



Heat shock protein 60 enhances CD4⁺CD25⁺ regulatory T cell function via innate TLR2 signaling

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CD4⁺CD25⁺ Tregs regulate immunity, but little is known about their own regulation. We now report that the human 60-kDa heat shock protein (HSP60) acts as a costimulator of human Tregs, both CD4⁺CD25^{int} and CD4⁺CD25^{hi}. Treatment of Tregs with HSP60, or its peptide p277, before anti-CD3 activation significantly enhanced the ability of relatively low concentrations of the Tregs to downregulate CD4⁺CD25⁻ or CD8⁺ target T cells, detected as inhibition of target T cell proliferation and IFN- γ and TNF- α secretion. The enhancing effects of HSP60 costimulation on Tregs involved innate signaling via TLR2, led to activation of PKC, PI3K, and p38, and were further enhanced by inhibition of ERK. HSP60-treated Tregs suppressed target T cells both by cell-to-cell contact and by secretion of TGF- β and IL-10. In addition, the expression of ERK, NF- κ B, and T-bet by downregulated target T cells was inhibited. Thus, HSP60, a self-molecule, can downregulate adaptive immune responses by upregulating Tregs innately through TLR2 signaling.

Introduction

CD4⁺CD25⁺ T cells, termed regulatory T cells (Tregs), have been found to control effector T cells in vitro and in animal models of autoimmune diseases in vivo (1, 2). Tregs constitute 5–10% of peripheral CD4⁺ T cells in healthy animals and humans (1, 2). Although Tregs are anergic in vitro, they suppress the proliferation of CD4⁺CD25⁻ T cells in coculture; this suppression is detected only when the Tregs are activated through their TCR (3). Decreased numbers and effector functions of Tregs have been reported in persons manifesting autoimmune diseases such as type 1 diabetes and multiple sclerosis (4, 5). Tregs are characterized by the constitutive expression of several activation markers, including the glucocorticoid-induced TNF receptor family-related protein (6), OX40 (CD134) (7), L-selectin (CD62L) (8), and CTL antigen-4 (CTLA4; CD152) (9). In addition, the transcription factor Foxp3 plays a key role in the development and function of Tregs (10, 11). The suppressive mechanisms deployed by Tregs on their target T cells are not clear. In some systems, direct cell-to-cell contact seems to be required (12); in other systems, suppression seems to involve TGF- β and IL-10 cytokines (13–15).

Recently, we reported that the human 60-kDa heat shock (HSP60) molecule, via innate TLR2 signaling, can downregulate T cell migration (16) and inhibit the secretion of proinflammatory cytokines by activated T cells (17). However, the subtype of T cells that mediated the innate antiinflammatory effects of HSP60 was undefined.

In this study we investigated the population of human T cells responsive innately to human HSP60. We now report that CD4⁺CD25⁺ Tregs, both CD4⁺CD25^{int} and CD4⁺CD25^{hi}, are the T cells innately responsive to HSP60; HSP60-treated Tregs are significantly more effective than untreated Tregs in downregulating CD4⁺CD25⁻ or CD8⁺ T cells. The p277 peptide of HSP60 also is effective as a coactivator of Tregs. No APCs were needed for this HSP60 coactivation.

Results

HSP60 inhibits IFN- γ and TNF- α secretion and upregulates IL-10 secretion by activated CD4⁺ T cells. We analyzed the effects of HSP60 on the cytokine secretion profile of freshly isolated human T cells purified into CD3⁺, CD4⁺, or CD8⁺ populations and activated by mitogenic anti-CD3 mAb. HSP60 alone (at a range of concentrations of 0.01–1,000 ng/ml) did not induce cytokine secretion by the T cells (data not shown). Pretreatment of the CD3⁺ and CD4⁺ T cells with HSP60 (1 ng/ml), however, inhibited by about 50% the secretion of the Th1-related cytokines IFN- γ and TNF- α induced by anti-CD3 (Figure 1, A and B); in contrast, IL-10 secretion was enhanced 2- to 3-fold by HSP60 treatment (Figure 1C). Purified CD8⁺ T cells, in contrast, were unresponsive to HSP60 (Figure 1). Recently, we reported that the innate effects of HSP60 on T cells manifested a biphasic bell-shaped dose-response curve (16, 17); here too the dose-response curve of the CD3⁺ or CD4⁺ T cells was biphasic and bell-shaped. Significant effects were achieved with relatively low concentrations of HSP60 (0.1 ng/ml and 1 ng/ml; $P < 0.05$), while a higher concentration (10 ng/ml) was less effective. However, cytokine secretion was again affected significantly at yet higher concentrations of HSP60 (100 and 1,000 ng/ml; $P < 0.05$). Thus, HSP60 specifically affected cytokine secretion in the CD3⁺ and CD4⁺ populations, but not in CD8⁺ T cells.

Depletion of CD4⁺CD25⁺ T cells abrogates inhibitory effects of HSP60. To test whether the CD4⁺CD25⁺ T cells were essential for the effects of HSP60 on T cell cytokine secretion, we depleted CD25⁺ T cells by magnetic beads and incubated the CD4⁺CD25⁻ T cells with HSP60 (1 ng/ml, 2 hours). Figure 2A shows that removal of the CD4⁺CD25⁺ T cells from the CD4⁺ population completely prevented the inhibition of IFN- γ and TNF- α secretion by HSP60 treatment of the residual CD4⁺CD25⁻ T cells.

HSP60-treated CD4⁺CD25⁺ T cells manifest augmented regulatory effects on CD4⁺CD25⁻ T cells. CD4⁺CD25⁺ T cells composed about 5% of the total CD4⁺ T cells responsive to HSP60. We purified the CD4⁺CD25⁺ T cells by positive selection (greater than 92% CD4⁺CD25⁺). Intracellular staining revealed that about 82% of the CD4⁺CD25⁺ T cells expressed the CTLA4 marker; 57% were CTLA4^{hi}, and another 25% were CTLA4^{lo} (Figure 2B). Moreover,

Nonstandard abbreviations used: CTLA4, CTL antigen-4; HSP60, 60-kDa heat shock protein; int, intermediate; PMB, polymyxin B.

Conflict of interest: The authors have declared that no conflict of interest exists.

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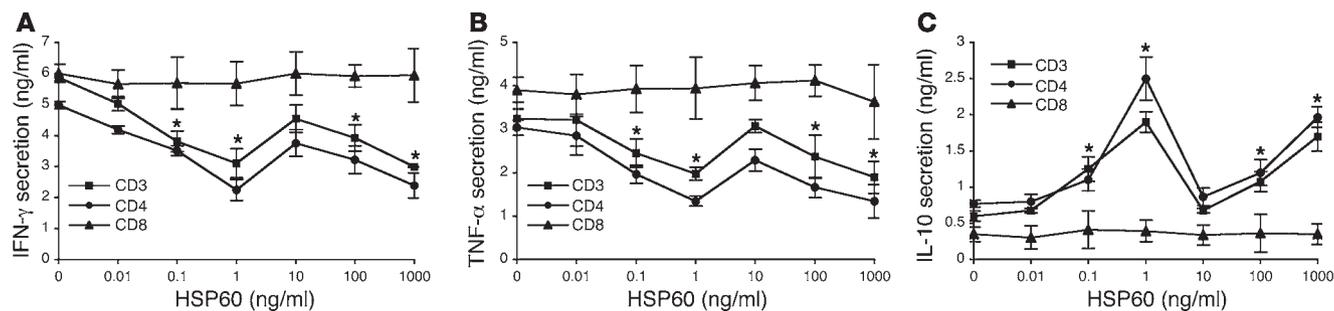


Figure 1

HSP60 pretreatment inhibits anti-CD3–induced IFN- γ and TNF- α secretion and upregulates IL-10 secretion by activated CD3⁺ and CD4⁺ T cells, but not by CD8⁺ T cells. Purified CD3⁺, CD4⁺, or CD8⁺ T cells were preincubated with the indicated concentrations of HSP60 for 2 hours, washed, and transferred to 24-well plates coated with anti-CD3 mAb (OKT; 0.5 μ g/ml) in serum-free medium. The supernatants were collected after 24 hours and analyzed for IFN- γ (A), TNF- α (B), and IL-10 (C). The means \pm SD of 5 different donors are shown. * P < 0.05.

the CD4⁺CD25⁺ population was strongly positive for the transcription factor Foxp3 (Figure 2B). The CD4⁺CD25⁻ T cells, in contrast, expressed considerably less CTLA4 and Foxp3.

To test whether the CD4⁺CD25⁺ T cells functioned as mediators of the inhibitory effect of HSP60 on IFN- γ and TNF- α secretion in CD4⁺ T cells, HSP60-treated or untreated CD4⁺CD25⁺ T cells in various proportions (1%, 10%, or 25%) were cocultured with untreated CD4⁺CD25⁻ T cells on mitogenic anti-CD3 for 24 hours, and the cytokine levels were measured in the culture media. Figure 2C shows that the untreated CD4⁺CD25⁺ T cells mediated a relatively moderate, but significant and concentration-dependent inhibition of IFN- γ and TNF- α secretion by the CD4⁺CD25⁻ T cells. However, HSP60 pretreatment of the CD4⁺CD25⁺ T cells significantly enhanced their downregulation of IFN- γ and TNF- α at the 10% and 25% concentrations.

The suppressive activity of Tregs was initially demonstrated by their ability to inhibit proliferation of effector cells (18). We found that direct HSP60 treatment of the CD4⁺CD25⁺ T cells induced significant augmentation of inhibition of CD4⁺CD25⁻ T cell proliferation (Figure 2D). Thus, HSP60 treatment of CD4⁺CD25⁺ T cells enhanced their ability to inhibit untreated CD4⁺CD25⁻ T cells, both proinflammatory cytokine secretion detected at 24 hours and proliferation detected at 96 hours; the HSP60-responsive CD4⁺CD25⁺ T cells functioned as Tregs for activated CD4⁺CD25⁻ T cells.

HSP60-treated CD4⁺CD25⁺ T cells regulate CD8⁺ T cell cytokine secretion. HSP60 did not directly affect the cytokine secretion of CD8⁺ T cells free of CD4⁺ T cells (Figure 1). It has been reported, however, that CD4⁺CD25⁺ T cells can suppress CD8⁺ T cells (19) as well as CD4⁺CD25⁻ T cells. We therefore cocultured purified CD8⁺ T cells with HSP60-treated or untreated CD4⁺CD25⁺ T cells in various proportions (1%, 10%, or 25%) on mitogenic anti-CD3 for 24 hours and measured the resulting cytokine secretion into the medium. Figure 2E shows that these concentrations of untreated CD4⁺CD25⁺ T cells did not significantly affect IFN- γ or TNF- α secretion by the CD8⁺ T cells. Nevertheless, treatment of the CD4⁺CD25⁺ T cells with HSP60 upregulated 3- to 4-fold their ability to inhibit significantly this cytokine secretion, even at a concentration as low as 1% (Figure 2C). Thus, HSP60 can affect CD8⁺ T cells indirectly through its activation of CD4⁺CD25⁺ Tregs.

Both CD4⁺CD25^{int} and CD4⁺CD25^{hi} T cells directly respond to HSP60 treatment. The results reported above were based on bead-sorted CD4⁺CD25⁺ T cells, containing mixtures of CD4⁺CD25^{intermediate} (CD4⁺CD25^{int}; 40%) and CD4⁺CD25^{hi} T cells (60%; data not shown).

It has been reported that the CD4⁺CD25^{hi} population mediates the most potent suppressive activity in vitro (18); we therefore FACS-sorted CD4⁺ T cells according to their CD25 expression and cocultured the CD4⁺CD25^{int} or CD4⁺CD25^{hi} T cells with target CD4⁺CD25⁻ T cells. As expected, the untreated CD4⁺CD25^{hi} T cells manifested significantly greater (P < 0.05) suppressive activity (Figure 3A). However, the suppressive activity on IFN- γ and TNF- α secretion (Figure 3A) and proliferation (data not shown) of both populations was enhanced significantly by treatment with HSP60. Although the inhibitory effect of the HSP60-treated CD4⁺CD25^{hi} T cells was significantly greater, nevertheless, the presence of CD4⁺CD25^{int} in the bead-sorted population did not reduce the overall suppressive effect of HSP60; this made it possible to study the effects of HSP60 using the bead-sorted population of CD4⁺CD25⁺ T cells.

Studies of the suppressive effects of CD4⁺CD25⁺ Tregs in coculture are often done at a concentration of 50% (1:1) or more to obtain strong suppression (18). In addition to the concentration of Tregs, the concentration of mitogenic anti-CD3 in coculture can also influence the degree of Treg activity (18). We therefore preincubated the CD4⁺CD25⁺ T cells with HSP60 and cocultured them with CD4⁺CD25⁻ T cells at proportions of 10% or 50%, in both lower (0.5 μ g/ml) and higher (5 μ g/ml) concentrations of immobilized mitogenic anti-CD3 antibodies. Figure 3B shows that the addition of HSP60-treated CD4⁺CD25⁺ T cells at a ratio of 1:1 as well as 1:10 in both concentrations of anti-CD3 resulted in significantly augmented inhibition of proliferation of the CD4⁺CD25⁻ T cells. Thus, treatment of CD4⁺CD25⁺ T cells with HSP60 enhances their regulatory activity even at relatively high concentrations of the cells and at different degrees of stimulation.

The effects of HSP60 on CD4⁺CD25⁺ T cells depend on TLR2 signaling. We previously reported that HSP60 can affect T cell behavior innately via TLR2 signaling (16, 17, 20). Moreover, a recent study indicates that TLR2 signaling via a non-self ligand can control expansion and function of Tregs (21). Here, we tested whether the enhancing effects of self-HSP60 on the regulatory CD4⁺CD25⁺ T cell population might require TLR2 or TLR4 signaling. We pretreated CD4⁺CD25⁺ T cells with neutralizing anti-TLR2 or anti-TLR4 mAb (both mouse IgG2a antibodies) before treatment with HSP60 and found that the mAb against TLR2, but not that against TLR4, blocked the enhancing effect of HSP60 (Figure 4, A and B). Thus, TLR2 appears to function in mediating the augmenting effects of human HSP60 on the regulatory activity of human CD4⁺CD25⁺ T cells.



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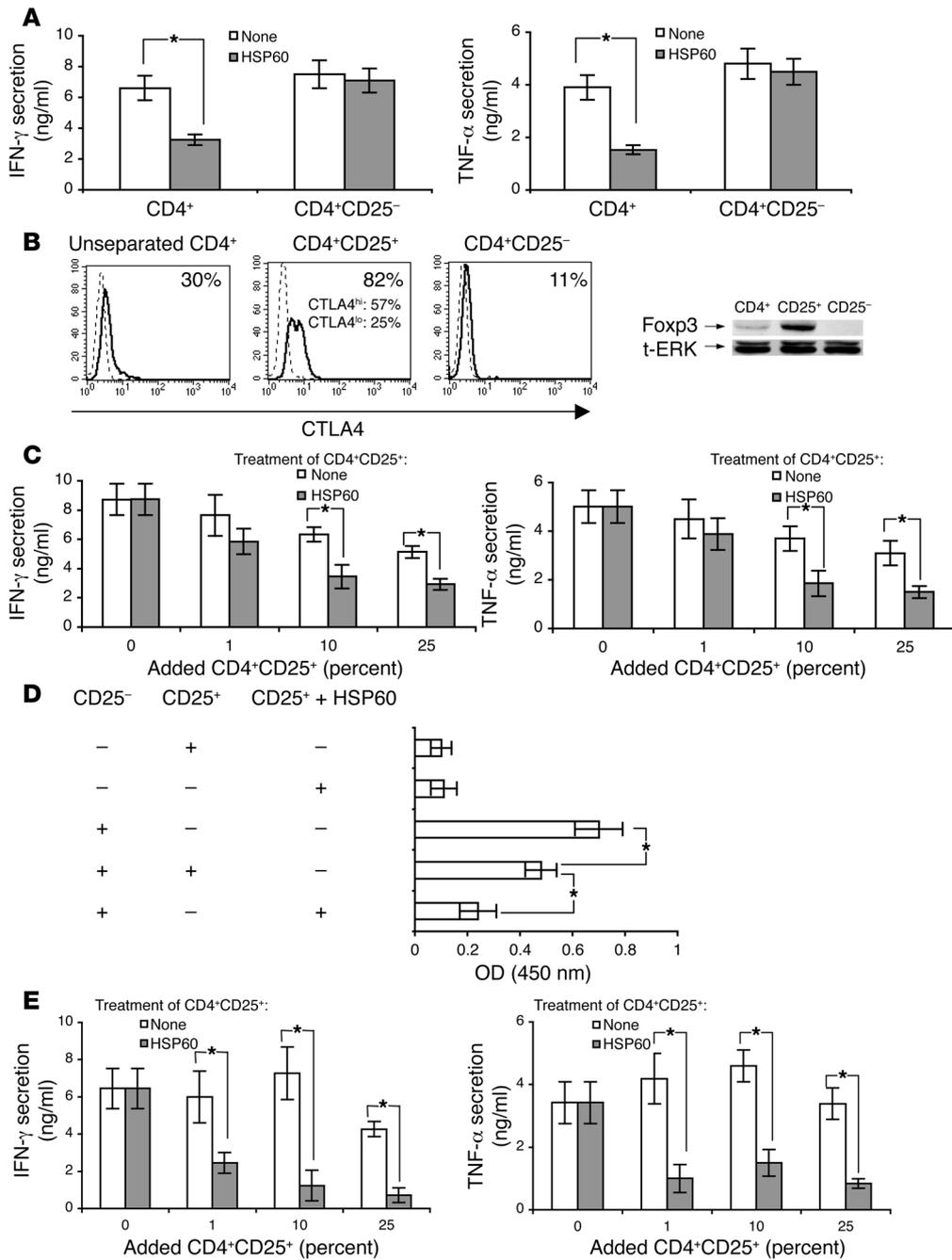


Figure 2

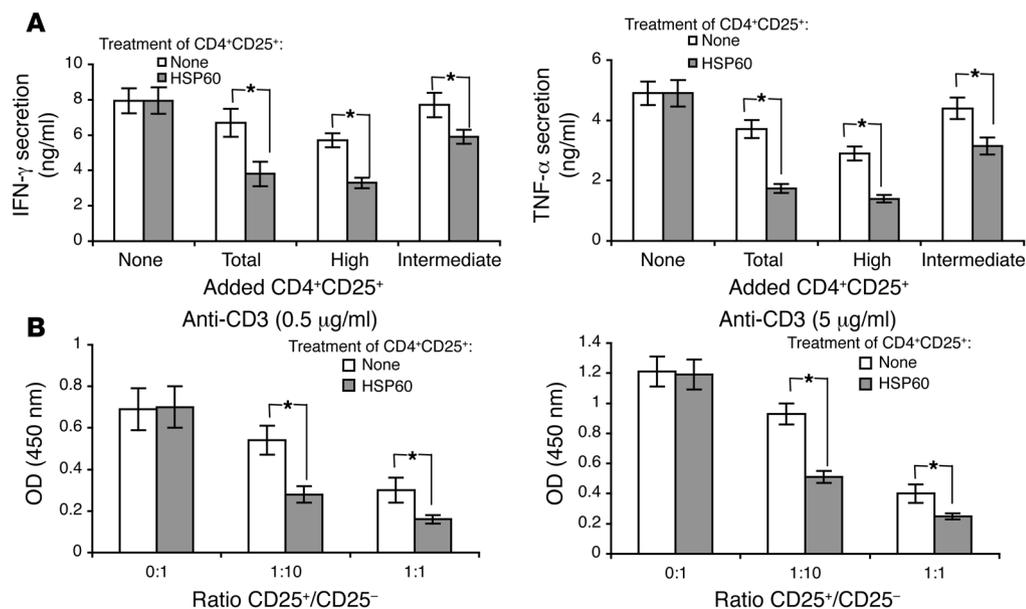
The CD4⁺CD25⁺ population mediates the inhibitory effect of HSP60 on IFN- γ and TNF- α secretion in T cells. (A) Unseparated CD4⁺ and CD4⁺CD25⁻ T cells were incubated with HSP60 (1 ng/ml) for 2 hours, washed, and transferred to 24-well plates coated with anti-CD3 mAb (OKT; 0.5 μ g/ml) in serum-free medium. The supernatants were analyzed for IFN- γ and TNF- α . (B) Unseparated CD4⁺, CD4⁺CD25⁺, or CD4⁺CD25⁻ T cells were checked for anti-CTLA4 and Foxp3 expression. One representative experiment of 5 is shown. (C–E) CD4⁺CD25⁺ T cells were incubated with HSP60 (1 ng/ml) for 2 hours, washed, mixed in the indicated proportions with CD4⁺CD25⁻ T cells (C) or with CD8⁺ T cells (E), and transferred to 24-well plates coated with anti-CD3 mAb (OKT; 0.5 μ g/ml) in serum-free medium. The supernatants were analyzed for IFN- γ and TNF- α (C and E). Cell proliferation was determined after 96 hours at a concentration of 10% of CD4⁺CD25⁺ T cells (D). The means \pm SD of 6 different donors are shown. * P < 0.05. t-ERK, total ERK.

In contrast to the results reported in mouse cells (22), we found that human CD4⁺CD25⁺ T cells were much richer in TLR2 than in TLR4 surface expression. However, no significant differences were found between human CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells in the proportions of cells expressing TLR2 (Figure 4C). Thus the nonresponsiveness of CD4⁺CD25⁻ T cells to the innate effects of HSP60 cannot be attributed merely to a lack of TLR2.

The effects of HSP60 on CD4⁺CD25⁺ T cells are not due to contaminating LPS. Commercial LPS has been reported to activate various types of leukocytes via TLR2 signaling (23). However, polymyxin B-coupled (PMB-coupled) agarose beads have been shown to bind and remove LPS and other bacterial contaminants (24). To test for possible LPS contamination, we incubated the HSP60 preparation with PMB-coupled

agarose beads (24); the treated-HSP60 still maintained its activity (Figure 4, D and E). In contrast, the effects of HSP60 on Treg function were completely inhibited by boiling, which denatures proteins but not LPS (Figure 4, D and E). Most importantly, in contrast to the stimulatory effects of LPS on mouse Tregs (22), we found that LPS did not enhance the regulatory functions of human CD4⁺CD25⁺ T cells (Figure 4, D and E). Consequently, the effects of HSP60 on human CD4⁺CD25⁺ T cells are not likely to be due to LPS or to other TLR2-ligand contaminants that bind to PMB-coupled agarose beads.

HSP60-derived peptide p277 augments the inhibitory effects of Tregs. A synthetic peptide derived from HSP60, the modified amino acid sequence 437–460 called p277, was found to arrest β cell destruction in type 1 diabetes in mice and in human patients (25, 26).

**Figure 3**

HSP60-treated CD4⁺CD25⁺ T cells manifest augmented regulatory effects on CD4⁺CD25⁻ T cells. (A) Bead-sorted CD4⁺CD25⁺ T cells (total), or FACS-sorted CD4⁺CD25^{hi} (high) and CD4⁺CD25^{int} (intermediate) T cells, were incubated with HSP60 (1 ng/ml) for 2 hours, washed, and cocultured with CD4⁺CD25⁻ T cells (ratio 1:10) on anti-CD3 in serum-free medium. The supernatants were analyzed for IFN- γ and TNF- α . The means \pm SD of 5 different donors are shown. (B) CD4⁺CD25⁺ T cells were incubated with HSP60 (1 ng/ml) for 2 hours, washed, mixed in the indicated proportions with CD4⁺CD25⁻ T cells, and transferred to 96-well plates coated with anti-CD3 mAb (0.5 μ g/ml or 5 μ g/ml). Cell proliferation was determined after 96 hours. The means \pm SD of 6 different donors are shown. * P < 0.05.

Hence, we tested whether p277, like the whole HSP60 molecule, might modulate immune reactions via upregulation of Treg activity. Figure 5 shows that treatment with peptide p277 enhanced the ability of CD4⁺CD25⁺ T cells to inhibit IFN- γ and TNF- α secretion and proliferation by target CD4⁺CD25⁻ T cells. A partially homologous, immunogenic control peptide of *Mycobacterium tuberculosis* had no such effect. Peptide p277 had no direct effect on the CD4⁺CD25⁻ T cells (data not shown). We used neutralizing antibodies against TLR2 and TLR4, and we found that the effects of p277 involved TLR2 but not TLR4 signaling (Figure 5, D and E). Thus, peptide p277, like whole HSP60, functions as a coactivator of CD4⁺CD25⁺ T cell regulatory function via TLR2 signaling.

To confirm the involvement of TLR2 signaling, we tested the effect on CD4⁺CD25⁺ T cell regulatory function of a known exogenous agonist of TLR2, Pam₃Cys. It has been reported recently that Pam₃Cys can influence the function of Tregs (21). Figure 5, F and G, shows that Pam₃Cys too could augment Treg inhibition of cytokine secretion by CD4⁺CD25⁻ T cells.

HSP60-induced enhancement of CD4⁺CD25⁺ T cell function involves both contact-dependent and cytokine-dependent mechanisms. Transwell experiments were performed to investigate whether cell-to-cell contact or soluble mediators were involved in the HSP60-enhanced regulatory effect of CD4⁺CD25⁺ T cells on CD4⁺CD25⁻ T cells; separation of the cells reduced cytokine suppression by about 50% (Figure 6A).

Recently it was reported that the CTLA4 molecule is involved in the suppression mediated by mouse or human CD4⁺CD25⁺ Tregs (9, 14). Figure 6B shows that neutralizing anti-CTLA4 mAb blocked by about 50% the enhancement induced by HSP60. Thus, the enhanced regulatory effects of HSP60-treated

CD4⁺CD25⁺ T cells depend, in part, on cell contact and on the CTLA4 molecule.

To test the role of regulatory cytokines, we incubated the CD4⁺CD25⁺ T cells with or without HSP60 (1 ng/ml, 2 hours), washed the cells, and activated them on anti-CD3 mAbs for 24 hours. We then collected the supernatants, added them to test CD4⁺CD25⁻ T cells for an additional 24 hours of incubation on mitogenic anti-CD3 mAbs, and assayed the culture media for IFN- γ and TNF- α . We found that HSP60 treatment of the CD4⁺CD25⁺ T cells induced them to secrete soluble factors that inhibited IFN- γ and TNF- α secretion by the CD4⁺CD25⁻ T cells by about 30%; the supernatants from CD4⁺CD25⁺ T cells untreated by HSP60 had no effect (Figure 6C).

We used neutralizing anti-IL-10 and anti-TGF- β mAb to test whether IL-10

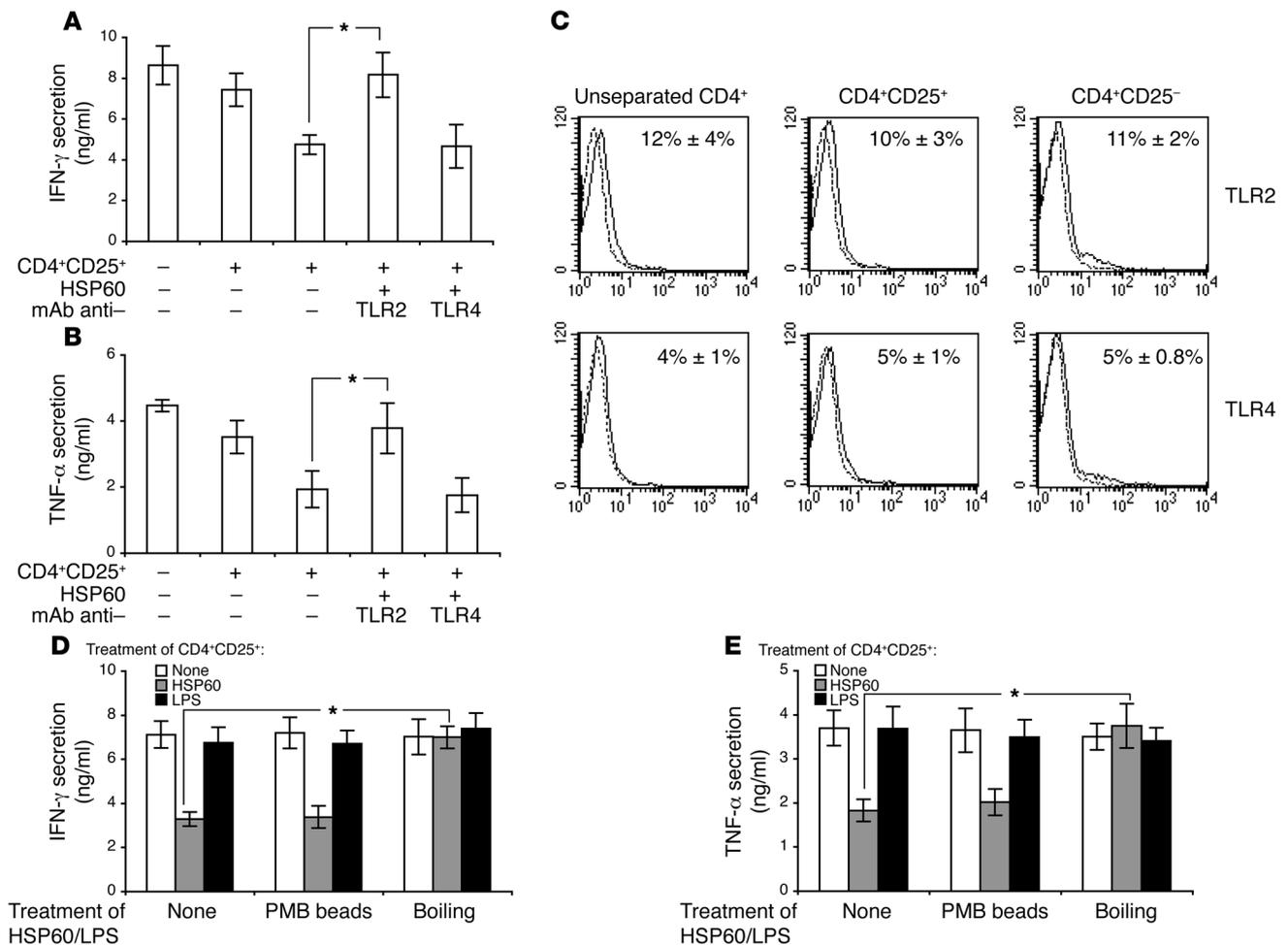
or TGF- β might be involved in the supernatant-mediated suppression of IFN- γ and TNF- α secretion. Each antibody alone reversed the HSP60-induced inhibition by about 40%; both antibodies together blocked about 80% of the inhibitory effect (Figure 7D). The isotype-matched control mAbs had no effect. Thus, HSP60 treatment enhanced the contact-dependent (CTLA4) regulatory activity of CD4⁺CD25⁺ T cells and induced cytokine-dependent (IL-10, TGF- β) regulation.

HSP60 upregulates IL-10 and TGF- β secretion in CD4⁺CD25⁺ T cells. Figure 7A shows that treatment with HSP60 (1 ng/ml, 2 hours) of unseparated CD4⁺ or separated CD4⁺CD25⁺ T cells significantly upregulated both IL-10 and TGF- β secretion. In contrast, CD4⁺CD25⁻ T cells secreted relatively low levels of these suppressive cytokines and were unresponsive to treatment with HSP60. We also monitored IL-10 secretion in the CD4⁺CD25⁺ T cells by FACS; Figure 7B shows that 1.5% of CD4⁺CD25⁺ T cells secreted IL-10 in response to activation by anti-CD3 antibodies. Pretreatment with HSP60 before anti-CD3 activation upregulated the percentage of IL-10-secreting CD4⁺CD25⁺ T cells 2- to 3-fold (Figure 7B).

HSP60-treated CD4⁺CD25⁺ T cells induce CD4⁺CD25⁻ cells to secrete IL-10. Do HSP60-treated regulatory CD4⁺CD25⁺ T cells also induce their target CD4⁺CD25⁻ T cells to secrete IL-10? To clarify the source of IL-10 in the coculture, we labeled the CD4⁺CD25⁻ T cells with CFSE and checked IL-10 secretion by FACS. Figure 7D shows that the addition of untreated CD4⁺CD25⁺ T cells to the CD4⁺CD25⁻ T cells augmented the percentage of IL-10-secreting CD4⁺CD25⁻ T cells from 0.4% to 1%. However, treatment with HSP60 of CD4⁺CD25⁺ T cells significantly upregulated the percentage of IL-10 secreted by both the CD4⁺CD25⁺ T cells (2- to



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**Figure 4**

The effects of HSP60 on CD4⁺CD25⁺ T cells depend on TLR2 signaling and are not due to contaminating LPS. (**A** and **B**) CD4⁺CD25⁺ T cells were pretreated with monoclonal anti-TLR2 or anti-TLR4 (20 μ g/ml, 30 minutes). Then, the cells were incubated with HSP60 (1 ng/ml, 2 hours), washed, and cocultured with CD4⁺CD25⁻ T cells (ratio 1:10) on anti-CD3 in serum-free medium. (**C**) The surface TLR2 or TLR4 expression on unseparated CD4⁺, CD4⁺CD25⁺, or CD4⁺CD25⁻ T cells was determined by FACS analysis. (**D** and **E**) CD4⁺CD25⁺ T cells were incubated (2 hours) with untreated, PMB-treated, or boiled (100°C, 30 minutes) HSP60 (1 ng/ml) or LPS (100 ng/ml). After washing, the CD4⁺CD25⁺ T cells were cocultured with test CD4⁺CD25⁻ T cells (ratio 1:10) on anti-CD3 in serum-free medium. The supernatants were collected after 24 hours and analyzed for IFN- γ (**A** and **D**) and TNF- α (**B** and **E**). The means \pm SD of 4 different donors are shown. * $P < 0.05$.

3-fold) and the CD4⁺CD25⁻ T cells (3- to 4-fold). Thus, HSP60 treatment of regulatory CD4⁺CD25⁺ T cells indirectly upregulates IL-10 secretion in cocultured CD4⁺CD25⁻ T cells.

HSP60 induces phosphorylation of AKT, Pyk2, and p38 and downregulates anti-CD3-induced ERK phosphorylation in regulatory CD4⁺CD25⁺ T cells. To gain some insight into molecular mechanisms, we preincubated the CD4⁺CD25⁺ T cells with inhibitors of signaling, exposed the CD4⁺CD25⁺ T cells to HSP60, and then examined their capacity to regulate cytokine secretion by cocultured CD4⁺CD25⁻ T cells. Treatment of regulatory CD4⁺CD25⁺ T cells with wortmannin (a PI3K inhibitor), GF109203X (a PKC inhibitor), or SB 203580 (a p38 MAPK inhibitor), but not pertussis toxin (a G protein inhibitor), completely blocked the HSP60-augmented downregulation of test CD4⁺CD25⁻ T cells (Figure 8A). Interestingly, PD 98059, an inhibitor of the MAP kinase ERK1/2, enhanced HSP60-augmented regulation of the CD4⁺CD25⁻ T cells (Figure 8A). Thus, the enhanced regulatory effects of HSP60 treatment appear to

require the activation of PI3K, PKC, and p38 MAPK signaling, and the downregulation of ERK-dependent signaling.

To extend these studies, we incubated unseparated CD4⁺ T cells or purified CD4⁺CD25⁺ or CD4⁺CD25⁻ populations with HSP60 (1 ng/ml, 10 minutes) and probed cell lysates for phosphorylation of AKT, Pyk2, p38, and ERK. Figure 8B shows that HSP60 treatment of CD4⁺CD25⁺ but not of unseparated CD4⁺ or CD4⁺CD25⁻ T cells induced significant phosphorylation of AKT, which is activated in a pathway involving PI3K.

The cytoplasmic tyrosine kinase 2 (Pyk2) is a member of the focal adhesion kinase (FAK) family of molecules phosphorylated upon PKC activation (27). We recently reported that HSP60 induces Pyk2 phosphorylation in human CD3⁺ T cells (16). Here, we found that HSP60 induced Pyk2 phosphorylation in both unseparated CD4⁺ and purified CD4⁺CD25⁺ T cells (Figure 8C). Figure 8D shows that HSP60 also induced phosphorylation of p38 MAPK in unseparated CD4⁺ and purified CD4⁺CD25⁺ T cells; CD4⁺CD25⁻ T cells were com-

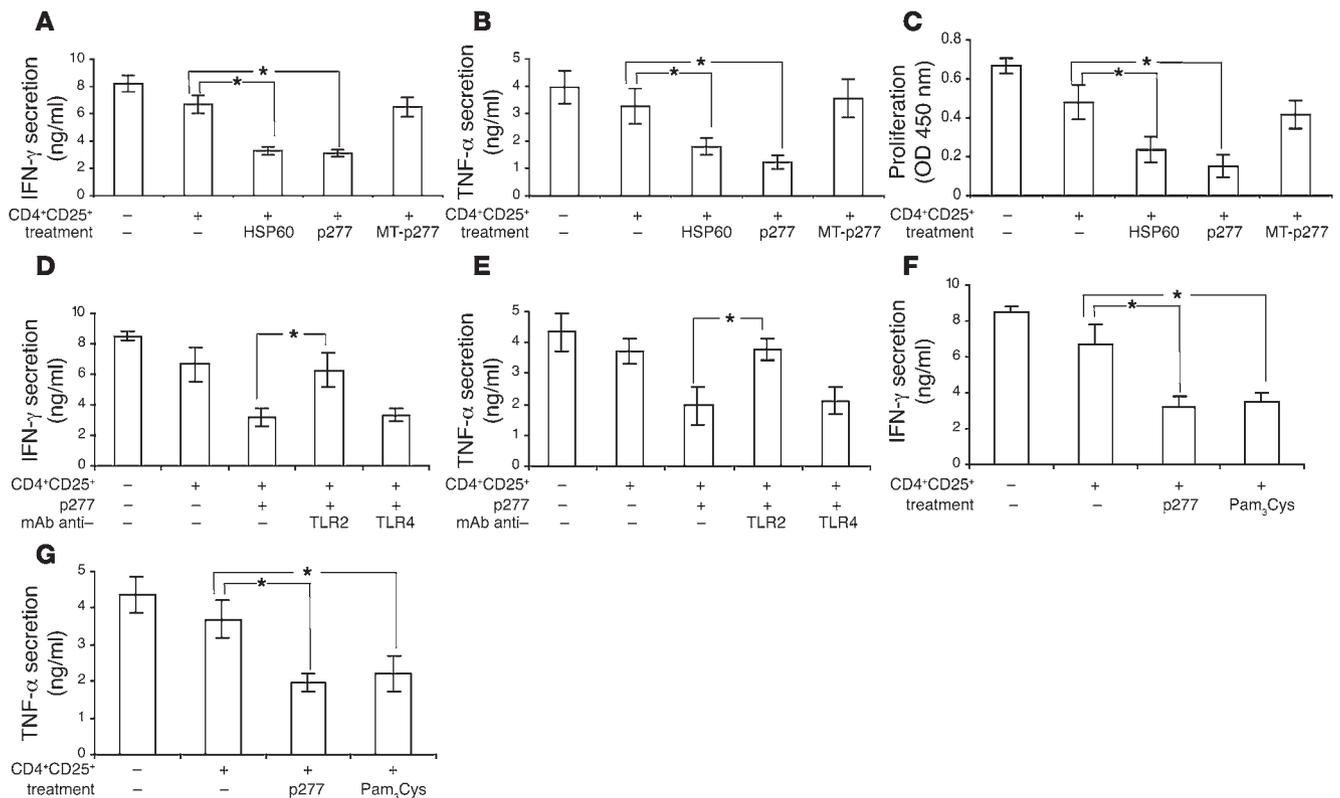


Figure 5

HSP60 peptide p277 or Pam₃Cys can augment the ability of CD4⁺CD25⁺ T cells to inhibit cytokine secretion and proliferation in coculture with target CD4⁺CD25⁻ T cells via TLR2. CD4⁺CD25⁺ T cells were incubated with HSP60, p277, MT-p277 (1 ng/ml), or Pam₃Cys (100 ng/ml) for 2 hours, washed, and then cocultured with untreated CD4⁺CD25⁻ T cells (ratio 1:10) on anti-CD3 in serum-free medium. The supernatants were analyzed for IFN- γ (A, D, and F) and TNF- α (B, E, and G). Proliferation was determined after 96 hours (C). In D and E, CD4⁺CD25⁺ T cells were pretreated with monoclonal anti-TLR2 or anti-TLR4 (20 μ g/ml, 30 minutes). The means \pm SD of 3 different donors are shown. * P < 0.05.

pletely unresponsive to HSP60 treatment. Moreover, HSP60 significantly inhibited ERK phosphorylation in the CD4⁺ and CD4⁺CD25⁺ populations, but not in the CD4⁺CD25⁻ T cells (Figure 8E). Thus, HSP60 induced phosphorylation of AKT, Pyk2, and p38, but inhibited ERK phosphorylation, in CD4⁺CD25⁺ T cells.

HSP60-treated CD4⁺CD25⁺ T cells downregulate ERK phosphorylation and inhibit nuclear translocation of NF- κ B and T-bet expression in regulated CD4⁺CD25⁻ T cells. To investigate signaling events induced in target CD4⁺CD25⁻ T cells, we labeled CD4⁺CD25⁻ T cells with CFSE, added HSP60-treated CD4⁺CD25⁺ T cells, and reisolated the CFSE-labeled CD4⁺CD25⁻ T cells by FACS sorting. We then analyzed the CD4⁺CD25⁻ T cell lysates for ERK phosphorylation, nuclear translocation of NF- κ B, and T-bet expression. Upon CD3 activation, ERK phosphorylation was significantly upregulated in CD4⁺CD25⁻ T cells, and adding 10% CD4⁺CD25⁺ T cells (untreated by HSP60) to the coculture led to a slight inhibition of ERK phosphorylation (Figure 9A). But treatment of the CD4⁺CD25⁺ Tregs with HSP60 significantly enhanced their ability to suppress ERK phosphorylation in the CD4⁺CD25⁻ T cells.

Moreover, treatment of CD4⁺CD25⁻ T cells with mitogenic anti-CD3 induced the nuclear translocation of NF- κ B (Figure 9B), but coculture of the CD4⁺CD25⁻ T cells with regulatory CD4⁺CD25⁺ T cells resulted in inhibition of the nuclear translocation of NF- κ B (Figure 9B). Indeed, an even more significant inhibition of the nuclear translocation of NF- κ B in the CD4⁺CD25⁻ T cells was

achieved by HSP60 treatment of the CD4⁺CD25⁺ T cells (Figure 9B). The nuclear protein lamin B was used as a constitutively expressed control protein.

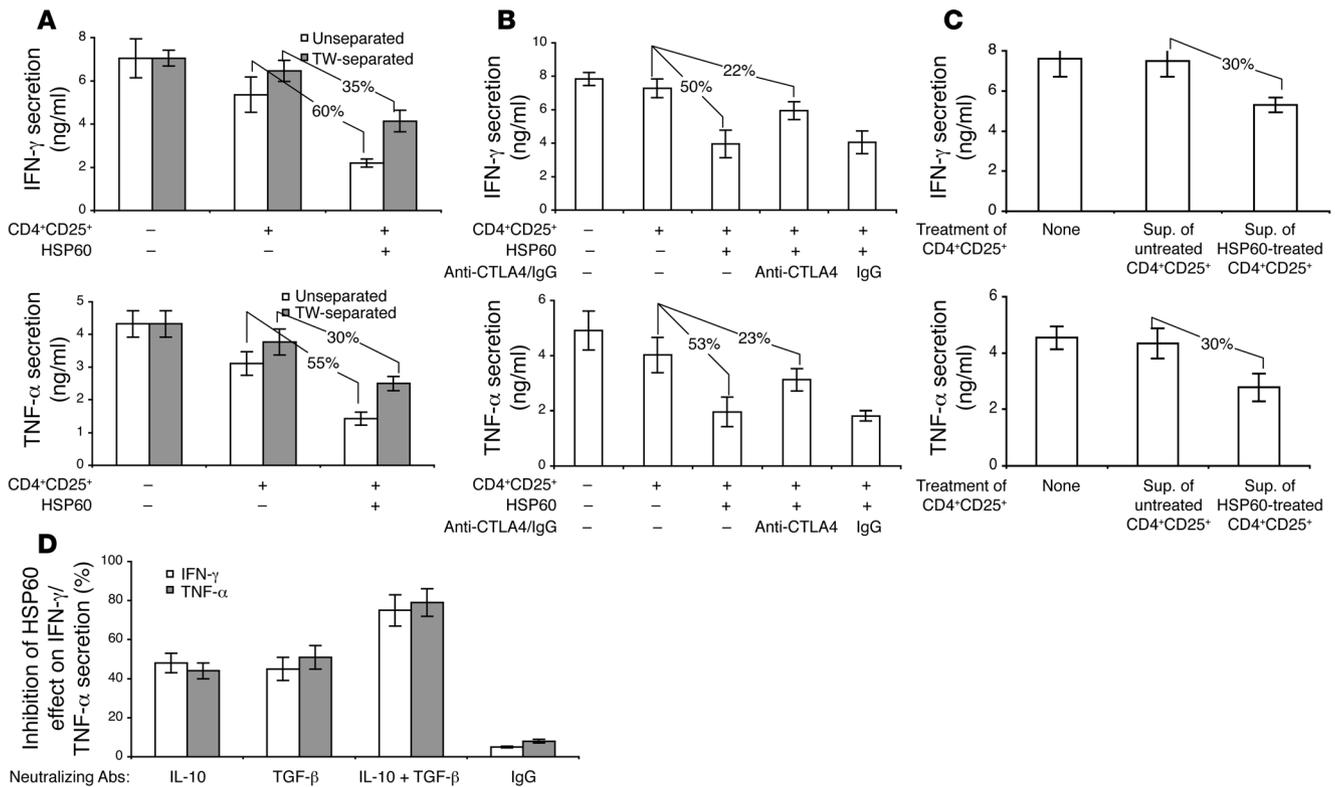
It has been recently reported that the level of T-bet can be augmented by TCR-mediated signals and that T-bet plays an essential role in IFN- γ production (28). Coculture of CD4⁺CD25⁻ T cells with untreated CD4⁺CD25⁺ T cells resulted in slight inhibition of T-bet expression in the CD4⁺CD25⁻ T cells (Figure 9C). However, HSP60 treatment of the CD4⁺CD25⁺ Tregs markedly enhanced their inhibition of T-bet in the CD4⁺CD25⁻ T cells (Figure 9C). In summary, HSP60-treated CD4⁺CD25⁺ Tregs induce in cocultured CD4⁺CD25⁻ T cells augmented downregulation of ERK phosphorylation, inhibition of nuclear translocation of NF- κ B, and decreased T-bet expression.

Discussion

This study connects two subjects that until now have been pursued separately: HSP60 as an innate, TLR2-dependent regulatory signal molecule (29) and the CD4⁺CD25⁺ population of T cells as mediators of immune system regulation (1, 2). The results presented here indicate that CD4⁺CD25⁺ T cells treated with HSP60 or its p277 peptide manifest enhanced regulatory functions dependent on innate TLR2. To obtain the amounts of CD4⁺CD25⁺ T cells needed for our studies, we used bead-separated CD4⁺CD25⁺ T cell populations containing both CD4⁺CD25^{hi} (60%) and CD4⁺CD25^{int} (40%) T cells.



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**Figure 6**

HSP60-induced enhancement of CD4⁺CD25⁺ Treg function involves both contact-dependent and cytokine-dependent mechanisms. (A) CD4⁺CD25⁺ T cells were incubated with HSP60 (1 ng/ml, 2 hours), washed, and cocultured with target CD4⁺CD25⁻ T cells (ratio 1:10) on anti-CD3 in the same lower well or separately in the upper chamber of the Transwell (TW). As indicated, some CD4⁺CD25⁺ T cells were pre-treated with control or monoclonal anti-CTLA4 (20 μ g/ml, 30 minutes) and washed (B), or blocking anti-IL-10 and/or anti-TGF- β (10 μ g/ml) mAbs (D) were added to the coculture. The supernatants were collected after 24 hours and analyzed for IFN- γ and TNF- α . The means \pm SD of 5 different donors are shown. (C) CD4⁺CD25⁺ T cells were incubated with HSP60 (1 ng/ml, 2 hours), washed, and plated on anti-CD3 for 24 hours in serum-free medium. Then the supernatants (Sup.) were collected and added to test CD4⁺CD25⁻ T cells in the presence of anti-CD3 mAbs. The supernatants from the CD4⁺CD25⁻ T cells were collected after an additional 24 hours and analyzed for IFN- γ and TNF- α . The means \pm SD of 4 different donors are shown.

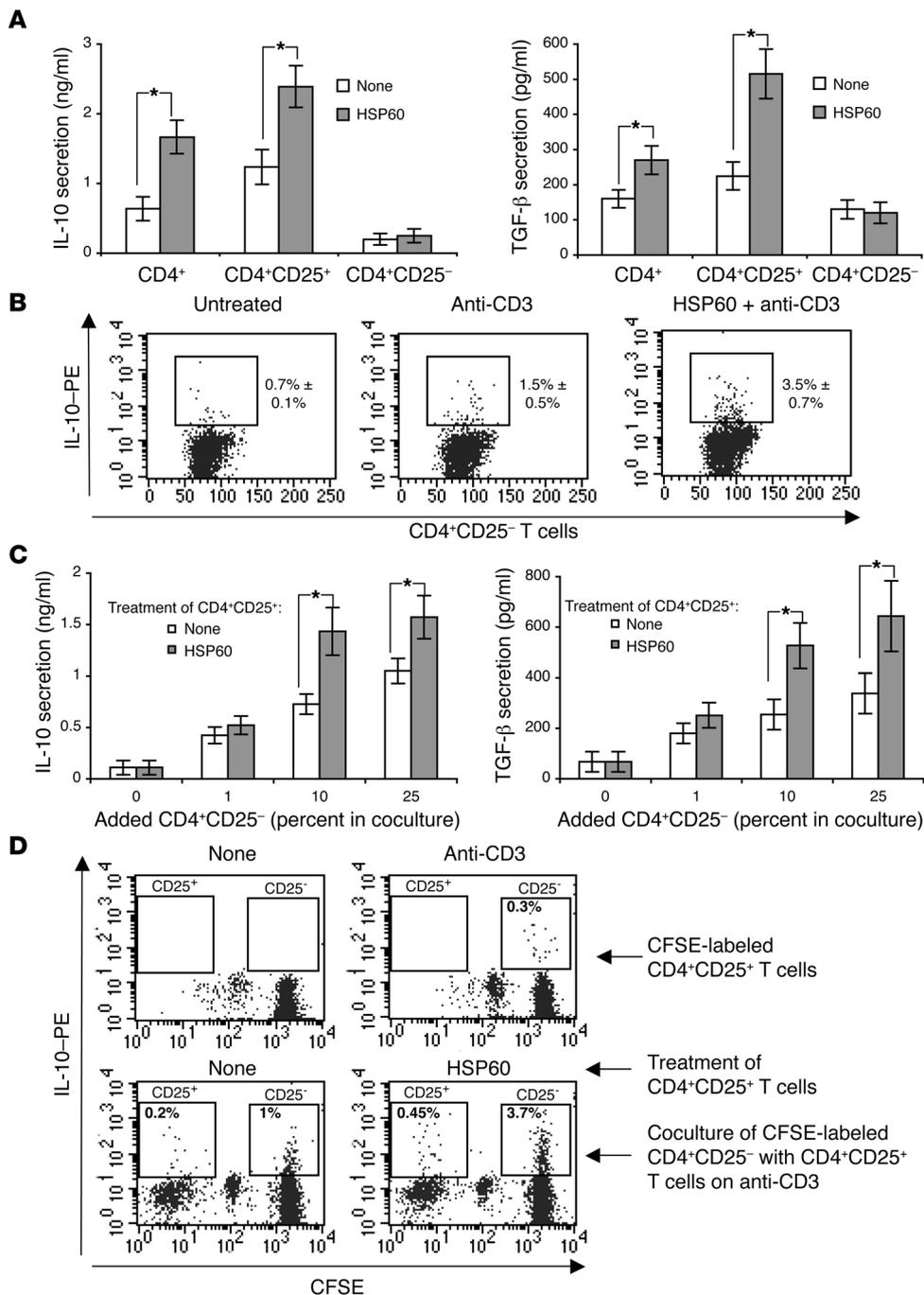
Nevertheless, highly purified FACS-separated CD4⁺CD25^{hi} T cells responded to HSP60 as did the CD4⁺CD25^{int} T cells (Figure 3), so our results using the bead-separated mixture of CD4⁺CD25^{hi} and CD4⁺CD25^{int} T cells reflect the enhancing effect of HSP60 on both sets of CD4⁺CD25⁺ T cells.

It has been reported that CD4⁺CD25⁺ Tregs can suppress proinflammatory functions of CD4⁺CD25⁻ T cells by more than 90% (12, 15, 18); but the coculture experiments in those reports have used a high ratio (1:1) of Tregs to CD4⁺CD25⁻ T cells. In reality, however, CD4⁺CD25^{hi} Tregs represent only a few percent of circulating CD4⁺ T cells (2, 18). In fact, in coculture experiments using proportions of CD4⁺CD25⁺ T cells of 10% or less, we observed only moderate regulatory effects in the absence of HSP60 pretreatment; pretreatment with HSP60 significantly upregulated the ability of relatively small numbers of the Tregs to inhibit IFN- γ and TNF- α cytokine secretion and proliferation in target CD4⁺CD25⁻ and CD8⁺ T cells (Figure 2, C–E). The actual concentration of Tregs at sites of regulation in vivo is presently unknown, and one may question the physiological relevance of the standard coculture systems now used to study Treg function; be that as it may, the present findings indicate that costimulation by HSP60 via innate TLR2 signaling can significantly enhance the effects of small numbers of Tregs. HSP60 performs complex adjuvant

roles in the regulation of inflammation (30), and the present results provide an additional mechanism by which self-HSP60 signaling can innately affect the expression of adaptive immunity (31).

Clinically, the upregulation of Treg function has important implications for the control of autoimmune and other inflammatory diseases (32, 33). Note that the p277 peptide fragment of HSP60 was able, like HSP60 itself, to act as a costimulator of Treg function (Figure 5). Peptide p277 is presently in advanced clinical trials as a treatment to arrest the β cell destruction responsible for type 1 diabetes (26); it is conceivable that the ability of p277 to enhance Treg function might contribute to its clinical effectiveness in shifting autoimmune T cell reactivity from a Th1 to a Th2 cytokine response (26).

The mechanism of regulation by CD4⁺CD25⁺ T cells has been controversial: some have reported that Treg function requires cell contact, but not CTLA4 or immunosuppressive cytokines (12, 15). Others, in contrast, have reported that neutralizing antibodies against CTLA4, IL-10, or TGF- β can block the function of CD4⁺CD25⁺ T cells (34, 35). We found here that HSP60-induced enhancement of CD4⁺CD25⁺ Treg function involves both contact-dependent and cytokine-dependent mechanisms (Figure 6). Blocking of CTLA4, IL-10, or TGF- β partially but significantly inhibited the augmenting effects of HSP60 (Figure 6,

**Figure 7**

HSP60 induces IL-10 and TGF- β secretion in CD4⁺CD25⁺ T cells. Unseparated CD4⁺ (A), CD4⁺CD25⁺ (A–D), or CD4⁺CD25⁻ T cells (A) were incubated with HSP60 (1 ng/ml) for 2 hours, washed, and transferred to 24-well plates coated with anti-CD3 mAb (OKT; 0.5 μ g/ml) separately (A and B), or cocultured in the indicated proportions (C and D) in serum-free medium. IL-10 and TGF- β secretion was analyzed after 24 hours by ELISA (A and C), or by FACS (B and D). (D) CD4⁺CD25⁺ T cells were labeled with CFSE and washed before coculture with CD4⁺CD25⁻ T cells. (A and C) The means \pm SD of 5 different donors are shown. * P < 0.05. (B and D) FACS histograms are representative of 3 different donors. Numbers indicate average percentage \pm SD of 3 different donors.

B and D). These results are consistent with the finding that HSP60 treatment markedly augments IL-10 and TGF- β secretion in CD4⁺CD25⁺ T cells (Figure 7). The finding that HSP60-treated

Tregs significantly upregulated their secretion of TGF- β and IL-10 (Figure 7A) suggests that costimulatory signals, such as HSP60, might account for the importance of these cytokines detected in Treg-mediated suppression in vivo (13–15). The discrepancies between the various Treg studies may be due to the possible heterogeneity of the CD4⁺CD25⁺ T cell population and to the previously undetected effects of coactivation reported here.

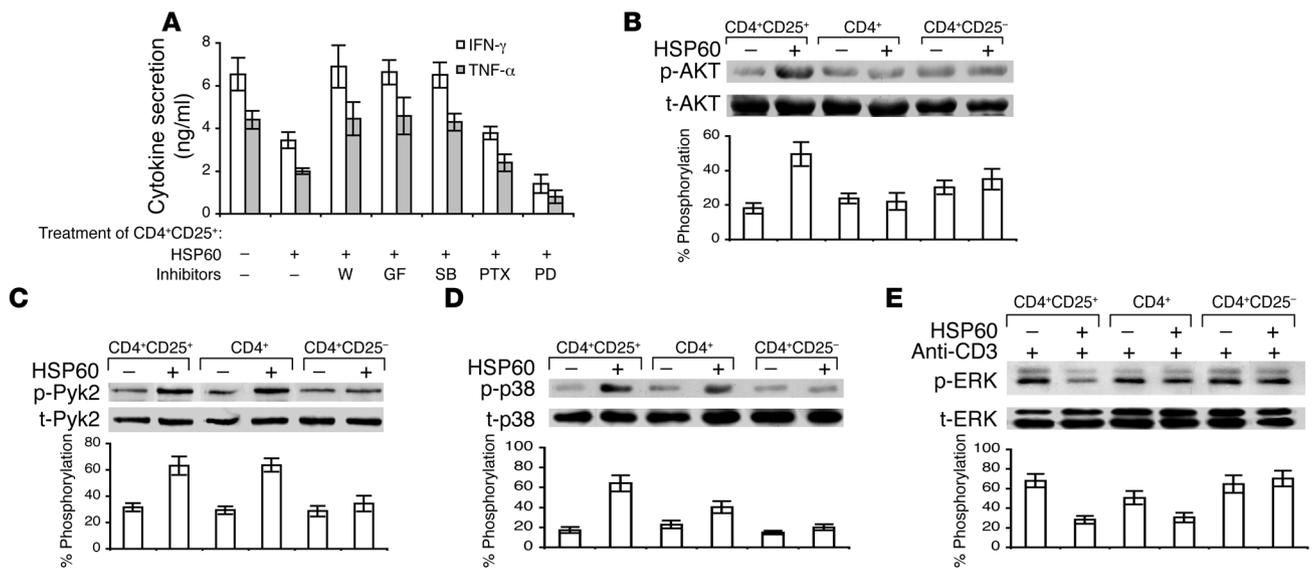
Our results indicate that the HSP60-treated Tregs can induce their target CD4⁺CD25⁻ T cells to secrete IL-10 (Figure 7D). It was recently proposed that Tregs direct the differentiation of effector CD4⁺CD25⁻ T cells toward an additional population of regulatory T cells (36). In contrast to primary Tregs, the regulatory T cells derived secondarily from CD4⁺CD25⁻ T cells inhibit T cell function through soluble mediators, such as IL-10 and TGF- β (36).

The signal transduction events responsible for CD4⁺CD25⁺ Treg function are largely unknown. Here we found that HSP60 treatment of Tregs significantly enhanced their phosphorylation of AKT, Pyk2, and p38 and inhibited ERK phosphorylation after mitogenic anti-CD3 stimulation (Figure 8). It is intriguing that the enhancing effects of HSP60 were accompanied by downregulation of ERK; the ERK response has been proposed to be required for strong T cell activation by antigen (37). Thus, Tregs may differ in their signaling pathways from other T cells in requiring inhibition of ERK to obtain full activity. It was recently reported that p38 phosphorylation was upregulated in anergic T cells in a TAB1-dependent manner (38). Moreover, in anergic, but not in naive, T cells, p38 activation led to inhibition of ERK phosphorylation and downregulation of IL-2 production (38). Moreover, the cross-talk between p38 and ERK

pathways in anergic T cells resulted in upregulation of IL-10 secretion in these cells (38–40). Thus we can see a parallel between the signaling pathways reported in anergic T cells (38) and the findings reported



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**Figure 8**

HSP60 pretreatment upregulates anti-CD3–induced phosphorylation of AKT, Pyk2, and p38 and downregulates ERK phosphorylation in CD4⁺CD25⁺ T cells. (A) CD4⁺CD25⁺ T cells were pretreated with 1 of the intracellular signal transduction inhibitors wortmannin (W; 5 nM), GF109203X (GF; 20 nM), SB 203580 (SB; 10 μ M), pertussis toxin (PTX; 2 μ g/ml), and PD 98059 (PD; 10 μ M). Then, the cells were incubated with HSP60 (1 ng/ml, 2 hours), washed, and cocultured with test CD4⁺CD25⁻ T cells (ratio 1:10) on anti-CD3 in serum-free medium. The supernatants were analyzed for IFN- γ and TNF- α . (B–E) Unseparated CD4⁺, CD4⁺CD25⁺, or CD4⁺CD25⁻ T cells were incubated with HSP60 (1 ng/ml) for 10 minutes and washed, and some cells (E) were exposed to anti-CD3 mAbs (60 minutes). Lysates of these cells were immunoblotted with antibodies: anti–phospho-AKT (p-AKT) and anti–total AKT (t-AKT) (B), anti–p-Pyk2 and anti–t-Pyk2 (C), anti–p-p38 and anti–t-p38 (D), or anti–p-ERK and anti–t-ERK (E). The blot of 1 experiment representative of 3 different donors is presented. Phosphorylation levels of the experiments were estimated by densitometry, and an average percentage of phosphorylation \pm SD was calculated as OD of p-AKT/t-AKT, p-Pyk2/t-Pyk2, p-p38/t-p38, or p-ERK/t-ERK \times 100%.

here for regulatory CD4⁺CD25⁺ T cells (Figures 2 and 8). Moreover, our results demonstrate that HSP60 treatment of CD4⁺CD25⁺ T cells augments their inhibition of ERK, NF- κ B, and T-bet in the target CD4⁺CD25⁻ population (Figure 9). Interestingly, downregulation of ERK activity occurs at two different points in the regulatory circuit: at the enhanced activation of the Tregs and at the downregulation they impose on the T effectors. Thus, HSP60 functions as a costimulator of regulatory CD4⁺CD25⁺ T cells expressed at the molecular level.

The present results, summarized in Figure 10, not only disclose the role of self-HSP60 in the innately controlled function of CD4⁺CD25⁺ Tregs but also provide some new insights into Treg signal transduction and Treg effects on the intracellular signaling and immune function of the regulated T cells.

Methods

Reagents. The following reagents and chemicals were obtained as indicated: RPMI-1640 from Invitrogen; FCS, HEPES buffer, antibiotics, and sodium pyruvate from Biological Industries; phosphatase inhibitor cocktail and LPS from Sigma-Aldrich; and Pam₃Cys from EMC Microcollections. Anti-CTLA4 mAb (BNI3) was purchased from BD Biosciences – Pharmingen, PE-conjugated anti-CD25 mAb was purchased from Miltenyi Biotec, and anti-TLR2 and anti-TLR4 mAbs were purchased from eBioscience. Anti-phosphorylated Pyk2 (clone py881), anti-phosphorylated p38, and anti-phosphorylated ERK1/2 antibodies were obtained from Biosource International; anti–total ERK1/2 antibody was obtained from Sigma-Aldrich; and anti-phosphorylated AKT and anti-AKT antibodies were obtained from Cell Signaling Technology. Anti–total ERK1/2 polyclonal antibody was obtained from Sigma-Aldrich; and anti-NF- κ B p65 (A), anti-T-bet (39D), anti–total

Pyk2 (clone N-19), anti–total p38, and anti-lamin B (C-20) polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. The anti-Foxp3 antibody (clone poly6238) was purchased from BioLegend. The inhibitors of intracellular protein kinases were obtained as follows: GF109203X from LC Laboratories; and wortmannin, PD 98059, SB 203580, and pertussis toxin from Calbiochem. Neutralizing antibodies anti-IL-10 (clone 23738.11) and anti-TGF- β (clone 9016) were purchased from R&D Systems. The recombinant HSP60 (StressGen Biotechnologies) used in this study contained less than 0.001 EU/ml (0.1 pg/ml) of bacterial endotoxin, as determined using a kinetic-turbidimetric limulus amoebocyte lysate test method (Biological Industries). The peptides used in this study were prepared using standard 9-fluorenylmethoxycarbonyl chemistry as previously described (26). The sequence of p277 is VLGGVALLRVIPALDSLTPANED, residues 437–460 of human HSP60 with V substituted for C at 442 and 447. The sequence of MT-p277 is VAGGGVTLTLLQAAPTLDELKLEG of HSP65 of *M. tuberculosis*.

Purified T cell populations. T cells were purified from the peripheral blood of healthy human donors (Blood Bank; Tel-Hashomer, Israel). The whole blood was incubated (20 minutes, 22°C) with RosetteSep human T cell enrichment cocktail (CD3⁺ T cells), or with RosetteSep human CD4⁺ and CD8⁺ T cell enrichment cocktail (StemCell Technologies). The remaining unselected cells were then loaded onto Lymphocyte Separation Medium (MP Biomedicals), isolated by density centrifugation, and washed with PBS. In a second round of purification, CD4⁺ T cells were separated into CD25⁻ and CD25⁺ populations with magnetically coupled mAb against human CD25 (Miltenyi Biotec). The purified cells obtained (>97% CD3⁺ T cells, >95% CD4⁺ T cells, >95% CD8⁺ T cells, >90% CD4⁺CD25⁺ or >99% CD4⁺CD25⁻ T cells) were cultured in RPMI containing antibiotics and 10% heat-inactivated FCS. CD4⁺CD25^{hi} T cells (about 2% of CD4⁺ T cells) and CD4⁺CD25^{int} T cells

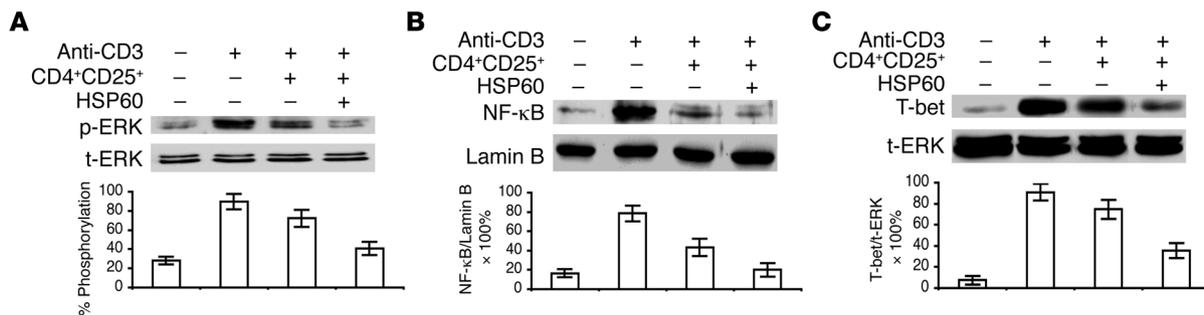


Figure 9 Coculture of CD4⁺CD25⁻ T cells with HSP60-treated CD4⁺CD25⁺ T cells downregulates ERK phosphorylation and inhibits nuclear translocation of NF-κB and T-bet expression in the CD4⁺CD25⁻ T cells. CD4⁺CD25⁻ T cells were labeled with CFSE, washed, and cocultured with HSP60-treated (1 ng/ml, 2 hours) CD4⁺CD25⁺ T cells (ratio 1:10) on anti-CD3 in serum-free medium for 6 hours. The CD4⁺CD25⁻ T cells were reisolated by FACS sorting, and cell lysates of these cells were immunoblotted with antibodies: anti-phospho-ERK (p-ERK) and anti-total ERK (t-ERK) (A), anti-NF-κB or anti-lamin B (B), or anti-T-bet or anti-t-ERK (C). The blot of 1 experiment representative of 4 different donors is presented. The levels of ERK phosphorylation, NF-κB, and T-bet were estimated by densitometry, and the average percentage derived from 4 different donors is shown.

(about 6% of CD4⁺ T cells) were separated from total CD4⁺ T cells according to their CD25 expression by means of high-speed FACS sorting. CD4⁺ T cells were incubated for 30 minutes with anti-CD25 PE-labeled antibodies (Miltenyi Biotec) at 4°C. Sorting was performed using a BD FACS Vantage. In the text, we refer to the bead-sorted Tregs as CD4⁺CD25⁻ T cells. The terms CD4⁺CD25^{hi} and CD4⁺CD25^{int} relate to FACS sorting.

Cytokine secretion. Cytokine secretion was determined by ELISA as previously described (17), using the appropriate mAb, according to the manufacturer's instructions (Biosource International).

Flow cytometry. The indicated populations of T cells (10⁶ cells per sample) were stained (60 minutes, 4°C) with anti-TLR2 or anti-TLR4 (30 μg/ml) and washed with PBS (containing 0.05% BSA and 0.05% sodium azide). For intracellular staining, cells were fixed with 3% paraformaldehyde (20 minutes, 25°C) and washed. Cell membranes were permeabilized with 0.5% Triton X-100 in PBS (10 minutes, 25°C), washed, stained (30 minutes, 4°C) with anti-CTLA4 (20 μg/ml), washed again, and incubated (30 minutes, 4°C) with a FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.). After washing, T cell staining was analyzed with a BD FACS Sort using CellQuest software.

Coculture and Transwell analyses. In coculture experiments, CD4⁺CD25⁺ T cells were incubated with HSP60 (1 ng/ml, 2 hours), washed, and added at different percentages (1%, 10%, 25%, or 50%) to test CD4⁺CD25⁻ T cells or CD8⁺ T cells. The cells (10⁶ cells per 0.5 ml in cytokine secretion assays and 2 × 10⁵ cells per 100 μl in proliferation assays) were cocultured on anti-CD3 mAb-precoated 24-well plates for 24 hours (cytokine secretion) or for 24 and 96 hours (proliferation). In Transwell analysis, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were cultured in Transwell plates (Corning). Both chambers of each Transwell were coated with anti-CD3 mAb (0.5 μg/ml). The supernatants for cytokine analysis were collected from the chambers after 24 hours.

CFSE labeling of CD4⁺CD25⁻ T cells. CD4⁺CD25⁻ T cells were labeled with a Vybrant CFDA SE Cell Tracer Kit (Invitrogen) according to the manufacturer's instructions. Labeled CD4⁺CD25⁻ T cells were cocultured with CD4⁺CD25⁺ T cells on anti-CD3 mAb, as described above, for 6 hours. After coculture, the CD4⁺CD25⁻ T cells were isolated by sorting using a BD FACS Vantage.

IL-10 secretion assay. The IL-10 secretion was evaluated with an IL-10 Secretion Assay Detection Kit (Miltenyi Biotec) according to the manufacturer's instructions.

Proliferation assay. Proliferation was assessed by the 2,3-bis-[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay (41).

Western blot analysis of T cell lysates. The indicated populations of T cells (5 × 10⁶) were pretreated with 1 ng/ml of HSP60 for 10 minutes (37°C in a 7% CO₂, humidified atmosphere). The cells were then washed and replated in the same concentration on anti-CD3 mAb-precoated 24-well plates for 4 hours (37°C in a 7% CO₂, humidified atmosphere). Total cell lysates and nuclear and cytoplasmic extracts were prepared as previously described (16, 17), and analyzed for protein content. 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 and probed with the specific mAb in the same buffer.

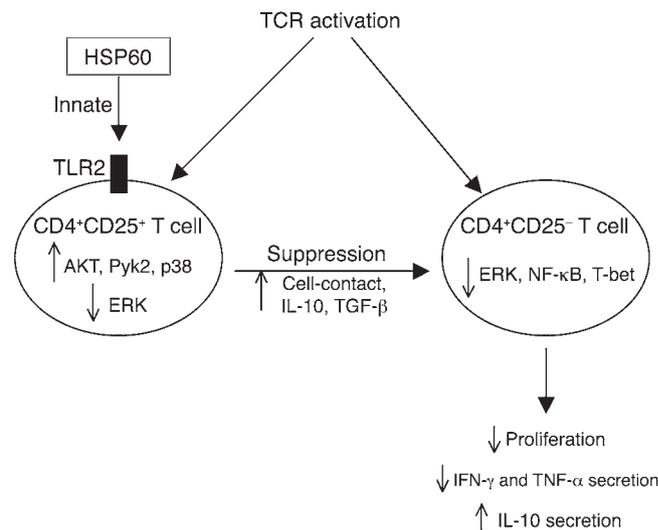


Figure 10 Schematic diagram of the innate effects of HSP60 on Treg function. The exposure of Tregs to HSP60 via innate TLR2 signaling amplifies their response to TCR-dependent (anti-CD3) stimulation, reflected by upregulation of AKT, Pyk2, and p38 and downregulation of ERK. These innate effects of HSP60 signaling amplify IL-10, TGF-β, and contact-dependent Treg suppressor mechanisms that impinge on TCR-activated CD4⁺CD25⁻ T cells to downregulate ERK, NF-κB, and T-bet, leading, in turn, to downregulated proliferation and secretion of the proinflammatory cytokines IFN-γ and TNF-α and to upregulation of IL-10 secretion.



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Immunoreactive protein bands were visualized using an HRP-conjugated goat anti-mouse antibody and the enhanced ECL system. Phosphorylation levels of the 3–5 independent experiments were estimated by densitometry.

Statistics. Data were analyzed by 2-tailed Student's *t* test. *P* < 0.05 was considered statistically significant.

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Ofer Lider is deceased.

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