Heat Shock Protein 60 Activates Cytokine-Associated Negative Regulator Suppressor of Cytokine Signaling 3 in T Cells: Effects on Signaling, Chemotaxis, and Inflammation[1]

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Previously, we reported that treatment of T cells with the 60-kDa heat shock protein (HSP60) inhibits chemotaxis. We now report that treatment of purified human T cells with recombinant human HSP60 or its biologically active peptide p277 up-regulates suppressor of cytokine signaling (SOCS)3 expression via TLR2 and STAT3 activation. SOCS3, in turn, inhibits the downstream effects of stromal cell-derived-1α (CXCL12)-CXCR4 interaction in: 1) phosphorylation of ERK1/2, Pyk2, AKT, and myosin L chain, required for cell adhesion and migration; 2) formation of rear-front T cell polarity; and 3) migration into the bone marrow of NOD/SCID mice. HSP60 also activates SOCS3 in mouse lymphocytes and inhibits their chemotaxis toward stromal cell-derived factor-1α and their ability to adoptively transfer delayed-type hypersensitivity. These effects of HSP60 could not be attributed to LPS or LPS-associated lipoprotein contamination. Thus, HSP60 can regulate T cell-mediated inflammation via specific signal transduction and SOCS3 activation. *The Journal of Immunology,* **2005, 175: 276–285.**

During their migration into inflammatory sites, T cells interact with tissue components and encounter a variety of immunomodulators, including cytokines, chemokines, acute phase proteins, and heat shock proteins (HSP)[4] (1–3). In addition to serving as a chaperone inside the cell, 60-kDa HSP (HSP60) is expressed by cells exposed to stress (3, 4) or immune activation (5), and is present in the blood and tissues during inflammation (6–9). HSP60 appears to down-regulate inflammation in models of type 1 diabetes (10, 11) and arthritis (5). Recently, we found that incubation of human T cells for 1 h with HSP60 inhibited cytoskeletal rearrangement and chemotaxis within the extracellular matrix in vitro induced by CXCL12 (stromal cell-derived factor (SDF)-1α) (12). Expression of the SDF-1α and EBV-induced molecule 1 ligand chemokine receptors, CXCR4 and CCR7, was also reduced by HSP60, but this effect required 18 h of incubation (12); at 1 h there was no detectible reduction of CXCR4 and CCR7 expression. Inhibition of chemotaxis to SDF-1α at 1 h suggested, therefore, that HSP60 might affect intracellular signaling downstream of CXCR4 ligation and activation by SDF-1α. The aim of the present study was to explore this possibility.

Immune cell migration mediated by chemotactic molecules is essential for a variety of physiological and pathological events, including inflammation (13–15). SDF-1α is expressed in a broad range of tissues and has multiple biological activities on diverse cell types (15). By binding to CXCR4, SDF-1α is chemotactic for B cells, T cells, monocytes, and hemopoietic cells (16–18). CXCR4-SDF-1α interactions are implicated in multiple signal transduction pathways, including activation of the p44/42 MAPK (19), PI3K, and AKT (18, 19), tyrosine phosphorylation of focal adhesion complex components, such as Pyk-2 and Crk (19), Rho-dependent phosphorylation of myosin L chain (MLC) (20), and the JAK/STAT pathway (21). CXCR4-mediated responses were found to be functionally inactivated through up-regulation of the protein suppressor of cytokine signaling (SOCS) (22).

In this study, we examined the effects of HSP60 in 1 h or less on the intracellular signaling and subsequent T cell functions induced by SDF-1α. Our results demonstrate that the inhibitory effects of HSP60 on SDF-1α-induced T cell responses, in vitro and in vivo, are mediated through up-regulation of SOCS3, and implicate a SOCS3-mediated molecular signaling mechanism for the innate effects of HSP60 on T cells. These findings provide a mechanism for the anti-inflammatory effect of HSP60 on T cell-mediated inflammation. Our experiments also indicate that these effects of HSP60 cannot be assigned to any contamination by LPS or other bacterial products; indeed, the results could be specifically reproduced with a peptide synthesized from the HSP60 sequence.

Materials and Methods

Reagents

The following reagents and chemicals were purchased as indicated: recombinant HSP60 (StressGen Biotechnologies); RPMI 1640 (Invitrogen Life Technologies); FCS, antibiotics, sodium pyruvate (Biological Industries); fibronectin (Chemicon International); SDF-1α (R&D Systems), phosphatase inhibitor mix, polymyxin B (PMB) and PMB-agarose beads (Sigma-Aldrich); AG9 and AG490 (Calbiochem), and $Na_2{}^{51}CrO_4$ (Amersham Pharmacia Biotech). Monoclonal Abs: anti-human CXCR4 (clone 12G5; R&D Systems); anti-SOCS3 (H-103; Santa Cruz Biotechnology); anti-

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[4] Abbreviations used in this paper: HSP, heat shock protein; SOCS, suppressor of cytokine signaling; PMB, polymyxin B; DTH, delayed-type hypersensitivity; SDF, stromal cell-derived factor; MLC, myosin L chain.

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TLR2 and TLR4 (eBioscience), and anti-human recombinant HSP60 (designated clone P5, IgM fraction; kindly provided by F. Quintana, The Weizmann Institute of Science, Rehovot, Israel). Abs anti-phosphorylated Pyk2 (clone py881) and anti-phosphorylated ERK1/2 (BioSource International); anti-total Pyk2 (clone N-19), anti-phosphorylated MLC and Ab anti-MLC (FL-172), anti-phosphorylated STAT3 (B7), and anti-total STAT3 (H-190; Santa Cruz Biotechnology); and anti-total ERK1/2 (Sigma-Aldrich); anti-phosphorylated AKT (pAKT) and anti-AKT (Cell Signaling Technology). 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 LAL test method (Biological Industries). The peptides used in this study were prepared using standard FMOC chemistry as previously described (23). The sequence of p277 is VLGGGVALLRVI PALDSLTPANED, residues 437–460 of human HSP60. The sequence of p30 is FNEETVSFWLRVPKVSASHLE, residue 947–967 of tetanus toxoid.

Human cells

T cells were purified from the peripheral blood of healthy human donors (Blood Bank, Tel-Hashomer Hospital) as previously described (2). Whole blood was incubated (20 min, 22°C) with RosetteSep Human T Cell Enrichment Cocktail (StemCell Technologies). After which, unsedimented cells were loaded onto Lymphocyte Separation Medium (ICN Biomedicals), and T cells were isolated by density centrifugation and washed with PBS. Purified cells (>97% $CD3^+$ T cells) thus obtained were cultured in RPMI 1640 containing antibiotics and 10% heat-inactivated FCS. HUVEC were isolated from umbilical cord veins and cultured as described (24). Primary cultures were serially passaged, and passages 2 and 3 were taken for experiments.

Mice and mouse T cells

Female C57BL/6J mice were obtained from Harlan Olac. TLR2 knockout mice on the C57BL/6J background were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan). $CD3^+$ T cells were isolated by negative selection with anti-mouse Ab mix (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. Similarly, T cells were purified from the lymph nodes of female BALB/c mice 1.5 mo of age.

T cell migration assay

Migration of ^{51}Cr-labeled human and mouse T cells through fibronectin or endothelial cells to recombinant human SDF-1α was examined in 48-well transwell chemotaxis apparatus (5-μm pore filters, 6.5-mm diameter; Corning), as previously described (2, 16). For transendothelial migration assays, 2×10^4 cells of HUVEC were layered on the filters and grown in 10% FCS M199 for 2 days before the performance of each assay. Then, HUVEC were stimulated with TNF-α (50 ng/ml) for 24 h and washed.

Western blotting of T cell lysates

T cells were incubated (24 h, 37°C) in starvation medium (RPMI 1640 medium without serum). Aliquots (5×10^6/sample) of the starved cells were preincubated (for various intervals) with different concentrations of HSP60 before exposure to SDF-1α (100 ng/ml, 10 min, 37°C, tissue culture conditions). These reactions were terminated by freezing the plates (−70°C, 10 min). The plates were thawed and the cells solubilized by incubating (60 min, 4°C) in lysis buffer (EDTA (0.5 mM), NaCl (150 mM), NaF (10 nM), Tris pH 7.5 (25 mM), Triton X-100 (1%), PMSF (200 μg/ml), and phosphatase inhibitor (1%) mixture). Lysates were cleared by centrifugation (30 min, 14×10^3 rpm, 4°C), and the resulting supernatants analyzed for protein content. Sample buffer was added, the samples were boiled, and equal amounts of proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with TBST buffer (Tris pH 7.5 (20 mM), NaCl (135 mM), and 0.1% Tween 20) containing low-fat milk (5%), and probed with the following mAb in the same buffer: anti-phosphorylated ERK (pERK) (0.2 μg/ml), anti-total ERK (tERK) (diluted 1/20,000), anti-pPyk2 (1.5 μg/ml), anti-tPyk2 (0.2 μg/ml), anti-pAKT (diluted 1/1000), anti-tAKT (diluted 1/1000), anti-pMLC (diluted 1/250), and anti-tMLC (diluted 1/1000), anti-pSTAT3 (diluted 1/250), and anti-tSTAT3 (diluted 1/500). Immunoreactive protein bands were visualized using an HRP-conjugated goat anti-mouse Ab and the enhanced ECL system. Phosphorylation levels of the three to five independent experiments were estimated by densitometry, and an average percentage of phosphorylation (±SD) was calculated as OD of pERK/tERK, or pPyk2/tPyk2, or pAKT/tAKT, pMLC/tMLC, or pSTAT3/tSTAT3 × 100%.

Cell morphology

T cells (3×10^6 cells/ml) were seeded in flat-bottom, 24-well plates in 500 μl of RPMI 1640 on coverslips coated with fibronectin (25 μg/ml). Some T cells were pretreated (1 h, 37°C, in a 7.5% CO_2 humidified atmosphere) with anti-TLR mAb (20 μg/ml), and then with HSP60 (1 h). After which, SDF-1α (200 ng/ml) was added, and the cells allowed to adhere (30 min, 37°C, in a 7.5% CO_2 humidified atmosphere). The cells were then fixed in 3.7% paraformaldehyde and permeabilized (5 min, room temperature) with 0.5% Triton X-100. For actin visualization, cells were stained with rhodamine-labeled phalloidin (diluted 1/100), and examined with a laser-scanning confocal microscope (LSM510; Zeiss).

In vivo homing assay

Purified human T cells were incubated (1 h, tissue culture conditions) with HSP60 (1 μg/ml) in tissue culture conditions. The cells were then washed with PBS, and where indicated, further incubated (30 min, 4°C) with mouse anti-human CXCR4 mAb (10 μg/3×10^6 cells), anti-human TLR2 or anti-TLR4 mAb (each at 20 μg/ml). The cells were then washed and injected (5×10^6 cells/0.5 ml per mouse) i.v. into the tails of irradiated (375 cGY from a cobalt 60 source), 8-wk-old NOD/SCID mice, and the entrance of human T cells into the bone marrow of recipient mice was evaluated as previously described (24, 25). Each tested sample contained 1.5×10^6 cells.

Delayed-type hypersensitivity (DTH) assays

Female BALB/c mice at 1.5 mo of age were sensitized by painting of 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. The cells were treated with HSP60 (1 μg/ml, 1 h, tissue culture conditions), washed, and injected (5×10^7 cells per mouse) i.v. into the tails of naive mice (at least six mice per group). Painting of the earlobes with oxazolone (10 μl of 0.5% in acetone/olive oil), which generates a DTH response, was performed immediately after cell inoculation. The area of the ears that was painted was measured before challenge and 24 h later with a micrometer. Changes in earlobe thickness are indicative of DTH reactivity. All animal studies were performed in compliance with and were approved by laws and animal welfare guidelines of The Weizmann Institute of Science (Institutional Animal Care and Use Committee).

RNA interference

We synthesized a silent RNA sequence targeting SOCS3 position 80–101 relative to the start codon: 5′-AAGAGCGAGTACCAGCTGGTG-3′; a dsRNA targeting luciferase (GL-2) was used as control (Dharmacon Research). Transfections of freshly purified T cells were performed using the human T cell Nucleofector kit (Amaxa Biosystems). In brief, 5×10^6 $CD3^+$ T cell were resuspended in 100 μl of human T cell Nucleofector solution, mixed with a total of 3 μg of silent RNA duplex, and pulsed using the Nucleofector program U-14. We controlled cell viability by trypan blue. Transfected cells were cultured in RPMI 1640 containing 10% heat-inactivated FCS and 10 ng/ml IL-2. Cell migration was evaluated as described 48 h after transfection. Transfection efficiency was controlled by evaluating SOCS3 levels using Western blot analysis.

Results

Exposure of T cells to HSP60 for 1 h inhibits SDF-1α-induced chemotaxis through fibronectin and endothelial cells

We incubated purified human T cells with various concentrations of HSP60 for 1 h and assayed the effect on chemotaxis. We observed a significant inhibition of T cell chemotaxis toward SDF-1α both through fibronectin (Fig. 1*A*) and through TNF-α-activated endothelial cells (Fig. 1*B*). These findings suggested that HSP60 might decrease the migration of T cells toward SDF-1α in vivo.

HSP60 decreases SDF-1α-mediated homing of human T cells to the bone marrow of NOD/SCID mice

Migration of T human cells in NOD/SCID mice is regulated primarily by interactions of SDF-1α with CXCR4; following exposure to sublethal doses of irradiation, the bone marrow of these mice express high levels of SDF-1α (24, 25). Therefore, we examined the homing of HSP60-treated human T cells into the bone

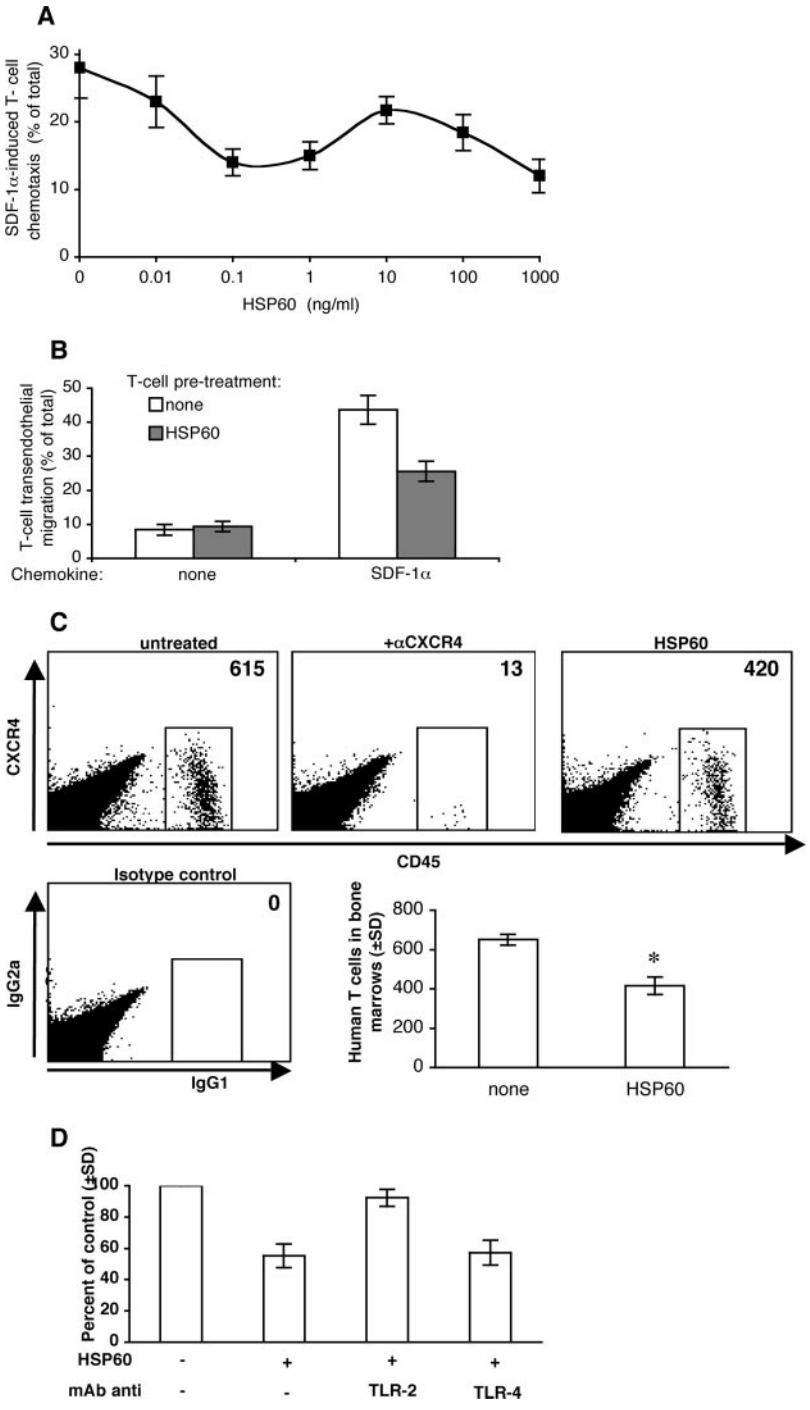

FIGURE 1. Inhibition of T cell chemotaxis toward SDF-1α. HSP60 inhibits SDF-1α-induced chemotaxis through fibronectin (*A*) and endothelial cells (*B*) of human T cells, and homing of such cells into the bone marrows of NOD/SCID mice in a TLR2-dependent manner (*C* and *D*). *A* and *B*, Human T cells were incubated with indicated concentrations of HSP60 for 1 h (*A*), or with 1 ng/ml HSP60 for 1 h (*B*), radioactively labeled, and T cell migration assays through fibronectin-coated (*A*), or through TNF-α-activated HUVEC monolayer (*B*) were performed (3 h, 37°C) in transwell apparati in the presence or absence of SDF-1α (100 ng/ml). Results are expressed as the percentage of T cells migrating toward SDF-1α. The average values $\pm$ SD of five experiments are depicted. *C* and *D*, Human T cells, some of which were pretreated with mouse anti-human CXCR4, TLR2, or anti-TLR4 mAb, were treated with HSP60 (1 μg/ml, 1 h) and injected into naive NOD/SCID mice. T cell homing to the bone marrows of mice was evaluated 16 h later by flow cytometry. The results are expressed as the number of homing human cells (*upper right corner*) per 10^6 acquired cells (*C*), and percentage of control (*D*). Combined data from five experiments are depicted. $*$, $p < 0.05$.

marrow of irradiated NOD/SCID mice. T cells were pretreated with HSP60 (1 μg/ml for 1 h) and/or anti-CXCR4, anti-TLR2, or anti-TLR4 mAb, and then the T cells were injected into irradiated NOD/SCID mice. After 16 h, homing of the T cells to the bone marrow of the mice was determined by assessing the expression of human CD45 and CXCR4 on bone marrow cells.

T cell homing into mouse bone marrow was significantly abrogated by pretreatment with anti-CXCR4 mAb (Fig. 1*C*); this indicates that CXCR4-SDF-1α interactions are indeed involved in the navigation of human T cells into this organ in vivo. Exposure of T cells to HSP60 for 1 h significantly ($p < 0.05$) decreased their homing to the bone marrow (Fig. 1*C*). Pretreatment of human T cells with neutralizing anti-TLR2 mAb, but not anti-TLR4 mAb (both are mouse IgG2a Abs), abrogated the inhibitory effect of HSP60 on T cell homing into recipient bone marrow (Fig. 1*D*). Thus, the down-regulation of SDF-1α-induced human T cell chemotaxis and homing by HSP60 requires functional TLR2.

HSP60 inhibits SDF-1α-induced mouse T cell chemotaxis in vitro and DTH in vivo

Recently, SDF-1α was shown to be involved in the recruitment of Ag-specific $CD4^+$ T cells to sites of DTH reactions in mice (26). Therefore, we examined whether HSP60 can inhibit chemotaxis of mouse T cells in vitro and a T cell-dependent DTH response in vivo. The effect of human HSP60 on SDF-1α-mediated chemotaxis of BALB/c lymph node cells in vitro was similar to that on human T cells: HSP60 inhibited migration of mouse lymph node

cells toward SDF-1α (Fig. 2*A*). We also studied the effect of human HSP60 on DTH in mice. Exposure in vitro of oxazolone-reactive mouse lymph node cells to HSP60 (1 μg/ml for 1 h) inhibited by 50% their ability to adoptively transfer DTH in vivo (Fig. 2*B*). Thus, HSP60, which inhibits SDF-1α-induced T cell chemotaxis, appears capable of down-regulating T cell homing to and function at inflammatory sites in vivo.

HSP60 inhibits SDF-1α-induced ERK phosphorylation via TLR-2

SDF-1α induces T cell migration by activating MAPKs (19). Activation of the MAPK designated ERK causes a cascade of events that involve Ras, Raf, and MAP/ERK kinase (27). Therefore, we examined the effect of HSP60 on ERK phosphorylation. T cells were pretreated (1 h) with various concentrations of HSP60 (Fig. 3*A*), or for various times with 0.1 ng/ml HSP60 (Fig. 3*B*). Some cells were then exposed to SDF-1α (100 ng/ml). The cells were lysed, and the lysates were subjected to SDS-PAGE and immunoblotting with mAb specific for pERK.

In contrast to SDF-1α, HSP60 alone did not induce ERK phosphorylation (data not shown). However, when HSP60-treated T cells were exposed to SDF-1α, ERK phosphorylation was significantly inhibited (Fig. 3*A*). The effect of HSP60 on SDF-1α-induced ERK phosphorylation was time-dependent; maximal levels of inhibition were achieved after 2 h of incubation (Fig. 3*B*).

HSP60 activates responsive macrophages via TLR4 or TLR2 (4, 9). Previously, we showed that T cells express TLR2, and that HSP60 can induce T cell adhesion and inhibit T cell migration toward SDF-1α by signaling through TLR2, but not through TLR4 (12). We tested whether TLR2 or TLR4 were involved in HSP60-mediated inhibition of SDF-1α-induced ERK phosphorylation by preincubating the T cells with anti-TLR neutralizing mAb. This inhibition by HSP60 was prevented by a mAb to human TLR2, but not by mAb to TLR4 (Fig. 3*C*). Thus, this effect of HSP60 on T cells too was mediated through TLR2.

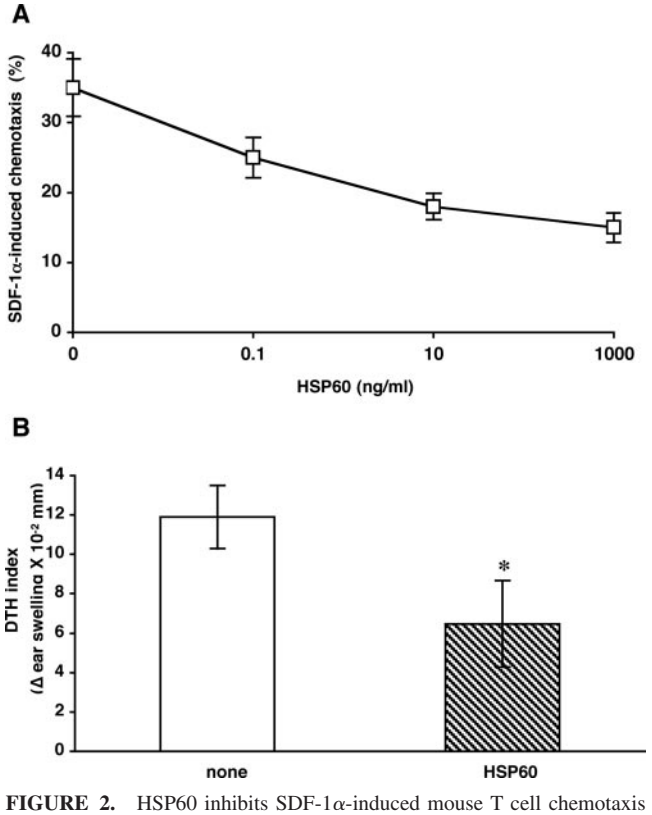

FIGURE 2. HSP60 inhibits SDF-1α-induced mouse T cell chemotaxis in vitro and DTH in vivo. *A*, Lymph node cells of BALB/c mice presensitized to oxazolone were incubated in vitro with HSP60 (1 h), washed, and their SDF-1α-mediated chemotaxis was determined. *B*, HSP60-treated mouse lymph node cells from oxazolone-sensitized mice were injected i.v. into naive recipients whose earlobes were then painted with oxazolone. DTH reactivity (ear swelling, 10^{-2} mm $\pm$ SD) was measured 24 h later. DTH index: $1 - [(\text{treated cells mice} - \text{control cells})] \times 100$. $*$, $p < 0.05$. One experiment representative of three is depicted.

HSP60 induces phosphorylation of Pyk2, but inhibits Pyk2 phosphorylation induced by SDF-1α

Pyk2 is a member of the focal adhesion kinase family. Tyrosine phosphorylation of Pyk2 (and related molecules) increases the activity of this kinase and links β_1 integrins to multiple signaling pathways that regulate cell adhesion and migration (27–30). Because phosphorylation of Pyk2 can be induced by SDF-1α (17), we examined whether pretreatment of T cells with HSP60 for 1 h might affect this phosphorylation. Although Pyk2 is constitutively activated in resting T cells, this activation was significantly increased ($p < 0.05$) by incubation with HSP60 (0.1–1 ng/ml for 1 h) in a bell-shaped dose-response pattern (Fig. 3*D*), similar to the previously observed increase that occurs after 10 min of incubation with the same concentrations of HSP60 (12). However, Pyk2 phosphorylation was inhibited when the T cells were preincubated with HSP60 and then exposed to SDF-1α for 10 min (Fig. 3*E*). This inhibitory effect occurred in a similar, but reversed, bell-shaped dose-response curve to that of Pyk2 activation by HSP60 alone (Fig. 3*D*); maximal inhibition was achieved with 0.1–1 ng/ml HSP60, decreased at 10–100 ng/ml, and increased again at 1 μg/ml. Thus, in addition to the ability of HSP60 to inhibit SDF-1α-induced phosphorylation of ERK (Fig. 3*A*), the inhibitory effect of HSP60 on T cell migration in response to SDF-1α could be attributed to HSP60-mediated down-regulation of SDF-1α-induced activation of Pyk2.

HSP60 inhibits SDF-1α-induced phosphorylation of AKT

Although the role of PI3K in chemokine-induced chemotaxis of various cell types is controversial (31, 32), SDF-1α does induce activation of PI3K type I via CXCR4. Furthermore, activation of PI3K is involved in the polarization and chemotactic responses of lymphocytes to SDF-1α (31). Previously, we showed that HSP60 induces T cell adhesion to fibronectin in a PI3K-dependent manner (12). Therefore, we measured the effect of HSP60 on the phosphorylation of AKT, which is activated in a pathway involving PI3K. In human T cells, HSP60 alone did not induce AKT phosphorylation (data not shown). However, when T cells were pretreated with 0.1–1000 ng/ml HSP60 and then exposed to SDF-1α (100 ng/ml, 10 min), AKT phosphorylation was significantly ($p < 0.05$) down-regulated (Fig. 3*F*). Thus, in addition to the effect of HSP60 on SDF-1α-induced phosphorylation of ERK and Pyk2, HSP60 also down-regulates SDF-1α-mediated activation of AKT.

HSP60 inhibits SDF-1α-induced phosphorylation of MLC

MLC phosphorylation is involved in the association of myosin with actin in the formation of actomyosin motors, which are essential for cell migration (33, 34). SDF-1α induces MLC phosphorylation by signaling via the RhoA-p160ROCK pathway (20). Therefore, we investigated whether the effects of HSP60 on human T cells involve activation of the RhoA-p160ROCK-MLC kinase pathway. Purified T cells were incubated (1 h, 37°C) with HSP60 (0–1000 ng/ml), and then exposed to SDF-1α. Fig. 4*A* shows that HSP60 induced phosphorylation of MLC in a dose-dependent manner, with a pattern similar to that of HSP60-induced activation of Pyk2 (Fig. 3*D*). However, when HSP60-treated T cells were exposed to SDF-1α, phosphorylation of MLC was significantly

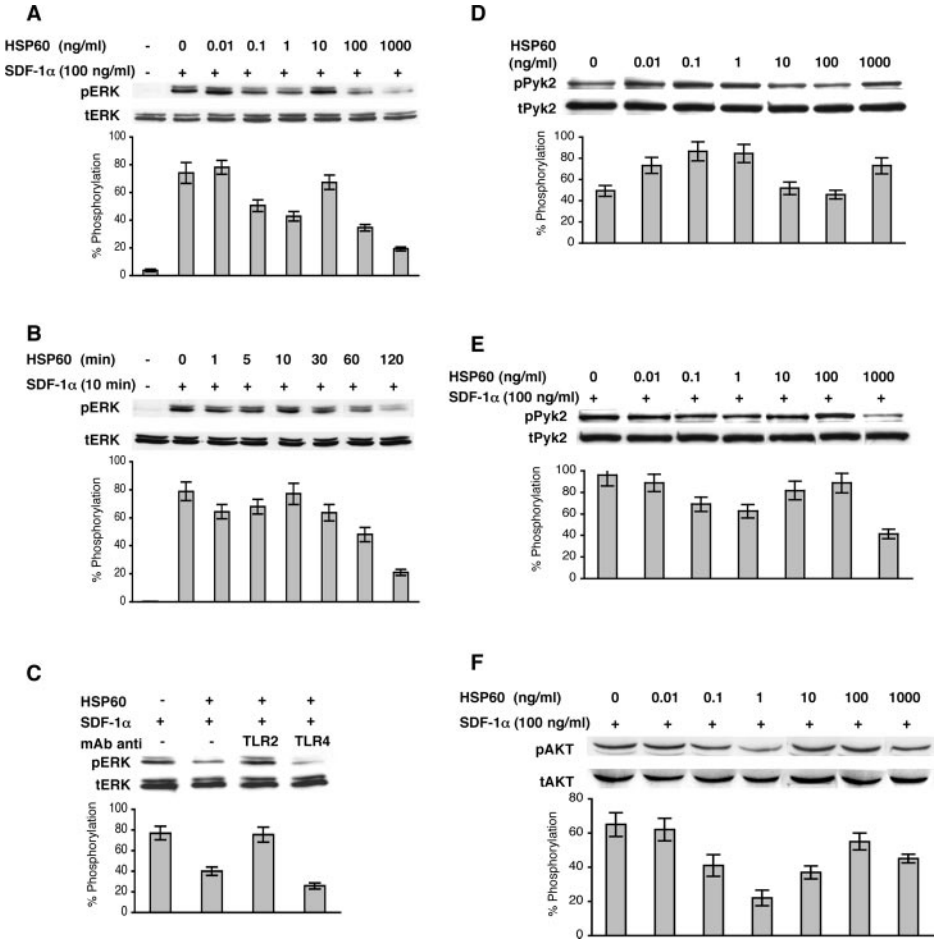

FIGURE 3. The effect of HSP60 on ERK, Pyk2, and AKT phosphorylation. HSP60 inhibits SDF-1α-induced ERK (*A–C*), Pyk2 (*D* and *E*), and AKT phosphorylation (*F*). Human T cells were exposed to HSP60 for 1 h (*A* and *C–F*) or to 1 ng HSP60/ml for 0–120 min (*B*), washed and then treated with SDF-1α (100 ng/ml, 10 min) (*A–C*, *E*, and *F*) or not (*D*). Lysates of these cells were immunoblotted with Abs: anti-phospho-ERK (pERK) and anti-total ERK (tERK) (*A–C*), anti-pPyk2 and anti-tPyk2 (*D* and *E*) or with anti-pAKT and anti-tAKT (*F*). *C*, T cells pretreated with mAb anti-TLR2 or anti-TLR4 mAb (20 μg/ml, 30 min) were then incubated with HSP60 (1 ng/ml, 1 h) and washed followed by SDF-1α (100 ng/ml, 10 min). The blot of one experiment representative of three (*A* and *B*) or five (*C–F*) is presented. Phosphorylation levels of the experiments were estimated by densitometry, and an average percentage of phosphorylation $\pm$SD was calculated as the OD of pERK/tERK, pPyk2/tPyk2, or pAKT/tAKT $\times$ 100%.

suppressed ($p < 0.05$) at most HSP60 concentrations (Fig. 4*B*). Thus, HSP60 inhibits SDF-1α-induced MLC phosphorylation in human T cells.

HSP60 inhibits T cell polarity

When migrating, lymphocytes acquire a shape with a defined rear-front polarity (34). SDF-1α-CXCR4 interactions also cause changes in the morphology of T cells (20, 31). Therefore, we studied the effects of HSP60 on the morphology of T cells in the presence or absence of SDF-1α. Immunofluorescence revealed that SDF-1α induced a change in T cell morphology, from a round to a spindle shape (Fig. 4*C*), as previously reported (20). Although exposure to HSP60 without subsequent exposure to SDF-1α induced some spreading of treated T cells, the human T cells did not acquire the rear-front polarity associated with cell motility. The findings that HSP60-induced T cell spreading is compatible with our recent report that HSP60 can induce T cell adhesion to fibronectin, but not chemotaxis (12), which requires the establishment of a polarized spindle shape. Pretreatment of human T cells with HSP60 abrogated SDF-1α-induced polarization of the cells. The inhibitory effects of HSP60 on SDF-1α-induced T cell polarization were specifically inhibited by mAbs to TLR2 (data not shown). Thus, inhibition by HSP60 of SDF-1α-induced intracellular phosphorylation of signaling elements involved in T cell activation and chemotaxis is accompanied by an inhibition of the morphologic changes required for T cell adhesion and migration.

HSP60 inhibits SDF-1α-induced activation and migration of T cells by up-regulation of SOCS3

SOCS family proteins have been identified as negative feedback regulators of cytokine-induced JAK/STAT activation, through their binding to JAK kinases (35–37). Chemokine activation also has been shown to be regulated by SOCS proteins; up-regulation of SOCS3 inhibited CXCR4 signaling and blocked chemotaxis to SDF-1α (22). Because T cell responses to SDF-1α are also attenuated by HSP60, we tested whether HSP60 might also induce SOCS3 activation. T cells were incubated (1 h) with HSP60 (0–1000 ng/ml) and intracellular activation of SOCS3 and tERK, which is constitutively expressed, were determined by Western blotting. Fig. 5*A* shows that HSP60 activated the expression of SOCS3 ($p < 0.05$). The effects of human HSP60 are not restricted to human T cells: HSP60 also induced the expression of SOCS3 in BALB/c lymph node cells (Fig. 5*B*). These effects were also dose-dependent, like the HSP60-induced expression of SOCS3 in human T cells (Fig. 5*A*). This effect of HSP60 on SOCS3 expression in BALB/c lymphocytes also explains the suppression of mouse T cell chemotaxis to SDF-1α previously noted in vitro (Fig. 2*A*) and in vivo (induction of DTH) (Fig. 2*B*).

The dose-response curve of the activation of SOCS3 in human T cells was biphasic, displaying maximal activities at HSP60 concentrations of 1 ng/ml and 1 μg/ml. With 1 ng/ml HSP60, activation was already apparent after 30 min and peaked at 1 to 2 h (Fig. 5*C*).

To test whether TLR2 or TLR4 might be functionally involved in the activation of SOCS3 by HSP60, we assayed the effect of preincubating T cells with blocking Abs to the TLR molecules. Fig. 5*D* shows that this activation of SOCS3 was TLR2-dependent because anti-TLR2 abrogated the activation by HSP60. Moreover, we confirmed the involvement of TLR2 by using T cells purified from TLR2 knockout and wild-type C57BL/6J mice. Fig. 5*E* shows that, as expected, treatment of wild-type C57BL/6J-derived

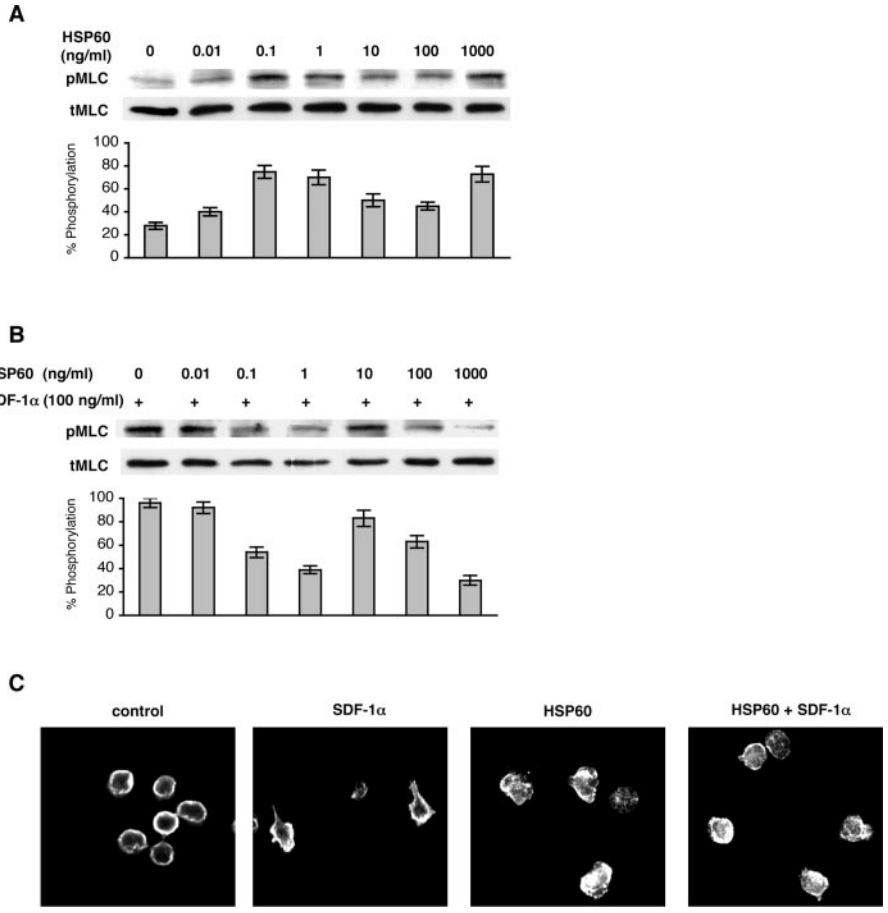

FIGURE 4. HSP60 induced phosphorylation of MLC in a dose-dependent manner. HSP60 induces phosphorylation of MLC (*A*), inhibits SDF-1α-induced phosphorylation of MLC (*B*) and polarized morphology (*C*) in T cells. Human T cells were treated with HSP60 (1 h) (*A*) followed by SDF-1α (100 ng/ml, 10 min) (*B*). Cell lysates were immunoblotted with anti-pMLC and anti-tMLC Ab. Phosphorylation levels of the five experiments were estimated by densitometry, and an average percentage of phosphorylation was calculated as the OD of pMLC/tMLC $\times$ 100%. *C*, T cells were plated onto fibronectin-coated coverslips in the absence or presence of HSP60 (1 μg/ml). After 1 h, some of the cells were exposed to SDF-1α (100 ng/ml). Immunofluorescent staining for actin in one experiment representative of four is shown.

T cells with HSP60 (1 ng/ml, 1 h) induced SOCS3 expression at levels similar to those induced by HSP60 in human T cells. In contrast, SOCS3 expression remained at the low levels of untreated cells when T cells from TLR2 knockout mice were treated with HSP60. Thus, HSP60 induces SOCS3 expression in T cells via signaling through TLR2.

Up-regulation of SOCS3 expression requires prior activation of the JAK/STAT pathway (35, 36). Therefore, we tested the effect of a specific blocker of JAK/STAT activation, AG490 (22, 38), on HSP60-induced up-regulation of SOCS3 in human T cells. Pretreatment (18 h) of T cells with AG490 (10 nM), but not with its control counterpart AG9, abrogated the effect of HSP60 on SOCS3 expression (Fig. 5*F*), whereas pertussis toxin, a Gα_i protein inhibitor, had no effect (data not shown). Furthermore, HSP60 induced phosphorylation of STAT3 (Fig. 5*G*), which is essential for SOCS3 up-regulation (35, 36). The dose-response curve of induction of STAT3 phosphorylation (Fig. 5*G*) was very similar to that of activation of SOCS3 by HSP60 (Fig. 5*A*). Thus, HSP60 induced SOCS3 expression through activation of intracellular signaling involving up-regulation of STAT3 phosphorylation.

Inhibition of SDF-1α-induced T cell chemotaxis by HSP60 is prevented by silencing SOCS3 gene expression

To confirm the conclusion that the inhibitory effect of HSP60 on SDF-1α-induced T cell chemotaxis is mediated through SOCS3 up-regulation, we specifically silenced *SOCS3* gene expression using RNA interference. This treatment abrogated the induction of SOCS3 expression by HSP60; transfection with control silent RNA had no effect (Fig. 5*H*). Moreover, the inhibitory effect of HSP60 on SDF-1α-induced T cell chemotaxis was completely prevented by specifically silencing the *SOCS3* gene (Fig. 5*I*).

Effects of HSP60 on SOCS3 expression are not due to contamination with LPS

The LPS-TLR2 interaction results in intracellular signals (39), and several batches of recombinant human HSP were shown to contain minimal residual LPS and LPS-lipoproteins, which are biologically active on macrophages (40). 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 exclude the possibility that such a minute amount of LPS could affect SOCS3 activation in T cells. First we examined whether LPS alone can affect SOCS3 expression by exposing T cells to increasing concentrations of LPS. LPS up-regulates SOCS3 expression in human T cells, but only at concentrations of 10–100 ng/ml (Fig. 6*A*). The amount of LPS possibly contaminating even the high concentration of HSP60 would be only 0.1 pg/ml. Thus, it is unlikely that LPS might contribute to either the effects of high concentrations or low concentrations of HSP60. Similarly, we previously found that 1000-fold greater concentrations of LPS were needed to induce T cell adhesion to fibronectin, an effect which was still less than that induced by human HSP60 (12).

The exclusion of LPS in activation of SOCS3 by HSP60 was further confirmed by using anti-HSP60 mAb, by boiling (which denatures HSP60, but not LPS), and by using an LPS inhibitor, PMB. The results, shown in Fig. 6, *B* and *C*, demonstrate that activation of SOCS3 by HSP60 (0.1 ng/ml) (Fig. 6, *upper panel*) was inhibited by boiling, but not by PMB (Fig. 6*B*), whereas activation of SOCS3 by LPS (100 ng/ml) (Fig. 6, *lower panel*) was inhibited by PMB, but not by boiling (Fig. 6*C*). Furthermore, anti-

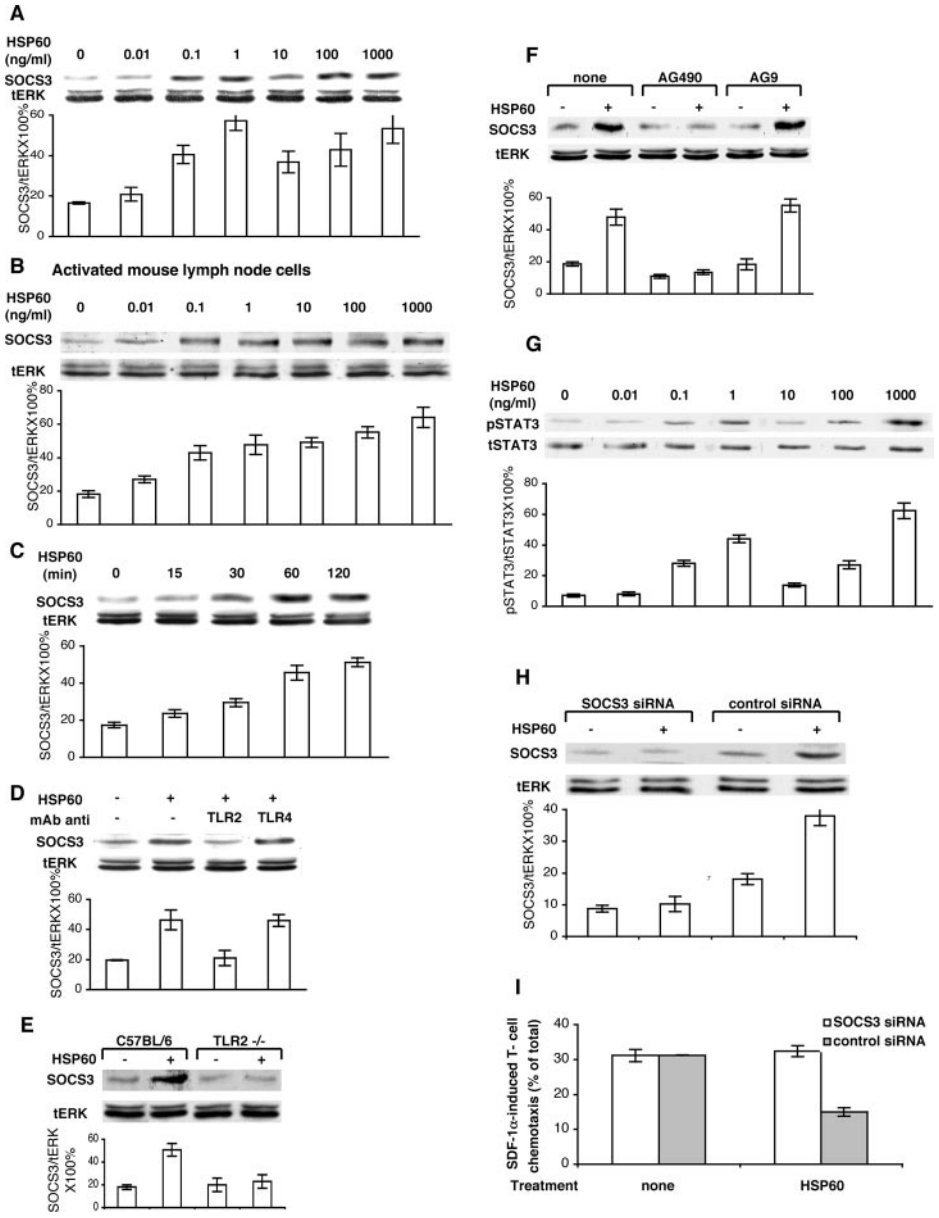

FIGURE 5. Activation by HSP60 of SOCS3 expression in T cells via TLR2 signaling and STAT3 activation mediates the down-regulation of SDF-1α-induced chemotaxis. Human T cells (*A*, *D*, *F*, *G*, and *H*), mouse lymph node lymphocytes (*B*) or mouse purified T cells (*E*) were incubated with HSP60 at 0.01–1000 ng/ml for 1 h, or at 1 ng/ml for 0–120 min (*C*). Some cells were pretreated with anti-TLR2 or anti-TLR4 mAb (*D*), the JAK/STAT inhibitor AG490 or control inhibitor AG9 (18 h) (*F*). *E*, Purified T cells from wild-type C57BL/6J and TLR2-knockout mice incubated with HSP60 1 ng/ml for 1 h. *H* and *I*, T cells were transfected with silent RNA targeting SOCS3, or with control silent RNA, and exposed to HSP60 (2 h). Cell lysates were immunoblotted with anti-SOCS3 and anti-total ERK (tERK; evaluation of total ERK served as a control) (*A–F* and *H*), or with anti-pSTAT3 and anti-STAT3 (*G*). The levels of SOCS3, tERK, pSTAT3, and tSTAT3 were estimated by densitometry and the average percentage ($\pm$SD) of the three experiments was calculated by OD of SOCS3/tERK (pSTAT3/tSTAT3) $\times$100. *H*, Cells were ^{51}Cr-labeled, washed, and their ability to migrate in response to SDF-1α was examined. Averages $\pm$ SD of three different experiments are shown.

human HSP60 mAb inhibited the activation of SOCS3 by HSP60, but not the activation induced by LPS.

Recently, lipoproteins extracted from the LPS of *Escherichia coli* were shown to activate macrophages via TLR-2 (40, 41). These lipoproteins can be removed from LPS by passage through PMB-coupled agarose beads (42). Preincubation with PMB-conjugated agarose beads did not block the efficacy of our HSP60 preparation in inducing SOCS3 expression by T cells. In contrast, the efficacy of the LPS preparation was abolished (Fig. 6*D*).

A synthetic peptide derived from HSP60, amino acid sequence 437–460 called p277, was shown to affect T cell function in a TLR-dependent manner (G. Nussbaum et al., manuscript in preparation), and to suppress ongoing diabetes in human patients and in mice (10, 23). The p277 peptide also inhibited human T cell chemotaxis to SDF-1α in vitro and T cell-mediated DTH responses in mice in vivo (G. Nussbaum et al., manuscript in preparation); this suggests that p277 possesses the immunomodulatory functions of intact HSP60 on T cells. We used this synthetic peptide to further rule out the possibility that the effects of HSP60 on SOCS3 expression could be due to contamination by LPS. Peptide p277 (1–1000 ng/ml), but not the control p30 peptide of tetanus toxoid, induced a marked expression of activated SOCS3 in T cells (Fig. 6*E*). Thus, the effect of our HSP60 preparation on SOCS3 expression was not due to LPS.

Discussion

In recent years it has been established that immune-cell migration to and function at inflammatory sites are controlled by the coordinated action of the cytokine-chemokine network (13–15). However, we have recently reported that stress proteins, particularly HSP60, also regulate T cell behavior in inflammation: HSP60 inhibits T cell chemotaxis (12) and shifts the cytokine secretion profile toward Th2 (43). The effect of HSP60 on the T cell cytokine profile was explained by the regulation of transcription factors: HSP60 down-regulates T-bet, NF-κB, and NFATp, and up-regulates GATA-3 (43). The present findings extend the effects of HSP60 via TLR2 on intracellular signal transduction pathways. We show that HSP60 activates SOCS3 expression in T cells via TLR2- and JAK/STAT-dependent signaling. This activation of

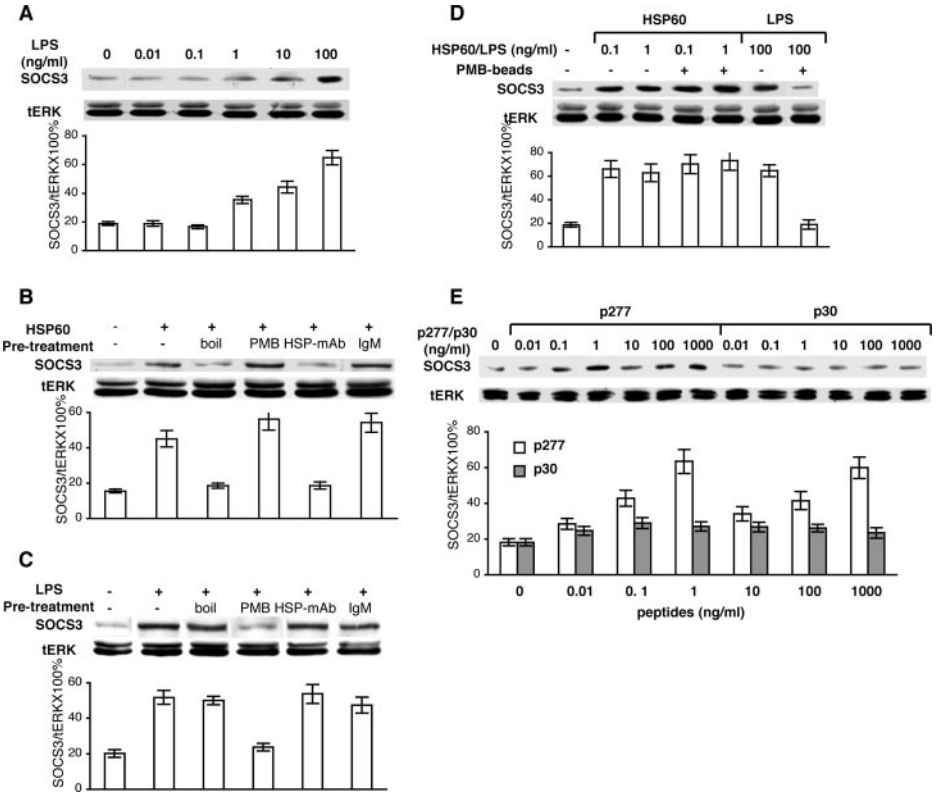

FIGURE 6. Effects of HSP60 on SOCS3 expression are not due to contamination with LPS. Human T cells were treated (1 h; 37°C) with LPS (different concentrations in *A*, 100 ng/ml in *C*), HSP60 (1 ng/ml) (*B*), LPS or HSP60 (*D*), or p30 or p277 (*E*). SOCS3 activation in the T cell lysates was measured by immunoblotting. *B*, T cells were pretreated (30 min) with anti-HSP60 mAb (20 μg/ml), isotype-matched mAb (IgM), or PMB (1 μg/ml). Alternatively, HSP60 or LPS were boiled (100°C, 30 min) before their addition to the T cell cultures. *D*, LPS and HSP60 were preincubated with PMB-conjugated agarose beads, the unbound material was collected, checked for protein amount, and used to pretreat the cells. One experiment representative of five (*A–C*), or three (*D* and *E*) is shown.

SOCS3 can explain the inhibitory effects of HSP60 on SDF-1α-induced phosphorylation of ERK, Pyk2, AKT, and MLC, along with rear-front polarity in human T cells. These findings provide new insights into the role of HSP60 in the regulation of the inflammatory process. Indeed, HSP60 regulates signaling pathways that until now have been primarily associated with cytokine-chemokine signaling.

Recently, cross-talk between TLR4 and the chemokine receptor CXCR2 in polymorphonuclear leukocytes was reported; signaling by LPS through the TLR4 pathway prevented desensitization of CXCR2 and markedly augmented migration of polymorphonuclear leukocytes (44). In our study, care was taken to rule out the possibility that the effects of recombinant human HSP60 on T cells were due to LPS or to LPS-associated contaminant.

The present study supports a model (Fig. 7) in which, in contrast to TLR4-CXCR2 interactions in neutrophils, HSP60 activates signaling through TLR2 in T cells to down-regulate the T cell chemotaxis induced by SDF-1α-CXCR4 interactions. TLR2 expression is regulated in immune cells at sites of infection or inflammation by bacterial components or cytokines (45, 46). Although a direct interaction between HSP60 and TLR2 on human T cells has not yet been shown, the results of this and our previous study (12) indicate that TLR2 is involved in transducing HSP60 signaling. These findings suggest that the TLR2 molecule of T cells can act as a "surveillance" transducer for an endogenous molecule, HSP60, which signals tissue injury by infection or other disease (47). Furthermore, treatment of human T cells with HSP60 inhibited their homing into the SDF-1α-rich bone marrow of NOD/SCID mice in a TLR2-dependent manner. Recently, bone marrow stroma was shown, under pathological conditions, to serve as a secondary immune organ by providing a site for T cell priming by Ags (48). Whether TLR2 actually binds HSP60 as a primary receptor, signaling through TLR2 seems to be critical for the effects of HSP60 on T cell-mediated inflammation.

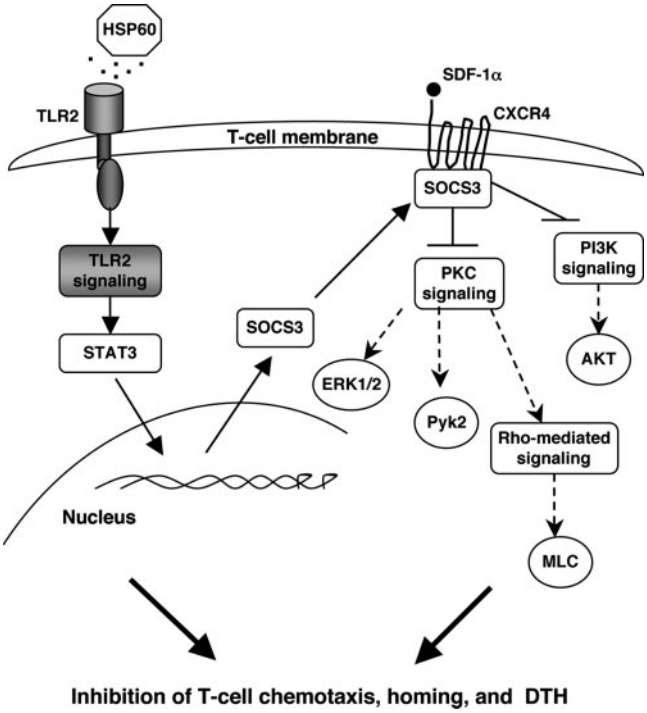

FIGURE 7. Paradigm for HSP60-induced inhibition of SDF-1α-mediated T cell chemotaxis, homing, and DTH. In human T cells, HSP60 activates SOCS3 expression through TLR2 signaling and STAT3 activation. SOCS3 then interacts with CXCR4, and thus inhibits SDF-1α-mediated phosphorylation of ERK1/2, Pyk2, AKT, and MLC. This down-regulation of SDF-1α-induced intracellular signaling impairs the establishment of a typically matrix-adherent polarized shape in T cells. As a result, HSP60 prevents the chemotactic responses of T cells and their homing in vivo to the spleen and bone marrow, or into inflamed sites, processes that require CXCR4-SDF-1α interactions.

Our results indicate that T cell responses to HSP60 are sensitive to specific concentrations: occurring at 0.1–1 ng/ml, negligible at lower concentrations (10–100 ng/ml), and increasing again at 1 μg/ml. This biphasic, dose-response curve of T cell responsiveness to HSP60 suggests that HSP60 might be recognized by multiple receptors, with high and low affinities. Thus, a fine balance of the amounts of HSP60 expressed by damaged tissues may participate in the dynamic process of inflammation, and so influence its intensity and duration. One may view the biphasic nature of the effects of HSP60 on T cells in the context of the complexity of cells, molecules and interactions needed to maintain homeostasis at sites of inflammation. However, until the receptor for HSP60 has been identified, the conclusion is only speculation.

The present findings indicate that activation of SOCS3 (35) is a key factor in the modulation by HSP60 of T cell responses to SDF-1α. SOCS3 activation, which follows HSP60-induced TLR2-signaling, appears to down-regulate CXCR4-mediated T cell functioning (Fig. 7). In fact, an association between SOCS3 and CXCR4 and a resulting impairment of cellular responses to SDF-1α were recently reported (22). Furthermore, under pathological conditions in which endogenous HSP60 is up-regulated (6–8, 49), SOCS3 appears to suppress inflammatory reactions in which IL-6 and related cytokines play decisive roles (36). SOCS3 activation has also been implicated in the regulation and maintenance of Th2-mediated allergic responses associated with asthma and atopic dermatitis (50). Whether inflammation-associated molecules other than HSP60 can activate SOCS3, and thus down-regulate T cell functioning and responses to SDF-1α is not yet known. Our findings imply that HSP60, at defined concentrations and time intervals, is capable via SOCS3 of exerting an anti-inflammatory effect on T cells. In fact, a brief exposure of murine T cells to a concentration of HSP60 that inhibits human and murine T cell chemotaxis toward SDF-1α and up-regulates SOCS3 expression, also abrogated the ability of Ag-reactive T cells to adoptively transfer DTH response in mice (Figs. 2 and 7), a reaction in which the CXCL12-CXCR4 interaction plays a pivotal role (26). This inhibition of T cell-mediated inflammation by HSP60 is due to down-regulation of T cell chemotaxis (Fig. 1) and secretion of major proinflammatory cytokines (43).

Overall, the results presented demonstrate that the inhibitory effects of HSP60 on SDF-1α-induced T cell responses, in vitro and in vivo, are mediated through up-regulation of SOCS3, and implicate a SOCS3-mediated molecular signaling mechanism for the innate effects of HSP60 on T cells.

Disclosures

The authors have no financial conflict of interest.

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