264-HIV-LTR-LUC cells in duplicate for 2 h with 100 μg/ml Hsp10 (*Hsp10*) significantly reduced the RLU induced by LPS compared with cells treated with buffer (*Control*). Calculation of the percent reductions in RLU after subtraction of background were 29.7 ± 0.8 (S.D.), 50 ± 4.6, and 71 ± 7.7 for 5, 1, and 0.2 ng/ml, respectively ($p < 0.001$ by two factor ANOVA, which included a term for LPS concentration). Compared with the control, treatment with trypsin-treated Hsp10 (*Trypsin Hsp10*) gave 0.1 ± 8.8, 11.6 ± 4.2, and 21 ± 7.4, and trypsin-treated buffer (*Trypsin Buffer*) gave 1.4 ± 2.1, 5.8 ± 1.1, and 14.9 ± 2.4% reduction for 5, 1, and 0.2 ng/ml LPS, respectively (neither was significantly different from control or each other). Trypsin treatment of the stimulating LPS did not affect LPS activity (*Trypsin LPS*, $p > 0.05$). *C*, LPS pretreatment 2 h before LPS stimulation did not inhibit LUC activity. RAW264-HIV-LTR-LUC cells were pretreated with the indicated LPS concentrations in duplicate, after 2 h the cells were stimulated with 5, 1, 0.2, and 0 ng/ml LPS, and the LUC activity was measured 2 h later. *D*, Hsp10-mediated reduction in LPS-induced LUC activity is dose-responsive. The experiment was set up as in Fig. 3*A*, except the Hsp10 concentration was varied as indicated. For each LPS concentration the percent inhibition in LUC activity over control cells not pretreated with Hsp10 is indicated. *E* and *F*, Hsp10-mediated inhibition of LPS-induced RANTES and IL-6 secretion. RAW264.7 cells were incubated with the indicated Hsp10 concentration for 2 h followed by the addition of 1 ng/ml LPS. After 6 h triplicate supernatants were analyzed for RANTES and IL-6 by ELISA. The mean percentage reduction in RANTES and IL-6 secretion mediated by 100 μg/ml Hsp10 is indicated. Significance was calculated using a paired *t* test.

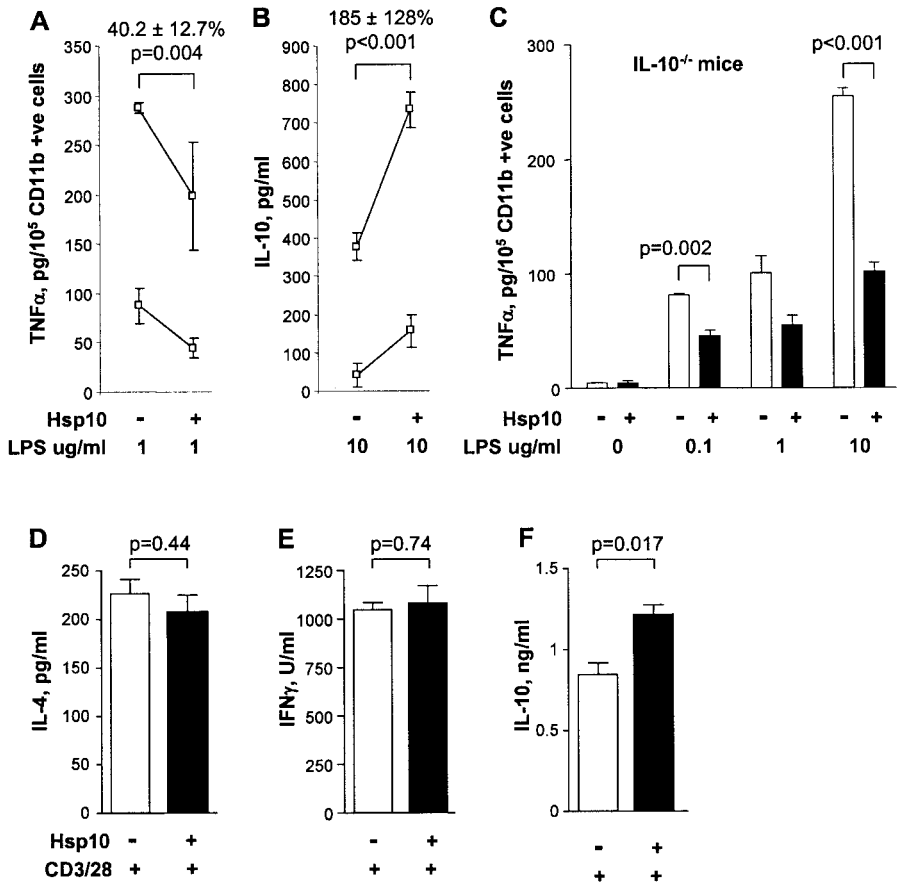

FIG. 4. **Effect of Hsp10 on cytokine secretion in murine systems.** *A*, Hsp10 treatment reduced the capacity of LPS-stimulated peritoneal macrophages to produce TNF-α. C57BL/6 mice (n = 3 per group) were treated with Hsp10 (*Hsp10*+) or buffer (*Hsp10*−). Peritoneal macrophages were harvested by peritoneal lavage on day 6 and pooled from individual animals within the treatment group. Cells were plated at 2×10^5 cells/well in the presence of LPS (1 μg/ml). Culture supernatants were collected at 5 h, and levels of TNF-α were assessed by ELISA (wells without LPS produced no detectable TNF-α; data not shown). The mean ± S.E. of triplicate wells are shown and are normalized to production per 10^5 macrophages based on CD11b staining. Data from two repeat experiments are shown, the average percentage reduction in TNF-α (± S.D.) is indicated, and the significance was calculated using a two way ANOVA, which takes account of the different experiments. *B*, Hsp10 treatment augmented IL-10 production from splenocytes. C57BL/6 mice (n = 3 per group) were treated with either Hsp10 or control diluent as above. Splenocytes were harvested on day 6, pooled from individual animals within a treatment group, and cultured at 5×10^5 cells/well in the presence of LPS (10 μg/ml). Culture supernatants were collected at 48 h, and levels of IL-10 were determined by ELISA (wells without LPS produced no detectable IL-10; data not shown). Means ± S.E. of triplicate wells are shown. Average percentage increase is shown with statistics calculated as for A. *C*, Hsp10 treatment reduced TNF-α production from IL-$10^{-/-}$ peritoneal macrophages. IL-$10^{-/-}$ C57BL/6 mice (n = 3) were treated with Hsp10 (*black bars*) or control diluent (*white bars*) as above, and peritoneal macrophages were harvested as for *A*. After 5 h of culture in the presence of LPS (0.1, 1, or 10 μg/ml) TNF-α was determined in culture supernatants by ELISA. The mean ± S.E. of triplicate wells for one representative experiment is shown. TNF-α levels were compared for Hsp10-treated and control animals using a non-parametric t test. *D–F*, effect of Hsp10 treatment on T cell IL-4, IFN-γ, and IL-10 secretion. C57BL/6 mice were treated with Hsp10 as above. T cells (3×10^6 cells/well) from Hsp10-treated and control animals were stimulated in culture for 7 days with irradiated allogeneic B6D2F1 splenocytes (5×10^6 cells/well). On day 7 T cells were collected and plated in triplicate (10^5 CD3-positive cells/well) and re-stimulated with plate-bound antibodies to CD3 and CD28. Culture supernatants were harvested at 24 h, and concentrations of IFN-γ, IL-4, and IL-10 were determined by ELISA. Results are the mean ± S.E. of triplicate wells from one representative experiment, and statistics were calculated as for *C*.

from healthy donors were pretreated with Hsp10 or buffer for 1 h and then stimulated with 0.04 ng/ml LPS for 20 h. This dose of LPS was established as the lowest dose reliably able to stimulate significant TNF-α secretion (Fig. 1) and corresponds to the dose in humans that produces a mild transient syndrome similar to clinical sepsis (61, 62). In PBMC from 8 donors, 1 μg/ml Hsp10 mediated an average 23.7% reduction, and 10 μg/ml mediated an average 23.3% reduction in LPS-induced TNF-α secretion (Fig. 5*A*), illustrating that Hsp10 also reduces LPS-induced TNF-α secretion from PBMC. No significant reduction in TNF-α secretion was observed when 0.1 μg/ml Hsp10 was used (data not shown). To illustrate that tolerance induction was not operating in this system, PBMC were pretreated with a range of LPS concentrations, and 1 h later they were stimulated with 0.04 ng/ml LPS for 20 h. LPS pretreatment for 1 h did not inhibit TNF-α secretion stimulated by the second LPS treatment (Fig. 5*B*), illustrating that LPS tolerance does not account for the activity of Hsp10.

IL-6 is another well known inflammatory cytokine induced by LPS (63). To determine whether Hsp10 inhibited LPS-induced IL-6 secretion, PBMC from eight donors were treated with 1 or 10 μg/ml Hsp10 or buffer for 1 h followed by stimulation with 0.04 ng/ml LPS for 20 h. An average 18.6 and 24.4% reduction in LPS-induced IL-6 secretion was observed for 1 or 10 μg/ml Hsp10, respectively (Fig. 5*C*). No significant reduction in IL-6 secretion was observed when 0.1 μg/ml Hsp10 was used (data not shown). LPS pretreatment for 1 h did not inhibit IL-6 secretion stimulated by a second LPS treatment, again illustrating that tolerance induction was not operating in this system (Fig. 5*D*).

Hsp10 Treatment Inhibited LPS-induced TNF-α Secretion in Vivo—A modified endotoxemia model was used to determine whether Hsp10 delivered *in vivo* would reduce the levels of pro-inflammatory mediators in serum after LPS challenge.

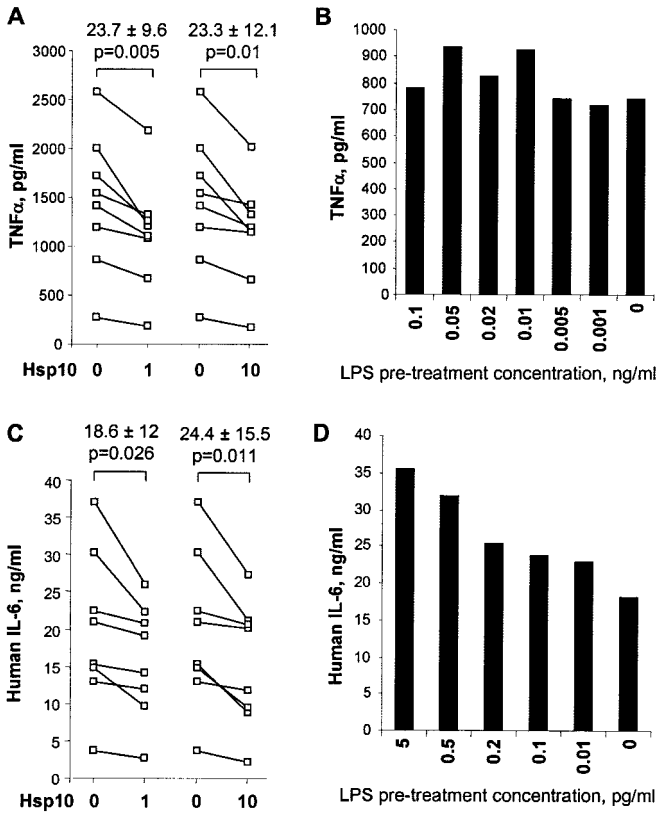

FIG. 5. **Hsp10 treatment of human peripheral blood mononuclear cells reduced LPS-induced TNF-α and IL-6 secretion.** *A*, Hsp10 reduced LPS-induced TNF-α secretion. Hsp10 (1 and 10 μg/ml) or buffer (0) was added to PBMC from eight different donors 1 h before the addition of 0.04 ng/ml LPS. Supernatants were removed after 20 h and analyzed for TNF-α. The percentage reductions and significance calculated using a paired *t* test are indicated. For all donors 10 μg/ml Hsp10 in the absence of LPS failed to induce TNF-α levels above the level of detection (31 pg/ml) (data not shown). *B*, pretreatment with LPS for 1 h was unable to induce tolerance to subsequent LPS-induced TNF-α secretion. PBMC were exposed to the indicated LPS pretreatment concentrations. After 1 h the PBMC were stimulated with 0.04 ng/ml LPS, and the supernatants were analyzed for cytokine 20 h later. *C*, Hsp10 reduced LPS-induced IL-6 secretion. Hsp10 (1 and 10 μg/ml) or buffer (0) was added to PBMC from eight different donors 1 h before the addition of 0.04 ng/ml LPS. Supernatants were removed after 20 h and analyzed for IL-6. The percentage reductions and significance (calculated as for A) are indicated. For all donors 10 μg/ml Hsp10 in the absence of LPS failed to induce IL-6 levels above the level of detection (9 pg/ml) (data not shown). *D*, pretreatment with LPS for 1 h was unable to induce tolerance to subsequent LPS-induced IL-6 secretion. PBMC were exposed to the indicated LPS pretreatment concentrations. After 1 h the PBMC were stimulated with 0.04 ng/ml LPS, and the supernatants were analyzed for cytokine 20 h later.

BALB/c mice were given 100 μg of intravenous Hsp10 30 min before injection of 10 μg of intravenous LPS, and blood was removed after 1.5 h. The Hsp10 treatment provided an average 47.6% reduction in serum TNF-α levels, an average 40.1% reduction in serum RANTES, and an average 43.3% increase in serum IL-10 levels in several repeat experiments (Fig. 6*A*). These data are consistent with the tissue culture experiments and illustrate the *in vivo* efficacy of Hsp10 in reducing pro-inflammatory mediators and increasing IL-10 production after challenge with LPS.

Hsp10 Reduced the Acute Symptoms of GVHD—Acute GVHD after allogeneic bone marrow transplantation is a T cell-mediated disease in which donor T cells recognize recipient alloantigens and differentiate in a Th1 dominant fashion. The resulting T cell-derived Th1-cytokines (primarily IFN-γ) prime donor mononuclear cells to release cytopathic quantities of inflammatory cytokines (*e.g.* TNF-α) when they are stimulated with LPS that has leaked through the radiation-damaged gastrointestinal mucosa. These cytokines and the allo-reactive T cells then contribute to increasing gastrointestinal damage and LPS leakage. GVHD mortality in bone marrow transplantation models is prevented if the donor mononuclear cells lack TLR4 (64), LPS is effectively blocked (by therapeutic antagonists) (65), or TNF-α itself is neutralized (51). The effect of Hsp10 administration during the peri-transplant period on GVHD was, therefore, investigated.

Hsp10 treatment of transplant donors and recipient animals before transplant significantly delayed GVHD mortality (Fig. 6*B*). In addition, the severity of GVHD as determined by the clinical score was also significantly reduced on day 7 after the bone marrow transplantation (Fig. 6*B*). Although Hsp10 treatment delayed GVHD mortality and reduced early morbidity, ultimately the animals succumbed to GVHD, consistent with the inability of Hsp10 to neutralize completely TNF-α secretion or to affect IFN-γ secretion (Fig. 4, *A* and *E*). Treatment of animals with Hsp10 post-transplant failed in isolation to ameliorate significantly GVHD (data not shown).

Hsp10 and Hsp60 in the Inhibition of LPS Signaling—In the mitochondria Hsp10 binds to Hsp60 in the presence of ATP and magnesium ions (1–3), and both ATP and magnesium ions are also present in the extracellular fluids or medium (66). To determine whether the inhibitory activity of Hsp10 on LPS is influenced by Hsp60, RAW264-HIV-LTR-LUC cells were incubated with polyclonal anti-Hsp60 antibodies before the addition of Hsp10 and LPS. Preincubation of RAW264-HIV-LTR-LUC cells with anti-Hsp60 serum, but not control serum, prevented the Hsp10-mediated reduction of LPS-induced NF-κB activation (Fig. 7), suggesting that Hsp60 may be required for Hsp10 activity. The anti-Hsp60 antibody by itself mediated a similar level of inhibition of LPS signaling to that seen for Hsp10 treatment (Fig. 7), indicating that anti-Hsp60 and Hsp10 individually mediate inhibitory activity of a similar magnitude in this system. However, when added together there was no additive inhibitory effect (the lack of detectable LUC activity when cells were incubated with either antisera alone illustrated that endotoxin contamination of the antibodies was below the level of detection; see Fig. 1*A*).

DISCUSSION

In addition to its critical role in protein folding within the mitochondria, Hsp10 appears to have an extracellular role in the modulation of specific inflammatory processes. In a number of different human and murine *in vitro* systems and in two murine disease models, Hsp10 consistently inhibited LPS-induced secretion of the pro-inflammatory cytokines TNF-α, IL-6, and the pro-inflammatory chemokine RANTES, and increased LPS-induced secretion of the anti-inflammatory cytokine IL-10. The Hsp10-mediated reduction in pro-inflammatory mediator secretion was never absolute; instead, Hsp10 mediated a 20–50% reduction in pro-inflammatory mediator levels, depending on the system and the dose of LPS and Hsp10. Hsp10 increased LPS-induced IL-10 secretion by ≈30–200% depending on the system; however, the reduction in TNF-α secretion was not dependent on this elevation of IL-10. We were unable to confirm previous reports (40, 41) that anti-Hsp10 antibodies reduced the growth of tumor cells *in vitro* or *in vivo*, nor were we able to demonstrate that Hsp10 is a growth factor for tumor cells.

Given that *E. coli*-derived LPS is a well described agonist for TLR4, the experiments described herein indicate that Hsp10 can reduce the magnitude of TLR4 signaling. The HIV LTR is highly responsive to NF-κB activation but whether Hsp10 also modulates signaling via the other pathways stimulated by the TLR4-complex remains to be established (13, 21). How Hsp10 mediates its inhibitory activity is unclear, although it appears

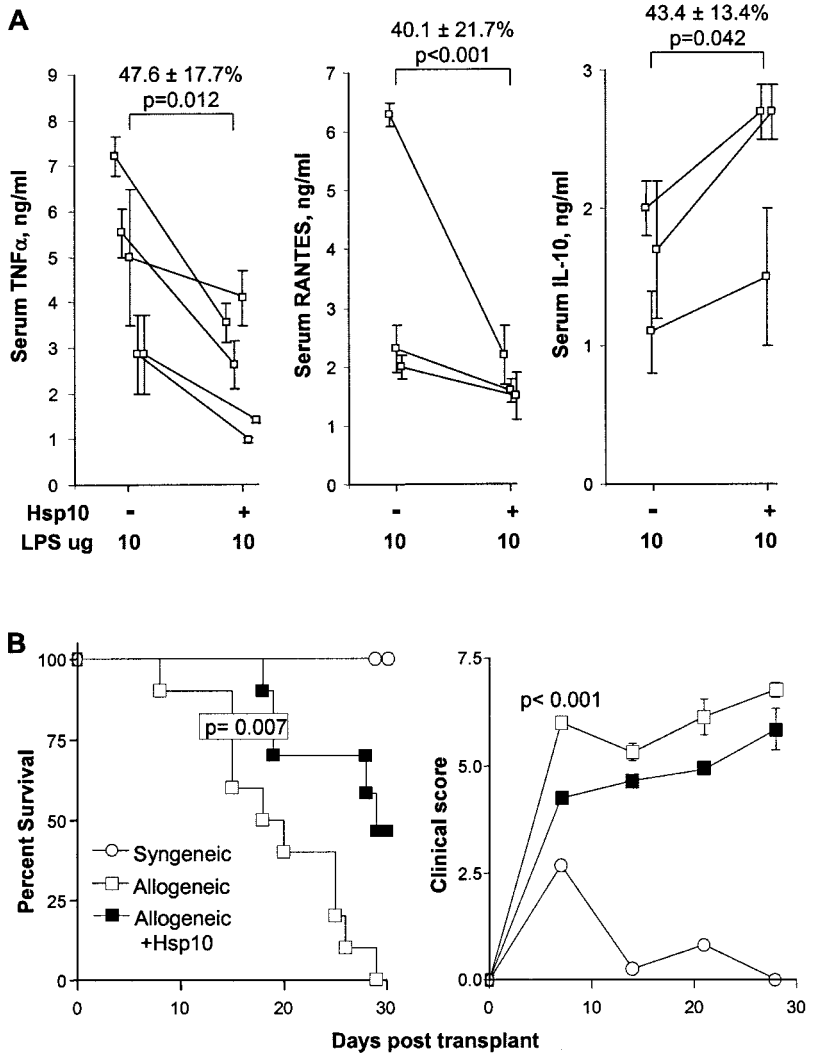

FIG. 6. **Hsp10 activity in murine inflammatory models.** *A*, Hsp10 reduced LPS-induced serum TNF-α and RANTES production and increased IL-10 levels. In five separate experiments BALB/c mice (n = 3 or 4 per group) were given buffer (*Hsp10*−) or 100 μg of Hsp10 (*Hsp10*+) intravenously 30 min before intravenous administration of 10 μg of LPS. After 1.5 h the animals were sacrificed, and serum TNF-α, RANTES, and IL-10 levels were determined (the latter two were assessed in 3/5 experiments). *Error bars* represent S.E. within each experiment. The percentage reduction in TNF-α and RANTES and increase in IL-10 (±S.D.) is indicated, and the significance was calculated using two way ANOVA, which took into account the separate experiments. No TNF-α or RANTES was detected in animals treated with Hsp10 alone (data not shown). *B*, pretransplant treatment with Hsp10 delayed GVHD mortality and reduced clinical severity of acute disease. Syngeneic negative controls (n = 8) (*white circles*) represent B6D2F1 mice transplanted with syngeneic B6D2F1 bone marrow and T cells. Allogeneic positive controls (n = 10) (*white squares*) represent diluent pretreated B6D2F1 recipient mice transplanted with cells from diluent pretreated B6 donor mice. Allogeneic + Hsp10 (n = 10) (*black squares*) represent B6D2F1 recipients receiving bone marrow and T cell grafts from B6 donor mice where both recipients and donors were pretreated with Hsp10 before transplantation. Kaplan-Meier survival curves and clinical scores are shown for the three groups, and the allogeneic groups treated with and without Hsp10 are compared by Log Rank Statistic and non-parametric t test, respectively. Clinical scores were only significantly different on day 7.

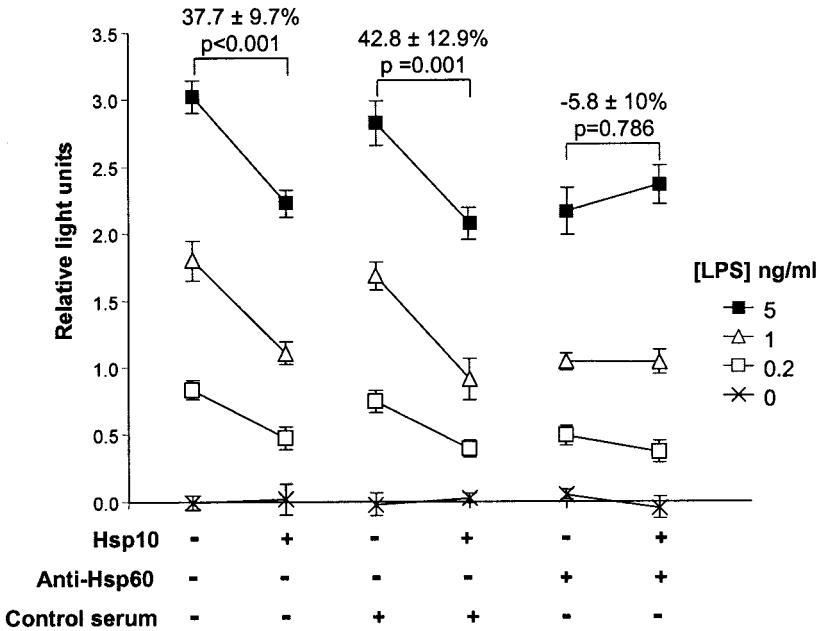

FIG. 7. **Hsp10 activity was blocked by anti-Hsp60 serum.** RAW264-HIV-LTR-LUC cells were treated with anti-Hsp60 or control serum for 1 h, and then Hsp10 (100 μg/ml) (*Hsp10*+) or buffer (*Hsp10*−) was added for a further 2 h. LPS was then added, and luciferase activity was measured 2 h later. The mean percentage reduction (±S.D.) in relative light units is indicated for 5, 1, and 0.2 ng/ml LPS taken together, and the significance was calculated using a two way ANOVA, which included a term for LPS concentration.

to effect inhibition very rapidly, within 30 min (Fig. 6*A*) to 2 h (Fig. 3*A*). This might implicate inhibition of early signaling events or activation of rapid negative feedback mechanisms such as phosphoinositide 3-kinase (28). However, we have been unable to prevent Hsp10 activity with the specific phosphoinositide 3-kinase inhibitor wortmannin (data not shown), suggesting this pathway is not involved in the Hsp10 mechanism of action.

The reported association of extracellular Hsp60 with TLR4 signaling (13) and the known interaction between Hsp10 and Hsp60 in the mitochondria suggested that Hsp10 might inhibit LPS-induced TLR4 signaling by interacting with Hsp60 in the extracellular milieu. This notion is supported by our finding that Hsp10 activity is blocked and mimicked by anti-Hsp60 antibodies (Fig. 7). Although Hsp60 was reported to stimulate TLR2 and TLR4 signaling, recent reports (22, 67) and our own studies (data not shown) indicate that such activity is likely due to LPS contamination of Hsp60 preparations. Nevertheless, in agreement with previous reports (24, 25), anti-Hsp60 antibodies were able to inhibit LPS-mediated signaling (Fig. 7). Thus, rather than being able by itself to stimulate TLR4, Hsp60 may be involved in augmenting LPS-induced signaling (7, 13, 68). A potentially similar role in the augmentation of inflammatory responses has been proposed for Hsp70 and Hsp90 (21, 69–71). Thus, Hsp10 may inhibit TLR4 signaling by inhibiting Hsp60-mediated augmentation of TLR4 signaling. Hsp60 is also reported to contribute to TLR2 signaling (19, 72), and we have recently shown that Hsp10 can also inhibit signaling by a TLR2 agonist, whereas Hsp10 was ineffective in inhibiting TLR9 signaling (data not shown). This line of reasoning might lead one to speculate that the physiological role of circulating Hsp10 during early pregnancy (38) may be to remove the Hsp60 danger signal (15) that has arisen from pregnancy (73, 74) rather than from pathogen-induced tissue damage. We are currently in the process of seeking formal proof of Hsp10 binding to extracellular Hsp60, as it remains possible that Hsp10 binds to an alternative receptor (75) and that Hsp10 and anti-Hsp60 exert similar suppressive (but non-additive) effects on TLR4 signaling (Fig. 7) but use distinct pathways.

The ability to reduce but not completely suppress TNF-α secretion would distinguish Hsp10 from other anti-inflammatory therapies, particularly those based on anti-TNF-α antibodies, which can accomplish efficient removal of TNF-α. However, such removal may not always be desirable (76). For instance, anti-TNF-α antibody treatment has been shown ultimately to increase the severity of multiple sclerosis (77). Multiple sclerosis has been suggested as a possible therapeutic target for Hsp10 since Hsp10 is reported to reduce clinical signs and delay onset of disease in a murine model of multiple sclerosis (experimental autoimmune encephalomyelitis) (35). The ability of Hsp10 to reduce the expression of inflammatory mediators indicates that Hsp10 may find therapeutic application in conditions where excessive LPS, TLR4 (and/or TLR2) signaling, and/or Hsp60 leads to pathology (11, 12, 21).

Acknowledgments—We thank Steve Mahler, Dave Chin, and Chris Marquis (Bioengineering Centre, University of New South Wales) for the production of recombinant Hsp10.

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J. Biol. Chem. 2005, 280:4037-4047.
doi: 10.1074/jbc.M411569200 originally published online November 16, 2004
