Heat Shock Protein 60 Inhibits Th1-Mediated Hepatitis Model via Innate Regulation of Th1/Th2 Transcription Factors and Cytokines[1]

Alexandra Zanin-Zhorov,* Rafael Bruck,† Guy Tal,* Shirly Oren,* Hussein Aeed,† Rami Hershkoviz,‡ Irun R. Cohen,[2]* and Ofer Lider[3]*

Extracellular heat shock protein 60 (HSP60) has been considered a proinflammatory danger signal. Yet, HSP60 can also down-regulate experimental immune arthritis and diabetes models by specific inhibition of Th1-like responses. We now report that HSP60 in vitro differentially modulates the expression of Th1/Th2 transcription factors in human T cells: HSP60 down-regulates T-bet, NF-κB, and NFATp and up-regulates GATA-3, leading to decreased secretion of TNF-α and IFN-γ and enhanced secretion of IL-10. These effects depended on TLR2 signaling and could not be attributed to LPS or to other contaminants. In BALB/c mice, HSP60 in vivo inhibited the clinical, histological, and serological manifestations of Con A-induced hepatitis associated with up-regulated T cell expression of suppressor of cytokine signaling 3 and GATA-3 and down-regulated T-bet expression. These results provide a molecular explanation for the effects of HSP60 treatment on T cell inflammation via innate regulation of the inflammatory response. ***The Journal of Immunology,*** **2005, 174: 3227–3236.**

The mammalian 60-kDa heat shock protein (HSP)[4] is a many-faceted molecule. In addition to serving as a chaperone, HSP60 is expressed by different types of cells following their exposure to stress (1, 2) or immune activation (3), is present in the blood during inflammation (4, 5), and has been found to be a target of autoantibodies and autoimmune T cells in healthy individuals, as well as in those suffering from autoimmune diseases (6). However, it was also shown that the same HSP60 molecule could inhibit inflammatory diseases, such as adjuvant arthritis and type I diabetes by shifting specific immune reactions to a Th2-like response (3, 7–9). These paradoxical effects on cells of the innate and adaptive arms of the immune system may be explained partially by the discovery that HSP60, via TLR2, can directly activate anti-inflammatory pathways, such as inhibition of T cell migration in vitro and in vivo in response to CXCL12 (stromal cell-derived factor 1α) and the expression of its receptor, CXCR4 (10). In the present study, we examined the effects of HSP60 on the specific activation of Th1 and Th2 cytokines and their associated signaling pathways and transcription factors in T cells.

Th cells differentiate into two distinct subsets, Th1 and Th2, as defined by their functional activities and cytokine secretion profiles. Th1 cells mediate delayed-type hypersensitivity responses and tissue damage in autoimmune diseases and allograft rejection and provide protection against intracellular pathogens and viruses. Th2 cells provide help to B cells and eradicate helminthes and other extracellular parasites (11). The functional differences between the two subsets are explained primarily through the activities of the cytokines they secrete. IFN-γ is the signature cytokine of Th1 cells, which also produce IL-2 and TNF-α, whereas Th2 cells preferentially secrete IL-4, IL-5, IL-10, and IL-13. Several transcription factors, including c-Maf, STAT6, NFAT, and GATA-3, are essential for the differentiation of T cells toward a Th2 phenotype (12, 13). T cell differentiation along the Th1 lineage is regulated similarly by specific transcription factors, among which are T-bet, which plays an essential role in IFN-γ production (14, 15). The activation of these transcription factors is regulated by signaling through the TCR and through costimulatory molecules (12–17).

In this study, we tested the effects of HSP60 on T cells in several systems; we studied human T cells activated by mitogenic anti-CD3 in vitro, and we also studied the expression of cytokines and their transcription factors in mouse T cells ex vivo and in vivo. We extended the studies in vivo to test the functional effect of HSP60 treatment on T cells in experimentally induced hepatitis in mice. We found that HSP60 inhibited anti-CD3-induced IFN-γ and TNF-α secretion and up-regulated IL-10 secretion from freshly isolated human T cells in a TLR2-dependent manner. We also found that HSP60 inhibited the expression of the Th1-associated factors, NF-κB and T-bet, inhibited NFATp activation, and induced the expression of GATA-3. HSP60 also inhibited Th1-mediated responses in vivo by suppressing TNF-α secretion and the clinical manifestations of Con A-induced hepatitis in mice. Thus, HSP60 can suppress Th1 activation and the subsequent functioning required for the fine-tuning of the inflammatory reaction.

*Department of Immunology, Weizmann Institute of Science, Rehovot, Israel; †Department of Gastroenterology, E. Wolfson Medical Center, Holon, Israel; and ‡Department of Internal Medicine D, Assaf Harofe, Zerifin, and Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

Received for publication July 9, 2004. Accepted for publication December 28, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by grants from the Center for the Study of Emerging Diseases (Jerusalem, Israel).

[2] Address correspondence and reprint requests to Dr. Irun Cohen, Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel. E-mail address: irun.cohen@weizmann.ac.il

[3] O.L. is deceased.

[4] Abbreviations used in this paper: HSP, heat shock protein; CHX, cycloheximide; t, total; SOCS, suppressor of cytokine signaling; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SDF-1α, stromal cell-derived factor 1α; PMB, polymyxin B.

0022-1767/05/$02.00

Materials and Methods

Reagents

The following reagents and chemicals were obtained as indicated: RPMI 1640 medium (Invitrogen Life Technologies), FCS, HEPES buffer, antibiotics, sodium pyruvate (Biological Industries), phosphatase inhibitor mixture, cycloheximide (CHX), PMA, and LPS (Sigma-Aldrich). mAbs anti-TLR2 and anti-TLR4 were obtained from eBioscience; anti-human recombinant HSP60 (designated clone P5, IgM fraction) was kindly provided by F. Quintana (Weizmann Institute of Science). Polyclonal Ab anti-total (t)ERK1/2 was obtained from Sigma-Aldrich; anti-suppressor of cytokine signaling (SOCS)3 (H-103), anti-NF-κB p65 (A), anti-T-bet (39D), anti-GATA-3 (HG3-31), and anti-lamin B (C-20) were purchased from Santa Cruz Biotechnology. Purified mouse anti-NFATp/NFATc2 (4G6-G5.1) was purchased from BD Pharmingen. The recombinant HSP60 (StressGen Biotechnologies) used in this study contained <0.001 EU/ml (0.1 pg/ml) of bacterial endotoxin, as determined using a kinetic-turbidimetric *Limulus* amebocyte lysate test method (Biological Industries).

Human T cells

T cells were purified from the peripheral blood of healthy human donors (Blood Bank). The whole blood was incubated (20 min, 22°C) with RosetteSepä human T cell enrichment mixture (StemCell Technologies). The remaining unsedimented cells were then loaded onto lymphocyte separation medium (ICN Biomedicals), isolated by density centrifugation, and washed with PBS. The purified cells (>95% $CD3^+$ T cells) so obtained were cultured in RPMI 1640 medium containing antibiotics and 10% heat-inactivated FCS. In a second round of purification, $CD3^+$ T cells were labeled for negative selection with a magnetically coupled mAb against $CD45RA^+$ and $CD45RO^+$ (Miltenyi Biotec). The purified cells obtained (usually >97% $CD45RO^+$ or $CD45RA^+$ T cells) were cultured in RPMI 1640 medium containing antibiotics and 10% heat-inactivated FCS.

Activated mouse lymph node cells

BALB/c mice (females, 1.5 mo of age) were sensitized by painting their shaved abdominal walls with oxazolone (2%) emulsified in 100 μl of acetone/olive oil (Sigma-Aldrich). On day 5, mice were sacrificed, their draining (inguinal, mesenteric, and cervical) lymph nodes were collected, and a single-cell suspension was prepared. Next, $CD3^+$ T cells were isolated by negative selection with anti-mouse Ab mixture (Pan T cell kit; Miltenyi Biotec). The labeled cells were then passed through separation columns (MidiMACS columns; Miltenyi Biotec). The purified cells (>97% T cells) were untreated or treated with HSP60 as described previously.

Cytokine secretion

T cells (2×10^6 cells/ml) were activated (1 h, 37°C) with the indicated concentrations of HSP60 in 24-well plates in RPMI 1640 medium containing 10% heat-inactivated FCS. The cells were then washed and replated at the same concentration on anti-CD3 mAb-precoated 24-well plates (0.5 μg/ml; nontissue culture grade plates) in serum-free medium containing 0.1% BSA at 4°C for 24–72 h. The supernatants were collected, and the cytokine content (TNF-α, IFN-γ, and IL-10) was determined by ELISA, using the appropriate mAb, according to the manufacturer's instructions.

Western blot analysis of T cell nuclear extracts

Purified T cells (5×10^6) were preincubated with different concentrations of HSP60 for the indicated periods of time (37°C in a 7% CO_2, humidified atmosphere). The cells were then washed and replated in the same concentration of HSP60 on anti-CD3 mAb-precoated 24-well plates for 2 or 24 h (37°C in a 7% CO_2, humidified atmosphere). T cells were lysed in 10 mM HEPES, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 0.5% Nonidet P-40. The lysates were incubated on ice for 10 min and centrifuged at 2000 rpm for 10 min at 4°C. The supernatants (cytoplasmic extracts) were transferred, and the pellet (nuclei) was suspended in buffer containing 30 mM HEPES, 450 mM NaCl, 25% glycerol, 0.5 mM EDTA, 6 mM DTT, 12 mM $MgCl_2$ 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1% phosphatase inhibitor mixture, and the suspension was incubated on ice for 30 min. The lysates were cleared by centrifugation (30 min, 14×10^3 rpm, 4°C), and the resulting supernatants were analyzed for protein content. For NFATp analysis, nuclear extracts were prepared with a NE-PER Nuclear and Cytoplasmic Extraction reagent (Pierce), according to the manufacturer's protocol. Sample buffer was then added, and, after boiling, the samples containing equal amounts of proteins were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with TBST buffer containing low-fat milk (5%), 20 mM Tris (pH 7.5), 135 mM NaCl, and 0.1% Tween 20 and probed with the following mAb in the same buffer: anti-NF-κB (diluted 1/1,000), anti-tERK (diluted 1/20,000), anti-NFATp (diluted 1/500), anti T-bet (diluted 1/1,000), anti-GATA-3 (diluted 1/1,000), and anti-lamin B (diluted 1/1,000). Immunoreactive protein bands were visualized using labeled secondary Abs and the enhanced ECL system.

Induction and evaluation of liver damage

BALB/c mice were maintained at the Animal Breeding Facility of the E. Wolfson Medical Center. Treatment of the animals was in accordance with institutional guidelines. Acute liver injury was induced by injecting 6- to 8-wk-old male mice with Con A (0.5 mg/0.3 ml; Sigma-Aldrich) in 250 ml of PBS via the tail vein. HSP60 (500 ng/ml) was administered i.p. 18 and 1 h before Con A. After 24 h, the mice were bled and euthanized with chloral hydrate anesthesia, their abdomens were opened by a midline incision, and sections from the left liver lobe were excised for histopathological examination. Liver sections were fixed in a 5% neutral formol solution and stained with H&E.

Enzymatic assessment of liver injury, determination of serum levels of TNF-α, and T cell purification

In addition to a histopathological examination, the extent of the liver damage was estimated by determining the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), with an automated Monarch Monoanalyzer 2000 (Allied) 24 h after Con A administration. For determination of TNF-α levels, blood was drawn from mice 2 h after administration of Con A. Serum TNF-α concentrations were assayed by ELISA kit (Genzyme), according to the manufacturer's instructions. Each sample was tested in duplicate. For T cell purification, spleens were removed, and a single-cell suspension was prepared. Next, $CD3^+$ T cells were isolated by negative selection with an anti-mouse Ab mixture (Pan T cell kit; Miltenyi Biotec). The labeled cells were then passed through separation columns (MidiMACS columns; Miltenyi Biotec). The purified cells (>97% T cells) so obtained were cultured in RPMI 1640 medium containing antibiotics and 10% heat-inactivated FCS.

Statistics

Data were analyzed by the Student's t test. A value of $p < 0.05$ was considered significant statistically.

Results

HSP60 inhibits T cell IFN-γ and TNF-α secretion and upregulates IL-10 secretion

We analyzed the effects of HSP60 on the cytokine secretion profile of freshly isolated and purified human T cells that were activated by the mitogenic anti-CD3 mAb. HSP60 alone (at a range of concentrations of 0.01–1000 ng/ml) did not induce cytokine secretion by the T cells (data not shown). However, when the T cells were pretreated with different concentrations of HSP60, washed, and then activated by anti-CD3, the secretion of the Th1-related cytokines IFN-γ and TNF-α was inhibited by 50% (Fig. 1, *A* and *B*), whereas IL-10 secretion was enhanced (Fig. 1*C*). Recently, we showed that the biological effects of HSP60 on T cells manifested a biphasic, bell-shaped, dose-response curve (10). It is interesting to note that both the inhibitory effects of HSP60 on IFN-γ and TNF-α secretion and the enhancing effect on activation of IL-10 secretion also manifested a biphasic, bell-shaped dose response. Significant effects were achieved with relatively low concentrations of HSP60 (0.1–1.0 ng/ml; $p < 0.01$), whereas higher doses (in the order of 10 ng/ml) did not appear to affect cytokine secretion. However, cytokine secretion was again affected by ~50% at higher concentrations of HSP60 (0.1–1 μg/ml; $p < 0.05$). In addition, we performed the same experiment without washing the T cells between HSP60 and anti-CD3 stimulation, and we found that the presence of HSP60 during anti-CD3 stimulation did not alter the biphasic, bell-shaped, dose-dependent curve (data not shown).

To study the effects of HSP60 treatment on cytokine secretion by T cells over time, we incubated the T cells with 1 ng/ml HSP60 for 1 h, washed the cells, and measured the cytokine accumulation at 24, 48, and 72 h of incubation with mitogenic anti-CD3 Abs.

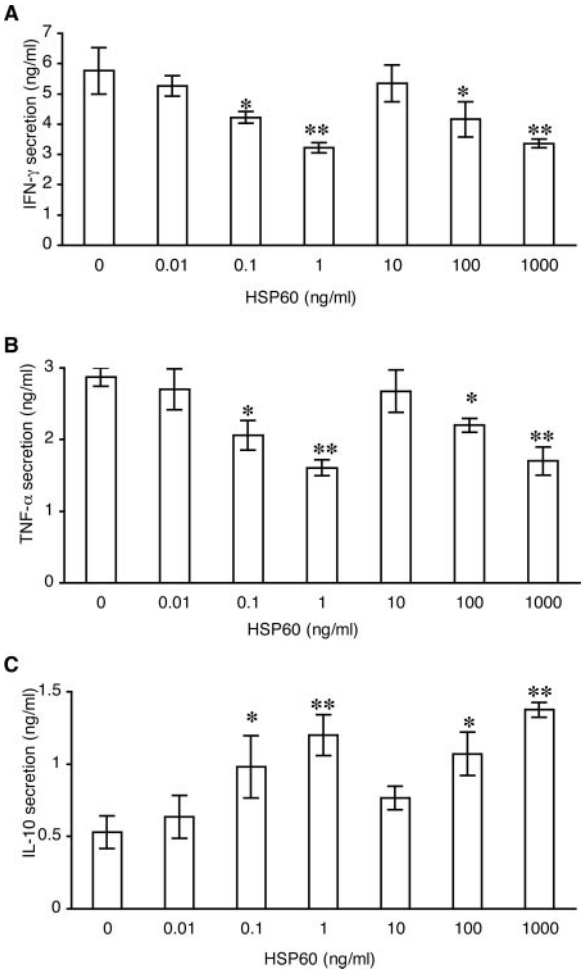

FIGURE 1. HSP60 inhibits anti-CD3-induced IFN-γ (*A*) and TNF-α (*B*) secretion and up-regulates IL-10 (*C*) secretion by T cells. Purified human T cells were preincubated with the indicated concentrations of HSP60 for 1 h, washed, and transferred to 24-well plates coated with mAb anti-CD3 (ortho-Kung-T cells; 0.5 μg/ml) in serum-free medium. The supernatants were collected after 24 h and analyzed for IFN-γ (*A*), TNF-α (*B*), and IL-10 (*C*) secretion. The means ± SD of five different donors are shown. *, $p < 0.05$; **, $p < 0.01$.

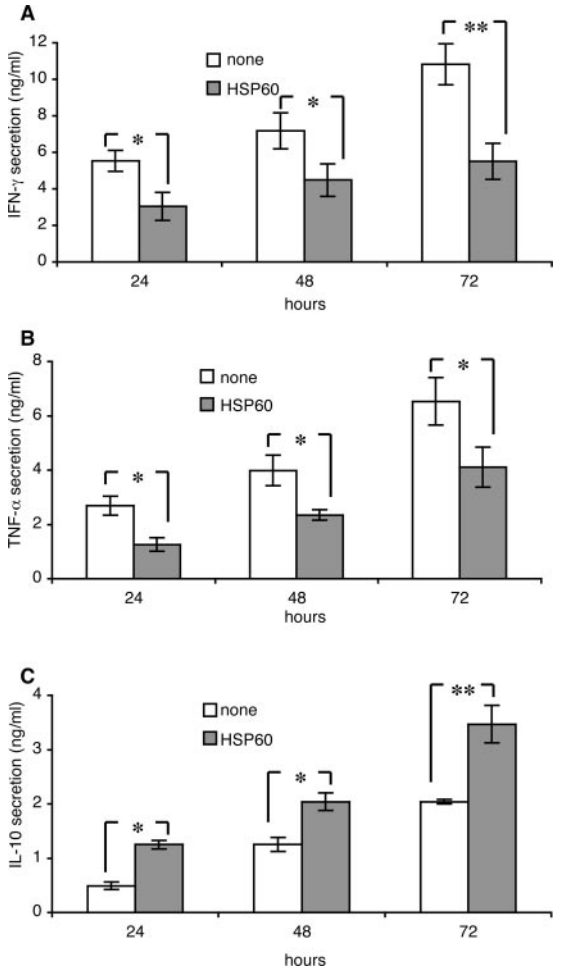

FIGURE 2. The effects of HSP60 on T cell cytokine secretion are consistent up to 72 h of exposure on anti-CD3 Abs. Purified human T cells were preincubated with 1 ng/ml HSP60 for 1 h, washed, and exposed to anti-CD3 mAbs for indicated periods of time. The supernatants were analyzed for IFN-γ (*A*), TNF-α (*B*), and IL-10 (*C*) secretion. The means ± SD of three different donors are shown. *, $p < 0.05$; **, $p < 0.01$.

Fig. 2 shows that the effects of HSP60 on cytokine secretion were manifested over the 24- to 72-h time period of incubation with the anti-CD3 Abs. Therefore, we elected to carry out the next experiments at 24 h of incubation.

The effects of HSP60 on cytokine secretion from CD45RA$^+$ and CD45RO$^+$ populations of human T cells

We have reported recently that the sensitivity of the T cell response to HSP60 was influenced by a T cell subset: CD45RA$^+$ T cells responded to lower concentrations of HSP60 than did CD45RO$^+$ T cells (10). In the present study, we tested whether HSP60 differently affects cytokine secretion of these subpopulations. We fractioned the T cells into CD45RA$^+$ and CD45RO$^+$ subpopulations and repeated the activation procedure as was described for unseparated T cells (Fig. 1). Fig. 3 shows that the CD45RA$^+$ population responded to relatively low concentrations of HSP60 (0.1–1.0 ng/ml), whereas higher doses (in the order of 10 ng/ml) did not appear to affect cytokine secretion. However, cytokine secretion was again affected by ~50% at higher concentrations of HSP60 (0.1–1 μg/ml; $p < 0.05$). In contrast, the CD45RO$^+$ population responded mostly to the higher concentrations (>100 ng/ml) of HSP60. Thus, CD45RA$^+$ and CD45RO$^+$ subpopulations have different sensitivities to HSP60.

The effects of HSP60 on T cell cytokine secretion are TLR2 dependent

HSP60 has been reported to activate responsive cells via TLR4 or TLR2 signaling (18, 19). It was found that human T cells express TLR2 and TLR4 (20, 21). We have previously reported that HSP60 via TLR2 signaling can induce T cell adhesion to fibronectin (extracellular matrix glycoprotein) and can inhibit T cell chemotaxis through fibronectin toward EBl-1 ligand chemokine and stromal cell-derived factor 1α (SDF-1α; CXCL12) (10). In the present study, we tested whether TLR2 or TLR4 was involved functionally in the effects of HSP60 on T cell cytokines. Human T cells were preincubated with anti-TLR2 or TLR4 mAbs (both are mouse IgG2a Abs), and the cytokine secretion profile induced by anti-CD3 was assessed. The inhibition of IFN-γ and TNF-α secretion (Fig. 4, *A* and *B*) and the activation of IL-10 secretion (Fig. 4*C*) by HSP60 were blocked by treating the T cells with the mAb to TLR2 but not by the mAb to TLR4. In addition, the effects of HSP60 on T cell cytokine secretion were blocked by anti-HSP60 mAb but not by an isotype-matched control mAb. Thus, TLR2

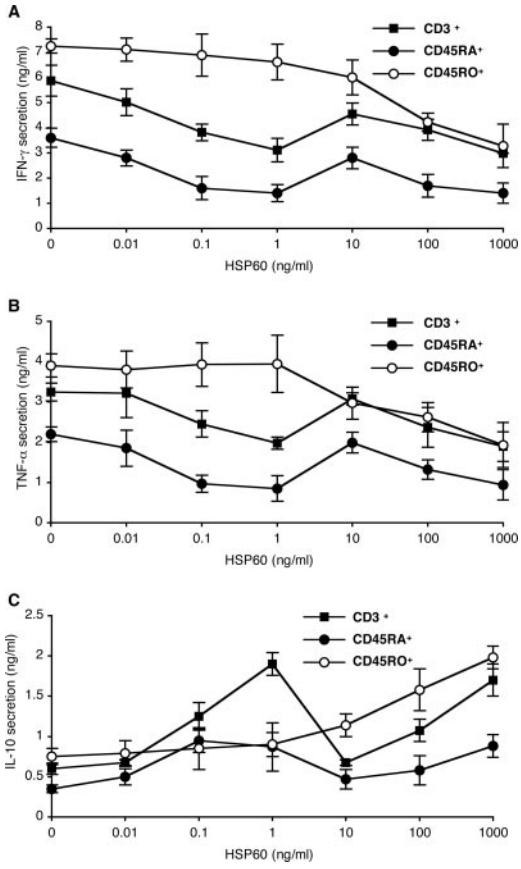

FIGURE 3. The effects of HSP60 on cytokine secretion from $CD45RA^+$ and $CD45RO^+$ populations of human T cells. Purified $CD45RA^+$ and $CD45RO^+$ human T cells were preincubated with the indicated concentrations of HSP60 for 1 h, washed, and transferred to 24-well plates coated with mAb anti-CD3 (ortho-Kung-T cells; 0.5 μg/ml) in serum-free medium. The supernatants were collected after 24 h and analyzed for IFN-γ (*A*), TNF-α (*B*), and IL-10 (*C*) secretion. The means $\pm$ SD of three different donors are shown.

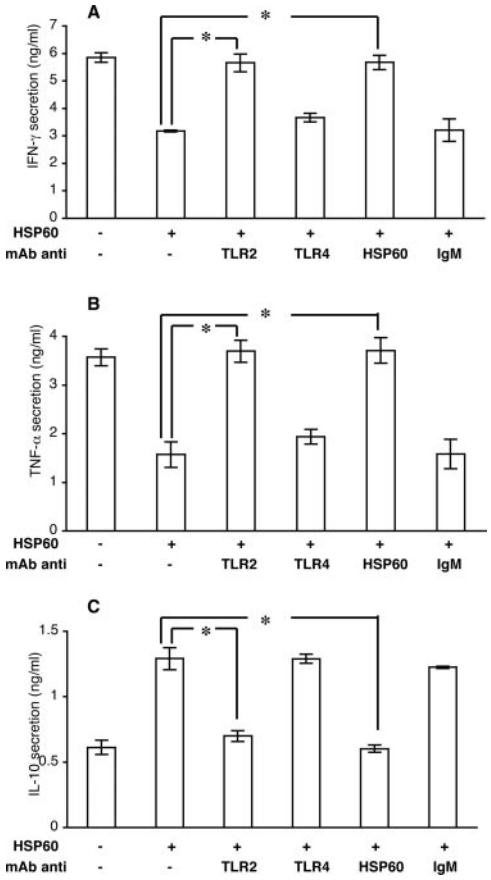

FIGURE 4. The effects of HSP60 on T cell cytokine secretion are TLR2 dependent. Purified human T cells were pretreated with monoclonal anti-TLR2, anti-TLR4, or anti-HSP60 (20 μg/ml, 30 min). Then, the cells were incubated with HSP60 (1 ng/ml, 1 h), washed, and exposed to immobilized monoclonal anti-CD3 in serum-free medium. The supernatants were collected after 24 h and analyzed for IFN-γ (*A*), TNF-α (*B*), and IL-10 (*C*) secretion. The means $\pm$ SD of three different donors are shown. *, $p < 0.05$.

appears to play a role in mediating the effects of HSP60 on activated human T cells.

The effects of HSP60 on T cell cytokine secretion are not due to contaminating LPS

LPS-TLR2 interactions can transmit intracellular activation signals in various types of leukocytes (22). Using a kinetic-turbidimetric test method, we found that the recombinant human HSP60 used in this study contained <0.001 EU/μg protein (0.1 pg/μg) of bacterial endotoxin. The following studies were performed to additionally exclude the possibility that even minute amounts of LPS might affect T cell cytokine secretion by using the LPS inhibitor polymyxin B (PMB) and by boiling the HSP60. Fig. 5 shows that the effects of HSP60 on cytokine secretion were completely inhibited by boiling (which denatures proteins, although not LPS) but not by PMB. Most importantly, in contrast to its effects on macrophages (22), we found that purified LPS did not modify T cell cytokine secretion. Consequently, the effects of HSP60 on T cell cytokine secretion could not be attributed to LPS.

Recently, lipoproteins extracted from the *Escherichia coli* were shown to interact with macrophages via TLR2 (23, 24). Such lipoproteins can be removed by binding to PMB-coupled agarose beads (23). Therefore, we incubated the HSP60 with PMB-coupled agarose beads, collected the unbound material, assayed the protein content, and tested the effect of the PMB-treated HSP60 on TNF-α secretion. Preincubation with PMB-conjugated agarose beads did not block the efficacy of our HSP60 preparation on TNF-α secretion, but the efficacy of LPS was completely abolished (data not shown). Thus, the effects of HSP60 on cytokine secretion by T cells could be attributed to HSP60 itself.

HSP60 inhibits nuclear translocation of NF-κB in T cells

Activation and nuclear translocation of NF-κB is an essential step in the regulation of gene expression and secretion of various proinflammatory cytokines in leukocytes, including T cells (25). We examined the effect of HSP60 on nuclear translocation of NF-κB by probing nuclear and cytoplasmic T cell extracts using mAb specific for the p65 subunit of NF-κB. The nuclear protein lamin B and the cytoplasmic ERK were used as constitutively expressed control proteins for the quantification of protein amounts (Fig. 6). Treatment of T cells with HSP60 for 1 h alone did not affect the nuclear translocation of NF-κB (Fig. 6*A*). However, pretreatment of T cells with HSP60, followed by exposure to anti-CD3 for 2 h, caused a significant down-regulation of NF-κB activation and translocation to the nucleus (Fig. 6*B*); the p65 subunit of this NF remained in the cytoplasmic compartment (Fig. 6*C*). Similar to the results in the cytokine secretion assays (Fig. 1), maximal effects on NF-κB ($p < 0.01$) were observed with 1 ng/ml and 1 μg/ml HSP60 (Fig. 6*B*).

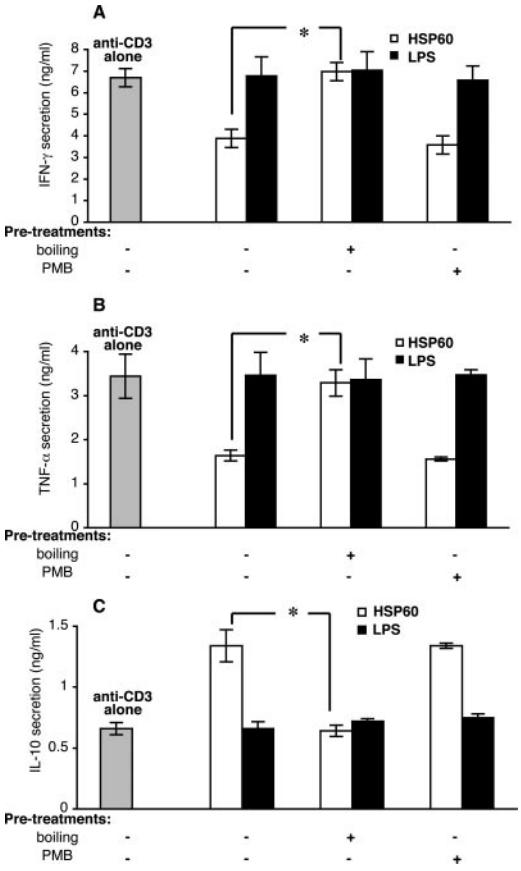

FIGURE 5. The effects of HSP60 on cytokine secretion are not due to contaminating LPS. Purified human T cells were treated (1 h) with HSP60 (1 ng/ml) or LPS (100 ng/ml) after pretreatment (30 min) with PMB (1 μg/ml). As indicated, the HSP60 and LPS were boiled (100°C, 30 min) in some samples before addition to the to the cell cultures. After washing, the T cells were exposed to immobilized anti-CD3 in serum-free medium. The supernatants were collected after 24 h and analyzed for IFN-γ (*A*), TNF-α (*B*), and IL-10 (*C*) secretion. The means $\pm$ SD of four different donors are shown. *, $p < 0.05$.

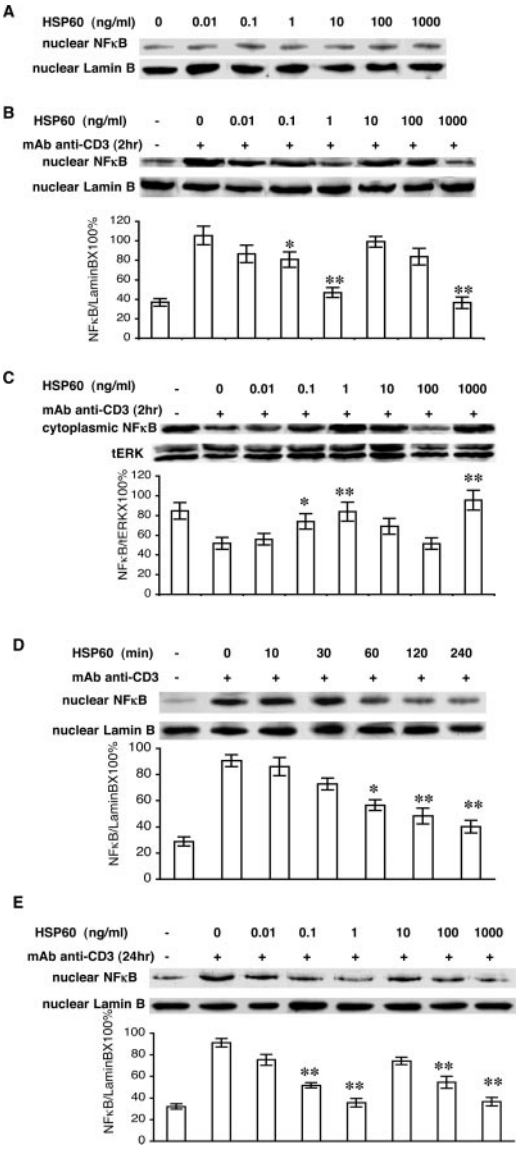

FIGURE 6. HSP60 inhibits anti-CD3-induced nuclear translocation of NF-κB in T cells. Purified human T cells were incubated with HSP60 at 0.01–1000 ng/ml for 1 h (*A–C* and *E*) or with 1 ng/ml for 0–240 min (*D*). Then, the T cells were washed and exposed to immobilized mAb anti-CD3 (*B–D*) for 2 h (*B* and *C*) or 24 h (*E*). Nuclear (*A*, *B*, *D*, and *E*) or cytoplasmic (*C*) lysates were immunoblotted with anti-NF-κB (*A–E*), anti-lamin B (*A*, *B*, *D*, and *E*), or anti-tERK (*C*). Abs against lamin B and tERK served as a control. One experiment representative of three is presented in each case. The levels of NF-κB, lamin B, and tERK were estimated by densitometry, and the average percentage of three different donors was calculated by OD of NF-κB/lamin B (tERK) $\times$ 100%. *, $p < 0.05$; **, $p < 0.01$.

Time-kinetic experiments showed that 10 and 30 min of exposure to HSP60 did not affect NF-κB activation induced by anti-CD3 (Fig. 6*D*). However, 60 min of incubation with HSP60 induced significant inhibition. This inhibition was even greater after 120–240 min (Fig. 6*D*). Note that the inhibitory effect of HSP60 on NF-κB activation also remained after 24 h of exposure of the T cells to the anti-CD3 Abs (Fig. 6*E*). This prolonged inhibition of NF-κB could account for the inhibition of TNF-α and IFN-γ induced by HSP60 (Fig. 2, *A* and *B*).

HSP60 inhibits NFATp activation in T cells

NFAT is a critical regulator of TCR-mediated signals involved in early events associated with gene transcription (26). Currently, four genes encoding NFAT-cytoplasmic subunits have been characterized: NFATc, NFATp, NFAT3, and NFAT4 (also called NFATc1, NFATc2, NFATc4, and NFATc3, respectively). Several studies indicate that NFATp negatively controls Th2 development (26–28). The activation of NFATp and its entry into the nucleus depend on the dephosphorylation of serine/threonine residues, which leads to a 10- to 20-kDa decrease in its apparent molecular mass, as visualized by the immunoblot (28). Treatment of T cells with HSP60 alone did not induce the nuclear translocation of NFATp (data not shown). To study the effects of HSP60 on NFATp activation, we incubated the T cells with HSP60 and mitogenic anti-CD3 Abs, prepared nuclear extracts, and analyzed the products of activation by immunoblotting. T cells that were pretreated with 1 ng/ml HSP60 and then exposed to immobilized anti-CD3 Abs for 2 h exhibited a significant down-regulation of their activation and dephosphorylation of NFATp (Fig. 7*A*). HSP60-mediated inhibition was apparent after 60 min and increased at 120 and 240 min (Fig. 7*B*).

Fig. 7*C* shows that the inhibitory effect of HSP60 on NFATp dephosphorylation and activation was blocked by the protein synthesis inhibitor CHX. This is compatible with the conclusion that inhibition of NFATp mediated by HSP60 is an active process. Similar to the inhibitory effects of HSP60 on NF-κB activation, the

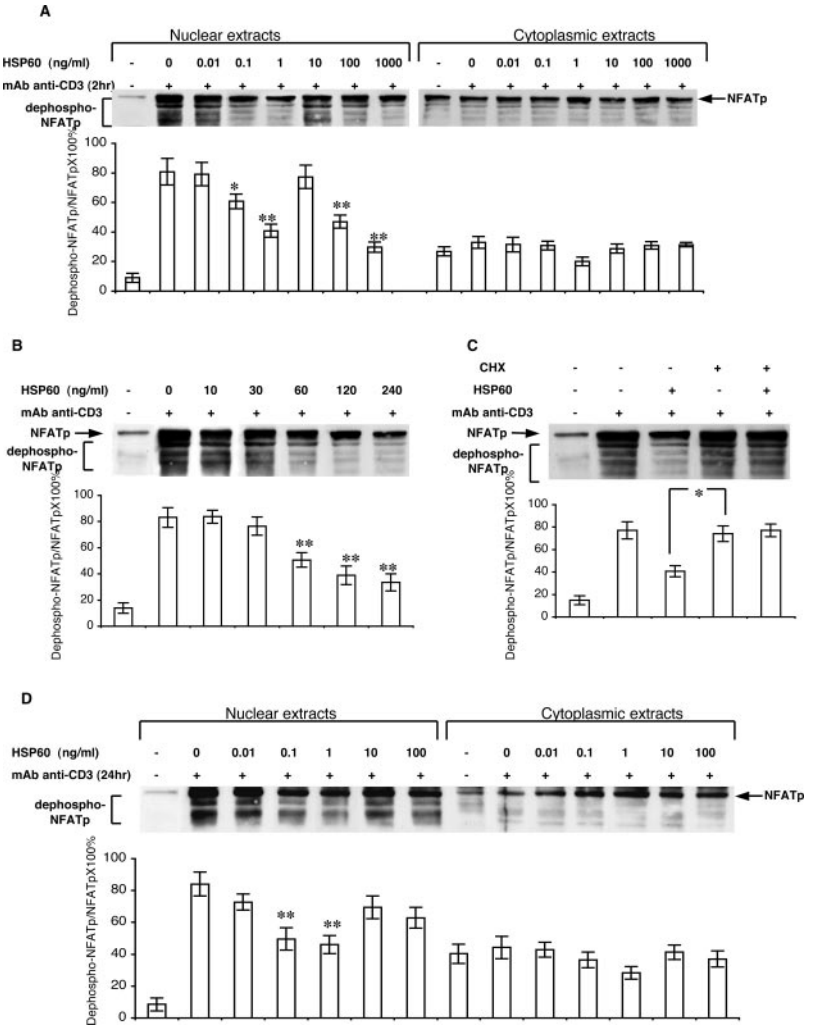

FIGURE 7. HSP60 inhibits anti-CD3-induced NFATp activation in T cells. Purified human T cells were incubated with HSP60 at 0.01–100 ng/ml for 1 h (*A* and *D*) or at 1 ng/ml for 0–240 min (*B*). *C*, The cells were pretreated with CHX (50 μM, 30 min) and exposed to HSP60 (1 ng/ml, 2 h). The T cells were then washed and exposed to immobilized anti-CD3 for 2 h (*A–C*) or 24 h (*D*). Nuclear (*A–D*) and cytoplasmic (*A* and *D*) lysates were immunoblotted with anti-NFATp. The *upper NFATp band* served as a control. One experiment representative of three is presented in each case. The levels of dephospho-NFATp were estimated by densitometry, and the average percentage of three different donors was calculated by OD of dephospho-NFATp/NFATp (*upper band*) $\times$ 100%. *, $p < 0.05$; **, $p < 0.01$.

down-regulation of NFATp activation also persisted after 24 h of exposure to the anti-CD3 Abs (Fig. 7*D*).

HSP60 inhibits T-bet and up-regulates GATA-3 expression via TLR2 in human T cells

We found that HSP60 inhibited secretion of Th1-associated cytokines IFN-γ and TNF-α and up-regulated secretion of the Th2-associated cytokines IL-10 (Fig. 1), IL-4, and IL-13 (data not shown). On the basis of these results, we considered that HSP60 might regulate differentially transcription factors associated with the Th1 and Th2 phenotypes, T-bet (16) and GATA-3 (12). We found that HSP60 significantly ($p < 0.01$) up-regulated the protein level of GATA-3 but not that of T-bet (Fig. 8*A*). To rule out the possibility that the differences in T-bet and GATA-3 expression were due to unequal amounts of protein loaded on the gel, we incubated the same blot with anti-T-bet and anti-GATA-3 Ab (separated by a stripping procedure). These experiments confirmed that the differences between GATA-3 and T-bet expression were not a result of a difference in protein loading.

The up-regulation of GATA-3 was TLR2 dependent because anti-TLR2, but not anti-TLR4, mAb abrogated the activation by HSP60 (Fig. 8*B*).

It has been reported recently that the level of T-bet can be augmented by TCR-mediated signals (16). It was also shown that the maximal expression of GATA-3 in T cells requires stimulatory signals from both TCR and CD28 (Th2-inducing conditions); signaling via TCR alone induces a lesser degree of the expression of GATA-3 (12, 29). To analyze the effect of HSP60 on CD3-induced T-bet and GATA-3 expression, we pretreated the cells with different concentrations of HSP60. The cells were then washed and seeded on immobilized anti-CD3. In the absence of HSP60, expression levels of T-bet were up-regulated markedly in anti-CD3-treated T cells. However, at 0.1–1 ng/ml and 1 μg/ml, HSP60 significantly ($p < 0.01$) inhibited the T-bet expression induced by the anti-CD3 (Fig. 8*C*). As expected, anti-CD3 alone up-regulated the expression of GATA-3 to a much lesser extent, compared with that of T-bet. Furthermore, preincubation of T cells with HSP60 significantly up-regulated the expression level of GATA-3. Thus, HSP60 inhibited the anti-CD3-induced expression of T-bet but up-regulated that of GATA-3.

HSP60 modulates expression of Th1/Th2 transcription factors in activated mouse T cells

The above findings indicated that HSP60 can down-regulate the molecular events leading to Th1 expression and can up-regulate those leading to Th2 expression in human T cells. To test whether HSP60 might shift the cytokine balance of already Th1-differentiated cells, we purified T cells from lymph nodes of mice immunized with oxazolone for 5 days, which induces Th1-like differentiation (11). We then examined the ability of HSP60 to modulate the expression levels of T-bet and GATA-3 in these committed T cells ex vivo. The T cells of the lymph nodes of the immunized mice expressed markedly higher levels of T-bet than we detected in nonactivated human T cells (compare the first bands in Fig. 8,

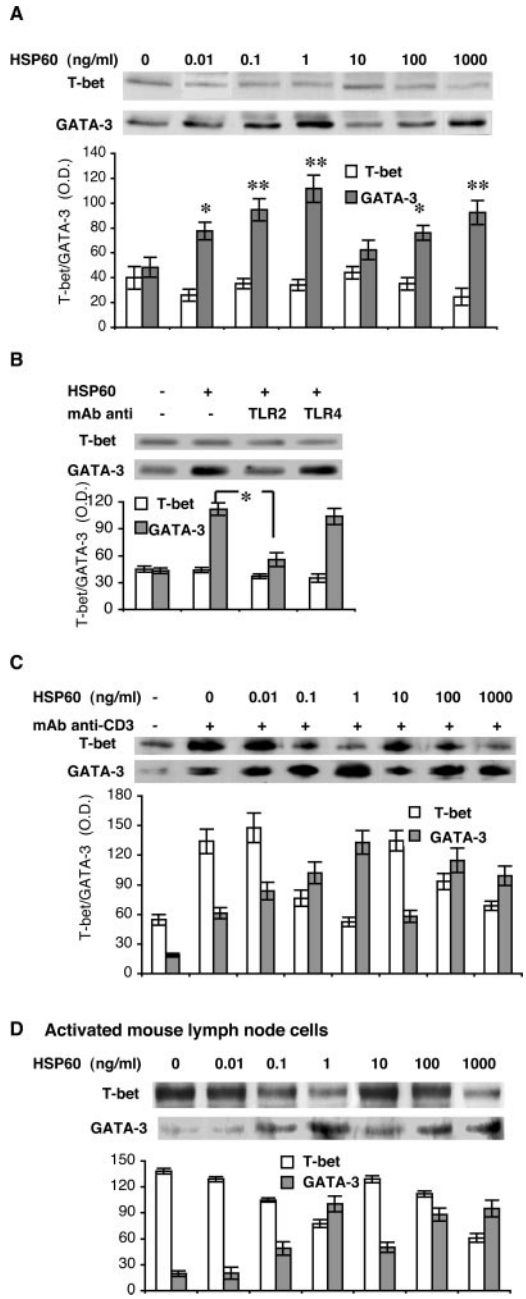

FIGURE 8. HSP60 inhibits T-bet and up-regulates GATA-3 expression in TLR-2-dependent signaling. Purified human T cells (*A–C*) or activated mouse lymph node cells (*D*) were incubated with HSP60 at 0.01–1000 ng/ml for 1 h (*A–C*). Some cells were pretreated with anti-TLR2 or anti-TLR4 (20 μg/ml, 30 min) and exposed to HSP60 (1 ng/ml, 2 h) (*B*). Then, the cells were washed and incubated in full medium (*A*, *B*, and *D*) or in the presence of immobilized mAb anti-CD3 (*C*). Nuclear lysates were immunoblotted with anti-T-bet Ab, stripped, and the same blot was incubated with anti-GATA-3 Ab. One experiment representative of three is presented in each case. The levels of T-bet and GATA-3 were estimated by densitometry, and the average percentage derived from three to five different donors was shown. *, $p < 0.05$; **, $p < 0.01$.

A and *D*). Nevertheless, these mouse T cells still down-regulated the expression of T-bet and up-regulated the expression of GATA-3 significantly upon treatment with 1 ng/ml HSP60 (Fig. 8*D*). Thus, HSP60 could modulate T-bet and GATA-3 expression both when applied in vitro to human T cells before activation by anti-CD3 Abs (Fig. 8*C*) and when applied ex vivo to mouse T cells already activated by immunization (Fig. 8*D*).

HSP60 suppresses Con A-induced hepatic injury in mice, which is associated with inhibition of T-bet and up-regulation of SOCS3 and GATA-3

To test whether HSP60 administration could affect an inflammatory disease in vivo, we examined the effect of HSP60 on BALB/c mice with acute liver injury induced by i.v. Con A; the liver injury has been shown to be caused by proinflammatory $CD4^+$ T cells (30). It has also been shown that the levels of expression of TNF-α and IL-4 are increased following Con A injection, whereas expression of IL-10 is decreased. Moreover, down-regulation of SOCS3 was observed in the hepatitis model (31), and SDF-1α-CXCR4 interactions were found to play a major role in the disease (32).

In this study, we administered HSP60 or saline i.p. twice (1 h and 18 h), before Con A injection. Changes in the secretion profiles of cytokines and a rise in serum levels of liver enzymes were used as markers for the severity of hepatitis; we measured serum cytokines at 2 h and liver enzymes at 18 h. Histological analysis of hepatic tissues was also performed 18 h after Con A administration. We found that HSP60 significantly ($p < 0.05$) inhibited the release of AST and ALT from the livers of the Con A-treated mice (Fig. 9*A*) and reduced the levels of TNF-α in the sera of the mice (Fig. 9*B*).

Histopathological examination of liver sections confirmed that HSP60 administration reduced liver damage. Con A induced a marked inflammatory-cell infiltrate around the central veins and large areas of necrosis in the liver lobules. In contrast, mice treated with HSP60 manifested only minimal liver damage; there were no areas of necrosis, and leukocyte infiltration was almost absent (Fig. 9*C*).

To assay the effects of HSP60 on the T cells of the mice, we purified T cells from the spleens and measured the expression of SOCS3, GATA-3, and T-bet in lysates of the T cells. T cell donor mice were either healthy, treated with HSP60, treated with Con A, or treated with both HSP60 and Con A. SOCS3 expression was minimal in healthy mice but became elevated following HSP60 treatment and was even more elevated in the mice treated with both Con A and HSP60 (Fig. 9*D*). Thus, the suppression of Con A-induced hepatitis was associated with augmentation of SOCS3 expression.

We also measured the expression of GATA and T-bet in these mouse T cells. HSP60 suppressed the Con A-induced expression of the Th1-associated transcription factor T-bet, while significantly augmenting the expression of the Th2-associated factor GATA-3 ($p < 0.05$; Fig. 9*E*). Thus, HSP60 down-regulates hepatitis in mice by down-regulating the expression of T-bet and up-regulating the expression of GATA-3 and SOCS3. These in vivo and ex vivo results in mice confirmed the findings induced by HSP60 in human T cells obtained in vitro.

Discussion

It has been shown that HSP60 can regulate the clinical signs of T cell-mediated experimental diabetes: administration of HSP60 or certain of its peptides led to the arrest of β-cell destruction in both the spontaneous diabetes of NOD mice and in human patients (3, 9, 33). HSP60 or a HSP60 peptide could also down-regulate adjuvant arthritis (7). The anti-inflammatory effects of HSP60 were marked by a shift in the autoimmune response from a damaging Th1 phenotype to a healing Th2 phenotype. In the current study, we found that treatment of T cells in vitro with HSP60 for $\geq$1 h induced changes in the expression and activation of transcription factors in response to mitogenic anti-CD3 Abs: HSP60 down-regulated the expression of the Th1 cytokine-associated transcription

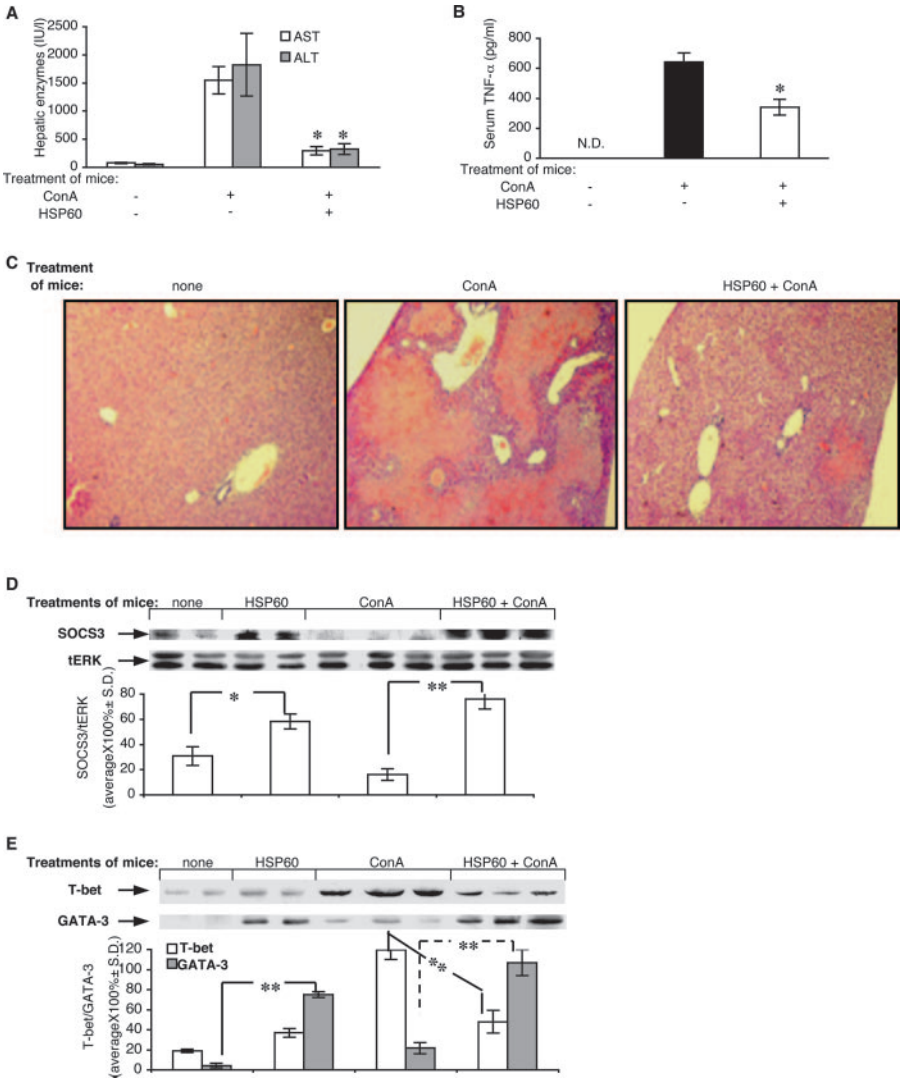

FIGURE 9. HSP60 inhibits Con A-induced hepatitis. The levels of hepatic enzymes, AST and ALT (*A*), and of TNF-α (*B*) were examined 24 and 2 h following Con A administration, respectively, in sera obtained from untreated, Con A-treated, and HSP60 and Con A-treated BALB/c mice (seven mice per group) (*C*). Histopathological analysis using H&E staining of liver sections of untreated and treated mice. T cells were purified from the spleens of untreated or treated mice, lysed, and immunoblotted with anti-SOCS3 (*D*), anti-T-bet, and anti-GATA-3 (*E*). Each band in the gels is composed of a pool of T cell lysate from two mice. The columns show the levels of SOCS3, T-bet, and GATA-3 expression were estimated by densitometry, and the average percentage ($\pm$SD) of the various pools was calculated. This experiment was repeated three times, and a representative experiment is shown. *, $p < 0.05$.

factors NF-κB, NFATp, and T-bet, while up-regulating the expression of a Th2-associated transcription factor, GATA-3. These early signaling events (2–24 h after anti-CD3) were followed later by a switch in cytokine secretion pattern (24–72 h after anti-CD3): down-regulation of TNF-α and IFN-γ secretion and augmentation of IL-10 secretion. Furthermore, the ability of HSP60 to modulate the T cell cytokine secretion pattern in vitro was reflected in vivo by down-regulation of Th1-mediated hepatitis in treated mice.

A number of recent papers have concluded that the effects of HSP60 reported on macrophages are likely to be caused by LPS contaminating the recombinant HSP60 (22–24). However, in contrast to macrophages, the effects of HSP60 on human T cells cannot be attributed to LPS contamination. The reasons are as follows: 1) our HSP60 preparation contained <0.001 EU/ml (<0.1 pg/ml) of bacterial endotoxin; 2) T cells are relatively unresponsive to the doses of LPS that are active on macrophages; 3) the effects of HSP60 on secretion of IFN-γ, TNF-α, and IL-10 by human T cells were inhibited by boiling, which denatures HSP60, but does not affect the biological activities of LPS; 4) treating the HSP60 preparation with PMB, which removes LPS, had no effect; 5) incubation of the HSP60 with PMB-coupled agarose beads, which removes macrophage-activating lipoproteins associated with LPS (23, 24), did not affect the activity of HSP60; 6) LPS, in contrast to HSP60, had no effect on T cell cytokine secretion, even when used at much higher concentrations (100 ng/ml) than those present in the HSP60 preparation (Fig. 5); and 7) the HSP60-specific mAb abrogated the effect of HSP60 on cytokine secretion but not that of LPS (data not shown). Macrophages, unlike T cells, are extremely sensitive to LPS, and any LPS contamination can be critical in macrophage experiments using HSP60. However, T cells are much less sensitive to LPS and much more sensitive to HSP60 than are macrophages; thus, contamination with LPS is not as serious a problem with regard to T cells.

Our results implicate TLR2 signaling in the action of HSP60 together with the regulation of expression of Th1 and Th2-associated transcription factors. The mechanistic responses and downstream effects following TLR ligation are partially understood only, but it is clear that all TLR activate MyD88-dependent pathways (34, 35), leading to activation of NF-κB and MAPK, ERK, JNK, and p38 (35, 36). It is reasonable to suppose that, by their ability to interact intracellularly with adaptor protein molecules such as MyD88, ligated TLR can signal the activation and expression of various cytokine regulatory factors and cytokines, including IFN-γ in APC (37, 38). It has recently been suggested that, in addition to its ability to interact with MyD88, TLR2 can recruit other adaptor molecules, such as Toll-IL-1R domain-containing adapter protein, and signal the secretion of anti-inflammatory cytokines (39). In addition, ligation of TLR2 on APC by microbial molecules induces a strong Th2 response implicated in asthma (40,

41). Interestingly, SOCS3, which is up-regulated in T cell responses to HSP60 (A. Zanin-Zhorov et al., manuscript in preparation), is expressed predominantly on Th2 cells and therefore has been suggested to play a pivotal role in the onset and maintenance of allergic diseases, such as asthma and atopic dermatitis (42).

In the present study, we extended our analysis of the effects of HSP60 to a model of acute inflammatory hepatitis. Con A-induced hepatitis in mice is a typical Th1-like disease, in which an elevation in the serum levels of IL-6, IFN-γ, and TNF-α and a parallel decrease in IL-10 have been observed (30, 43). Also, the level of the in situ activation of STAT1 and STAT3 appears to correlate with the amount of damage present in the inflamed liver, while activation of SOCS1 is decreased (34). We found that treatment of mice with HSP60 induced significant suppression of the cytological and pathological signs of hepatitis (Fig. 9). Moreover, HSP60 also increased the level of SOCS3 and GATA-3, while decreasing the level of expression of T-bet in the mouse T cells. Because the CXCR4-SDF-1α interaction also plays a critical role in regulating the entry of T cells to the inflamed liver (35), it is likely that the mechanism of suppression of experimental hepatitis by HSP60 involves at least two pathways: 1) inhibition of Con A-induced and SDF-1α-mediated T cell chemotaxis into the liver; and 2) a switch in the Th1/Th2 cytokine secretion profiles of the T cells that damage the liver.

The findings presented here and elsewhere indicate that HSP60 can inhibit Th1-mediated inflammatory disease (3, 7, 9, 33). Importantly, the in vitro studies indicate that modulation of cytokine secretion by HSP60 in human T cells is only partial: at most, we noted a 50% inhibition of TNF-α and IFN-γ and a 2- to 3-fold elevation of IL-10 (Fig. 1). Nevertheless, the effects of HSP60 in down-regulating inflammatory disease in vivo were significant; Con A-induced hepatitis was inhibited by 80–90% (Fig. 9).

In addition to our previous results, the results of this study indicate that HSP60, which is up-regulated by stress and inflammation, can function as a down-regulator of T cell-mediated inflammation. Thus, the HSP60 molecule acts both inside the cell, as a chaperone and as a signal molecule between immune cells, to activate the immune system and to reduce its inflammatory potential postactivation.

Acknowledgments

The anti-HSP60 mAb, clone P5, was kindly provided by Dr. Francisco Quintana.

Disclosures

The authors have no financial conflict of interest.

References

1. Wallin, R. P. A., A. Lundqvist, S. H. More, A. von Bonin, R. Kiessling, and H.-G. Ljunggren. 2002. Heat shock proteins as activators of the innate immune system. *Trends Immunol. 23:130*.
2. Young, D. B., J. Ivanyi, J. H. Cox, and J. R. Lamb. 1987. The 65 kD antigen of mycobacteria: a common bacterial protein? *Immunol. Today 8:215*.
3. Raz, I., D. Elias, M. Tamir, M. Metzger, and I. R. Cohen. 2001. β-Cell function in new-onset type I diabetes and immunomodulation with a heat shock protein peptide (DiaPep277): a randomized, double blind, phase II trial. *Lancet 258:1749*.
4. Hu, W., A. Hasan, A. Wilson, M. R. Stanford, T. Li-Yang, S. Todryk, R. Whiston, T. Shinnick, Y. Mizushima, R. van der Zee, and T. Lehner. 1998. Experimental mucosal induction of uveitis with the 60-kDa heat shock protein-derived peptide 336–351. *Eur. J. Immunol. 28:2444*.
5. Mor, F., and I. R. Cohen. 1992. T cells in the lesion of experimental autoimmune encephalomyelitis: enrichment for reactivities to myelin basic protein and to heat shock proteins. *J. Clin. Invest. 90:2447*.
6. Xu, Q., G. Schett, H. Perschinka, M. Mayr, G. Egger, F. Oberhollenzer, J. Willeit, S. Kiechl, and G. Wick. 2000. Serum soluble heat shock protein 60 is elevated in subjects with atherosclerosis in a general population. *Circulation 102:14*.
7. Quintana, F. J., P. Carmi, F. Mor, and I. R. Cohen. 2002. Inhibition of adjuvant arthritis by a DNA vaccine encoding human heat shock protein 60. *J. Immunol. 169:3422*.
8. Quintana, F. J., P. Carmi, and I. R. Cohen. 2002. DNA vaccination with heat shock protein 60 inhibits cyclophosphamide-accelerated diabetes. *J. Immunol. 169:6030*.
9. Elias, D., A. Meilin, V. Ablamuntis, O. S. Birk, P. Carmi, S. Konen-Weisman, and I. R. Cohen. 1997. Hsp60 peptide therapy of NOD mouse diabetes induces a Th2 cytokine burst and down-regulates autoimmunity to various β-cell antigens. *Diabetes 46:758*.
10. Zanin-Zhorov, A., G. Nussbaum, F. Franitza, I. R. Cohen, and O. Lider. 2003. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. *FASEB J. 17:1567*.
11. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol. 7:145*.
12. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell 89:587*.
13. Kuo, C. T., and J. M. Leiden. 1999. Transcriptional regulation of T lymphocyte development and function. *Annu. Rev. Immunol. 17:149*.
14. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell 100:655*.
15. Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. *Science 295:338*.
16. Glimcher, L. H., and K. M. Murphy. 2000. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev. 14:1693*.
17. Murphy, K. M., W. Ouyang, J. D. Farrar, J. Yang, S. Ranganath, H. Asnagli, M. Afkarian, and T. L. Murphy. 2000. Signaling and transcription in T helper development. *Annu. Rev. Immunol. 18:451*.
18. Ohashi, K., V. Burkart, S. Flohe, and H. Kolb. 2000. Heat shock protein 60 is a putative endogenous ligand of the Toll-like receptor-4 complex. *J. Immunol. 164:558*.
19. Kol, A., G. K. Sukhova, A. H. Lichtman, and P. Libby. 1998. Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-α and matrix metalloproteinase expression. *Circulation 98:300*.
20. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartman. 2002. Quantitative expression of Toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligonucleotides. *J. Immunol. 168:4531*.
21. Komai-Koma, M., L. Jones, G. S. Ogg, D. Xu, and F. Y. Liew. 2002. TLR2 is expressed on activated T cells as a costimulatory receptor. *Proc. Natl. Acad. Sci. USA 101:3029*.
22. Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signaling. *Nature 395:284*.
23. Gao, B., and M.-F. Tsan. 2003. Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor α from murine macrophages. *J. Biol. Chem. 278:22523*.
24. Lee, H.-K., J. Lee, and P. S. Tobias. 2002. Two lipoproteins extracted from *Escherichia coli* K-2 LCD25 lipopolysaccharide are the major components responsible for toll-like receptor 2-mediated signaling. *J. Immunol. 168:4012*.
25. Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol. 16:225*.
26. Shaw, J., P. Utz, D. Durand, J. Toole, E. Emmel, and G. Crabtree. 1988. Identification of a putative regulator of early T cell activation genes. *Science 241:202*.
27. Northrop, J. P., S. N. Ho, L. Chen, D. J. Thomas, L. A. Timmerman, G. P. Nolan, A. Admon, and G. R. Crabtree. 1994. NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature 369:497*.
28. Ranger, A. M., M. Oukka, J. Rengarajan, and L. H. Glimcher. 1998. Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. *Immunity 9:627*.
29. Skapenko, A., J. Leipe, U. Niesner, K. Devriendt, R. Beetz, A. Radbruch, J. R. Kalden, P. E. Lipsky, and H. Schulze-Koops. 2004. GATA-3 in human T cell helper type 2 development. *J. Exp. Med. 199:423*.
30. Tiegs, G., J. Hentschel, and A. Wendel. 1992. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. *J. Clin. Invest. 90:196*.
31. Hong, F., B. Jaruga, W. H. Kim, S. Radeava, O. N. El-Assal, Z. Tian, V.-A. Nguen, and B. Gao. 2002. Opposing roles of STAT1 and STAT3 in T cell-mediated hepatitis: regulation by SOCS. *J. Clin. Invest. 110:1503*.
32. Wald, O., O. Pappo, R. Safadi, M. Dagan-Berger, K. Beider, H. Wald, S. Franitza, I. Weiss, S. Avniel, P. Boaz, et al. 2004. Involvement of the CXCL12/CXCR4 pathway in the advanced liver disease that is associated with hepatitis C virus or hepatitis B virus. *Eur. J. Immunol. 34:1164*.
33. Bockova, J., D. Elias, and I. R. Cohen. 1997. Treatment of NOD diabetes with novel peptide of HSP60 molecule induces Th2-type antibodies. *J. Autoimmun. 10:323*.
34. Barton, G. M., and R. Medzhitov. 2003. Toll-like receptor signaling pathways. *Science 300:1524*.

35. Li, L. 2004. Regulation of innate immunity signaling and its connection with human diseases. *Curr. Drug Targets Inflamm. Allergy 3:81*.

36. Agrawal, S., A. Agrawal, B. Doughty, A. Gerwitz, J. Blenis, T. Van Dyke, and B. Pulendran. 2003. Different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J. Immunol. 171:4984*.

37. Into, T., K. Kiura, M. Yasuda, H. Kataoka, N. Inoue, A. Hasebe, K. Takeda, S. Akira, and K. Shibata. 2004. Stimulation of human Toll-like receptor (TLR) 2 and TLR6 with membrane lipoproteins of *Mycoplasma fermentans* induces apoptotic cell death after NF-κB activation. *Cell. Microbiol. 6:187*.

38. Coccia, E. M., M. Severa, E. Giacomini, D. Monneron, M. E. Remoli, I. Julkunen, M. Cella, R. Lande, and G. Uze. 2004. Viral infection and Toll-like receptor agonists induce a differential expression of type I and λ interferons in human plasmacytoid and monocyte-derived dendritic cells. *Eur. J. Immunol. 34:796*.

39. Netea, M. G., C. van der Graaf, J. W. M. Van der Meer, and B. J. Kullberg. 2004. Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system. *J. Leukocyte Biol. 75:749*.

40. Dillon, S., A. Agrawal, T. Van Dyke, G. Landreth, L. McCauley, A. Koh, C. Maliszewski, S. Akira, and B. Pulendran. 2004. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. *J. Immunol. 172:4733*.

41. Redecke, V., H. Hacker, S. K. Datta, A. Fermin, P. M., Pitha, D. H. Broide, and E. Raz. 2004. Activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. *J. Immunol. 172:2739*.

42. Seki, Y., H. Inoue, N. Nagata, K. Hayashi, S. Fukuyama, K. Matsumoto, O. Komine, S. Hamano, K. Himeno, K. Inagaki-Ohara, et al. 2003. SOCS-3 regulates onset and maintenance of T_H2-mediated allergic responses. *Nat. Med. 9:1047*.

43. Kato, M., N. Ikeda, E. Matsushita, S. Kaneko, and K. Kobayashi. 2001. Involvement of IL-10, an anti-inflammatory cytokine, in murine liver injury induced by Con A. *Hepatol. Res. 20:232*.