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*J Immunol* published online 4 January 2013
http://www.jimmunol.org/content/early/2013/01/04/jimmunol.1200593

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/01/07/jimmunol.1200593.DC1

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Heat Shock Protein 90 Is Critical for Regulation of Phenotype and Functional Activity of Human T Lymphocytes and NK Cells

Jooeun Bae,* Aditya Munshi,*† Cheng Li,*‡ Mehmet Samur,*† Prabhala Rao,*† Constantine Mitsiades,* Kenneth C. Anderson,* and Nikhil C. Munshi*†

The 90-kDa heat shock protein (Hsp90) has become an important therapeutic target with ongoing evaluation in a number of malignancies. Although Hsp90 inhibitors have a high therapeutic index with limited effects on normal cells, they have been described to inhibit dendritic cell function. However, its effect on human immune effector cells may have significant clinical implications, but remains unexplored. In this study, we have evaluated the effects of Hsp90 inhibition on human T lymphocyte and NK cells, including their Ag expression, activation, proliferation, and functional activities. These studies demonstrate that Hsp90 inhibition irreversibly downregulates cell surface expression of critical Ags (CD3, CD4, CD8), the costimulatory molecule (CD28, CD40L), and αβ receptors on T lymphocytes, as well as activating receptors (CD2, CD11a, CD94, NKp30, NKp44, NKp46, KARp50.3) on NK cells. Hsp90 inhibition significantly reduced CD4 protein expression on T lymphocytes at both the cell surface and intracellular level, which was shown to be associated with aberrant regulation of Src-kinase p56Lck. Downregulation of the Ags triggered by Hsp90 inhibition on CD3+ T lymphocytes, both in CD4+ and CD8+ T cell subsets, was associated with a disruption in their cellular activation, proliferation, and/or IFN-γ production, when the inhibition occurred either in activated or inactivated cells. In addition, downregulation of key activating receptors on NK cells following Hsp90 inhibition resulted in decreased cytotoxicity against tumor cells. Therefore, these observations demonstrate the need to closely monitor immune function in patients being treated with a Hsp90 inhibitor and may provide a potential therapeutic application in autoimmune diseases. The Journal of Immunology, 2013, 190: 000–000.

Hsp90 (90-kDa heat shock protein) is a ubiquitously expressed conserved molecular chaperone, which mediates the maturation and stabilization of various cellular proteins. It influences the activity of many client proteins that function as critical regulators of cellular growth, differentiation, and apoptotic pathways (1–5). Hsp90 client proteins include oncogenic proteins in human malignancies acting via multiple signal transduction pathways: steroid receptors, epidermal growth factor receptor family members, MET, Raf-1 kinase, AKT, Bcr-abl, mutant p53, CDK4, and many other molecules (6–8). Inhibition of Hsp90 causes the simultaneous degradation of multiple oncogenic proteins and thereby affects signal transduction pathways important for cancer cell proliferation and survival; therefore, Hsp90 inhibitors have attracted a great deal of attention as promising anticancer drugs (9–11).

Geldanamycin, the first known Hsp90 inhibitor, modulates the proteasomal degradation of Hsp90 client proteins and displays a potent antitumor activity in vitro, but has been shown to be too hepatotoxic for clinical use (12, 13). The anasamycin geldanamycin derivative, 17-allylaminogeldanamycin (tanespimycin, KOS-953), is less toxic and also induces the degradation of proteins that require the Hsp90 chaperone for conformational maturation, disrupts association with client proteins, and has shown potent antitumor activity in preclinical and clinical studies (14, 15). Additionally, several other types of geldanamycin derivatives, including 17-demethylaminoethylmamo-17-demethoxygeldanamycin hydrochloride (alvespimycin, KOS-1022) and IPI-504 (retaspimycin), are in evaluation as promising therapeutic candidates in various cancer trials (4, 16, 17). Besides geldanamycin derivatives, numerous other classes of Hsp90 inhibitors have recently been developed, including the macroline radicicol and purine-scaffold derivatives, pyrazoles and shepherdins, which bind to the N-terminal high-affinity ATP-binding domain of Hsp90. Additionally, other drugs such as cisplatin and novobiocin as well as histone deacetylase or proteasome inhibitors have recently been shown to modify Hsp90 posttranslationally through binding to the C-terminal dimerization domain (18–24). The first in-class Hsp90 inhibitor 17-allylaminogeldanamycin entered into Phase I clinical trials in 1999, and there are now 18 natural and synthetic Hsp90 inhibitors in a variety of chemical scaffolds targeting different types of cancer in clinical trials worldwide (15, 25, 26).

The anticancer effects of Hsp90 inhibitor have been well studied in malignant cells, but their impact on human effector cells has not been well characterized. In these studies, we demonstrate the...
potential modulation of immune responses in patients treated with Hsp90 inhibitor, focusing on changes in the phenotype and function of human T lymphocytes and NK cells. Our studies reveal that Hsp90 inhibition leads to a significant and irreversible decrease in expression of critical Ags on human T lymphocytes at protein and mRNA levels. The downregulation of these Ags was associated with impairment of cellular activation, proliferation, and IFN-γ secretion by T lymphocytes stimulated with a different type of Ag. Additionally, after Hsp90 inhibition, NK cells displayed decreased activating receptor expression at protein and mRNA levels, which correlated with a downregulation in their cytotoxic activity against tumor cells. Therefore, these results suggest that Hsp90 inhibition disrupts both the Ag expression profile and functional activities of both T lymphocytes and NK cells, and highlights the relevance of potential impact of Hsp90 inhibitors on effector cell function in cancer patients during clinical trials.

Materials and Methods

Cell lines

MM cell lines, McCAR and ARP, obtained from the American Type Culture Collection (Manassas, VA), were cultured in RPMI 1640 medium (Life Technologies-Life Technologies, Rockville, MD) supplemented with 10% FCS (BioWhittaker, Walkersville, MD).

Reagents

Recombinant cytokines, including human IL-2, IL-4, IFN-α, and TNF-α, were purchased from R&D Systems (Minneapolis, MN), and human GM-CSF was obtained from Immunex (Seattle, WA). Mouse anti-human CD2, CD3, CD4, CD8, CD11a, CD25, CD28, CD69, CD80, CD83, CD86, CD154, HLA-DPQR, IFN-γ, and TCR αβ mAbs labeled with FITC, PE, PerCP, or allophycocyanin were purchased from BD Biosciences (San Diego, CA). Mouse anti-human CD94, NKp30, NKp44, NKp46, KARp50.3, and KIRp70 mAbs labeled with FITC, PE, PerCP, or allophycocyanin were purchased from Beckman Coulter Immunotech (Miami, FL). Hsp90 inhibitor (geldanamycin) was obtained from National Cancer Institute (Bethesda, MD).

Generation of human monocyte-derived dendritic cells

PBMCs were isolated by standard density gradient centrifugation over Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden) from leukopaks obtained from normal donors. In brief, dendritic cells (DC) were generated from monocytes isolated as the adherent cell fraction following overnight incubation of PBMC in tissue culture flasks at 37°C and 5% CO₂. The monocytes were cultured in the presence of 1000 U/ml GM-CSF and 1000 U/ml IL-4 in RPMI 1640 medium supplemented with 10% FCS and 2 mM l-glutamine (Life Technologies-Life Technologies) for 7 d to obtain immature DC. Fresh media plus GM-CSF and IL-4 was added to the cultures every other day. On day 7, the cultures were supplemented with IFN-α (1000 U/ml) plus TNF-α (10 ng/ml) to induce the maturation of the DC. After an additional 3 d of incubation, mature DC (mDC) were harvested and evaluated for their phenotype (CD80, CD83, CD86, HLA-DPQR) by flow cytometry.

Isolation human T lymphocytes and NK cells

Human T lymphocytes or NK cells were isolated from leukopaks using a Pan T cell or NK cell isolation kit (Miltenyi Biotec, Auburn, CA), respectively. In brief, CD3+ T cell enrichment was accomplished by an indirect magnetic labeling system for the isolation of T cells from human leukopaks. Non-T cells, that is, B cells, NK cells, DC, monocytes, granulocytes, and erythroid cells, were labeled with a mixture of biotin-conjugated Abs against CD14, CD19, CD36, CD56, CD123, and glycophorin A. These cells were subsequently incubated with antibiotin microbeads and magnetically depleted. In addition, CD4+ Th or CD8+ Tc cells were purified using an indirect magnetic isolation method. Alternatively, NK cell enrichment was achieved using an indirect magnetic labeling system. Additionally, CD3+ T cell or NK cell isolation kit was purchased from Miltenyi Biotec (Auburn, CA). Human T lymphocytes were isolated from normal donors using a Pan T cell or NK cell isolation kit (Miltenyi Biotec, Auburn, CA), respectively. In brief, CD3+ T cell enrichment was accomplished by an indirect magnetic labeling system for the isolation of T cells from human leukopaks. Non-T cells, that is, B cells, NK cells, DC, monocytes, granulocytes, and erythroid cells, were labeled with a mixture of biotin-conjugated Abs against CD14, CD19, CD36, CD56, CD123, and glycophorin A. These cells were subsequently incubated with antibiotin microbeads and magnetically depleted. In addition, CD4+ Th or CD8+ Tc cells were purified using an indirect magnetic isolation method. Alternatively, NK cell enrichment was achieved using an indirect magnetic labeling system.

FIGURE 1. No apoptosis induction or decrease in human T lymphocyte viability after treatment with geldanamycin. Human CD3+ T lymphocytes isolated from normal donors were treated with 1 μM geldanamycin for 24 h, washed, and evaluated for cellular apoptosis/necrosis and viability. (A) Untreated (left panel) or Hsp90 inhibitor–treated (right panel) lymphocytes were stained with annexin V-FITC and PI and analyzed by flow cytometry. Geldanamycin treatment did not induce apoptosis (annexin V-FITC−, PI−). Results are shown from enriched CD3+ T lymphocytes isolated from three normal individuals (donor 1, 2, or 3). (B) T lymphocytes, either untreated (●) or treated (■) with Hsp90 inhibitor, were analyzed by trypan blue dye exclusion using a hemacytometer and light microscopy. Hsp90 inhibitor–treated T lymphocytes showed no significant difference in viable cell numbers as compared with the untreated control cells (mean ± SE, n = 3).
FIGURE 2. Hsp90 inhibition irreversibly downregulates key Ags on and in human T lymphocytes. Human CD3+ T lymphocytes isolated from normal donors were treated with 1 μM geldanamycin for 24 h, washed, and evaluated for cell surface or intracellular expression of key Ags. (A) T lymphocytes, either untreated or treated with Hsp90 inhibitor, were analyzed for expression of key cell surface Ags by flow cytometry. Hsp90 inhibition results in the downregulation of key cell surface Ags on T lymphocytes. Results are shown as the MFI (mean ± SE) of three separate experiments using T cells obtained from different individual donors. (B) Hsp90 inhibitor–treated T lymphocytes were washed three times and recultured in a fresh media for an additional 3 d prior to the analyses. Surface Ag expression (MFI; mean ± SE, n = 3) was not recovered on the T lymphocytes treated with the Hsp90 inhibitor as compared with the untreated cells. (C) Hsp90 inhibitor–treated T lymphocytes were analyzed for their intracellular protein expression by flow cytometry. Both CD4 and CD8 expression was decreased in T lymphocytes by Hsp90 inhibition compared with untreated control cells to a great extent on CD4+ T lymphocytes (upper panel) than CD8+ T lymphocytes (lower panel).
**Treatment with geldanamycin**

Titration and kinetic studies for geldanamycin treatment were examined by flow cytometry. Downregulation of cell surface CD4 and CD8 Ags measured by mean fluorescence intensity (MFI) was observed in dose-and time-dependent manners (Supplemental Fig. 1). These results supported the dose and time length of geldanamycin (1 μM concentration for 24 h) used in these studies.

**Cell survival and apoptosis by treatment of cells with geldanamycin**

The effect of the Hsp90 inhibitor treatment on induction of apoptosis was examined by flow cytometry using the annexin V/propidium iodide (PI)-apoptosis kit (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. Cell viability measurement was also performed manually using a hemacytometer and light microscopy using trypan blue dye exclusion.

**Examination of cell surface Ags on T lymphocytes or NK cells**

Expression of surface proteins was analyzed by staining with fluochrome-conjugated mouse anti-human mAbs specific to CD3, CD4, CD8, CD25, CD28, CD40L, or TCR αβ on purified T lymphocytes, and CD2, CD11a, CD94, NKp30, NKp44, NKp46, KARp50.3, or KIRp70 on purified NK cells. Following staining, the cells were analyzed using a FACSCalibur flow cytometer and CellQuest v2.1 software (BD Biosciences) after gating on the respective purified cell population. To examine the recovery of the cell surface protein expression, Hsp90 inhibitor–treated cells were extensively washed and cultured in fresh complete media. The level of cell surface expression was re-evaluated on day 3 of incubation.

**Analyses of intracellular protein expression**

Enriched CD3+ T cells, either untreated or treated with Hsp90 inhibitor, were incubated with CD28/CD49d mAb mixture, and the intracellular expression of CD4 or CD8 Ag was evaluated by flow cytometry. For the intracellular expression of IFN-γ, CD3+ T cells were stimulated with 1 μg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich) for 6 h in the presence of brefeldin A (1 μg/ml; Sigma-Aldrich) to block protein secretion. Cells were washed three times in FACS buffer (0.1% BSA/PBS/0.05% sodium azide), fixed in 2% paraformaldehyde, permeabilized in buffer containing 0.1% saponin, and stained with mAbs specific to CD4, CD8, and CD69 at 4°C for 30 min, followed by flow cytometry analysis.

**[3H]Thymidine incorporation assay**

The proliferation of T lymphocytes was assessed in an allogeneic MLR. Allogeneic mDC (5 × 10^3 cells/well; stimulator cells) were irradiated at 10,000 cGy and cocultured with untreated or Hsp90 inhibitor–treated T lymphocytes (5 × 10^3 cells/well; responder cells) in 96-well U-bottom microtiter plates. Control cell cultures established in the absence of stimulators or responders were prepared to monitor background proliferation. The cells were cultured in AIM-V media (Life Technologies-Life Technologies) supplemented with 10% human AB serum (BioWhittaker) and 50 U/ml IL-2. After 5 days of culture, the cells were pulsed with 1 μCi [3H]thymidine for 18 h and harvested on day 6 to measure T cell proliferation. The results were evaluated for CPM ± SE of T lymphocytes was calculated from three separate experiments using CD3+ T cells obtained from different individual donors.

**Membrane labeling of T cells with CFSE in mitogen stimulation assay**

Untreated or Hsp90 inhibitor–treated CD3+ T lymphocytes were washed three times and resuspended at 1 × 10^6 cells/ml in AIM-V media supplemented with 10% human AB serum and 50 U/ml IL-2. The cells were labeled with CFSE (Molecular Probes, Eugene, OR) at a final concentration of 5 μM and incubated for 10 min at 37°C in a CO2 incubator protected from light. Five volumes of ice-cold PBS with 2% FCS were added to the cells to quench the reaction, incubated for 5 min on ice, centrifuged, and resuspended in fresh PBS with 2% FCS after a total of three washings. CFSE-labeled T lymphocytes were adjusted to 2 × 10^5 cells/ml with AIM-V media containing 1 μg/ml Con A (Sigma-Aldrich), and then incubated in a 24-well plate at 37°C and 5% CO2. The Con A-stimulated CFSE-labeled T lymphocytes were examined by flow cytometry on day 3 or 4.

**Cytotoxicity assay**

The cytotoxic activity of NK cells was measured using a calcein-release assay. Briefly, target cells were incubated in serum-free culture medium containing 10 mM calcine-AM (Molecular Probes) for 30 min at 37°C, washed three times in cold PBS with 5% FCS, and incubated with effector cells at various E:T cell ratios in 96-well U-bottom microtiter plates (triplicates/sample) for 3 h at 37°C and 5% CO2. After incubation, the cells were pelleted by centrifugation at 1000 rpm for 5 min, and 100 μl of the supernatant was transferred from each well to 96-well flat-bottom plates; calcine release was then measured using a VICTOR2-1420 multilabel counter (PerkinElmer, Wellesley, MA). Maximum release was obtained from detergent-released target cell counts and spontaneous release from target cell counts in the absence of effector cells. Cellular cytotoxicity was calculated as follows: percentage of specific lysis = ([experimental release – spontaneous release]/[maximum release – spontaneous release]) × 100%.

**FIGURE 3.** The level of CD25 is decreased on activated T lymphocytes by Hsp90 inhibition. Enriched human CD3+ T lymphocytes were activated by stimulation with Con A (1 μg/ml) prior to Hsp90 inhibition, and expression of the IL-2Ra (CD25) was then analyzed on T lymphocytes by flow cytometry. Expression of CD4 cell surface Ag was completely abrogated by Hsp90 inhibition. The CD4+ and CD8+ T cell population expressing IL-2Ra (CD25+/CD4+, CD25+/CD8+ cells) was dramatically reduced by Hsp90 inhibition.
FIGURE 4. Hsp90 inhibition decreases T cell proliferation induced by allogeneic mDC or Con A. (A) Untreated (○) or Hsp90 inhibitor–treated (■) CD3+ T lymphocytes (5 × 10^5 cells/well, 1 × 10^5 cells/well) were cultured with allogeneic mDC (5 × 10^5 cells/well) in triplicate wells at 37°C for 5 d, pulsed overnight with 1 μCi/well [3H]thymidine, harvested, and analyzed for T cell proliferation. Hsp90 inhibition induced a significant (*p < 0.05) reduction in T cell proliferation in response to allogeneic mDC at both responder:stimulator ratios tested (10:1, 20:1). The values represent the mean cpm ± SE of three separate experiments using T cells obtained from different individual donors. (B) Untreated or Hsp90 inhibitor–treated CD3 T lymphocytes were washed, labeled with CFSE, and stimulated with 1 μg/ml Con A for 3 or 4 d prior to measuring T cell proliferation by flow cytometry. The proliferating cell population (M1) was identified by a decrease in CFSE intensity. Con A stimulation (middle panel) induced cell proliferation as compared with unstimulated (left panel), untreated T cells. However, Hsp90 inhibition (right panel) significantly decreased T cell proliferation induced by Con A. (C) Con A–induced CD4+ or CD8+ T cell proliferation was evaluated in Hsp90 inhibitor–treated T lymphocytes on day 3 of culture. The specific proliferating CD4+ or CD8+ T cell populations (Q1) are identified by a decrease in CFSE intensity. Hsp90 inhibition (right panels) decreased both CD4+ and CD8+ T cell proliferation induced by Con A as compared with control T cells (left panels).
Oligonucleotide microarray analysis of gene expression

Total RNA was extracted from untreated or Hsp90 inhibitor (1 μM for 24 h)-treated T lymphocytes using a RNeasy kit (Qiagen, Valencia, CA). RNA concentration was determined by absorbance at 260 nm, and 15 μg high-quality total RNA was used in a CDNA synthesis reaction using the Megascript T7 kit (Ambion, Austin, TX). The resulting cdNA was subsequently used to generate biotin-labeled antisense cRNA for hybridization with Human Genome HG-U133A Affymetrix gene chips. Scanning of image output files and analysis of gene expression data sets were followed by filtering of upregulated or downregulated transcripts based on conventional criteria for statistical significance, as well as by hierarchical and functional clustering algorithms (27–29). In statistical analysis, expression values and fold change values were calculated for the groups (treated versus untreated samples) of CD4+ T cells, CD8+ T cells, and NK cells. Expression value ≥100 and fold change ≥1.2 were taken as threshold and used to report upregulated and downregulated genes, because they are more likely to correspond to biologically relevant changes rather than expression or technical noise. Microarray raw data files and normalized expression values are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE42253.

Results

Geldanamycin-induced Hsp90 inhibition irreversibly downregulates expression of critical T cell Ags at both the cell surface and intracellular levels

We first evaluated the effect of geldanamycin (1 μM concentration for 24 h) on T cell survival using annexin V/PI staining and flow cytometry. Hsp90 inhibition did not induce apoptosis (annexin V-FITC+, PI-) in enriched CD3+ T lymphocytes from three separate normal donors (Fig. 1A). We also confirmed >90% viability in treated cells by light microscopy using trypan blue dye exclusion staining, which was similar to the untreated control cells (Fig. 1B).

Expression of critical cell surface Ags on enriched CD3+ T lymphocytes following Hsp90 inhibition was evaluated by flow cytometric analyses after gating for the enriched T cell population. Hsp90 inhibitor-treated T cells (1 μM geldanamycin for 24 h) showed a significant (p < 0.05) decrease in the expression of CD3, CD4, and CD8 T cell Ags, CD28 and CD40L (CD154) costimulatory molecules, and the TCR αβ as compared with untreated control T cells obtained from the same donors (Fig. 2A). Recovery of downregulated T cell surface Ags was next evaluated after washing of the cells extensively to remove free Hsp90 inhibitor, followed by 3 d of culture in fresh media. Whereas overall cell viability remained high (>80%) in the cultures on day 3 following removal of the inhibitor, the downregulated surface Ags CD3, CD4, CD8, CD28, CD40L, and TCR αβ were not recovered on T lymphocytes treated with Hsp90 inhibitor (Fig. 2B). These results demonstrate that inhibition of Hsp90 irreversibly interferes with the expression of critical cell surface Ags on CD3+ T lymphocytes. In addition, intracellular protein expression of the CD4 and CD8 Ags, evaluated using enriched CD3+ T cells, was also decreased after Hsp90 inhibition (Fig. 2C). Taken together, CD4 expression on T cells showed the most significant downregulation after Hsp90 inhibition at both the cell surface and intracellular levels.

Geldanamycin downregulates CD25 on activated T lymphocytes

The effect of Hsp90 inhibition was next evaluated on activated CD4+ or CD8+ T lymphocytes. For the analyses, CD3+ T lymphocytes were stimulated with a lymphocyte mitogen (Con A) and then treated with the Hsp90 inhibitor. Modification of IL-2R α-chain CD25 was examined on T lymphocytes by flow cytometry. Complete abrogation of CD4 Ag was detected on activated T cells following Hsp90 inhibition (Fig. 3); therefore, the double-positive population was dramatically reduced, including CD4+/CD25+ T cell subset (untreated versus treated; 18% versus 1%). In contrast to CD4, CD8 Ag was still present after Hsp90 inhibition on the activated T cells, although the expression level was reduced. The CD8+ T cells expressing CD25 decreased from 9 to 1% after

![FIGURE 5](http://www.jimmunol.org/) Hsp90 inhibition decreases SEB-induced T lymphocyte IFN-γ production and cellular activation. Human CD3+ T lymphocytes stimulated with SEB (1 μg/ml) were treated with geldanamycin and then evaluated for intracellular IFN-γ production and cell activation by flow cytometry. Hsp90 inhibition resulted in a dramatic decrease in IFN-γ production and activation (CD69+) in both CD4+ and CD8+ T lymphocyte subsets.
Hsp90 inhibition (Fig. 3). Thus, these results demonstrate the Hsp90 inhibition downregulates a critical cell surface molecule, CD25 on activated T cells, which may impair their ability to respond to exogenous IL-2.

Geldanamycin inhibits proliferation of T lymphocytes

Next, we evaluated the impact of Hsp90 inhibition on T cell proliferation in response to either allogeneic mDC or a mitogen. T cells were initially treated with the Hsp90 inhibitor geldanamycin for 24 h, washed, and then cultured with irradiated (10 Gy) allogeneic mDC. T cell proliferation measured by [³H]thymidine incorporation was significantly (*p < 0.05) reduced in Hsp90 inhibitor–treated T cell cultures as compared with the untreated control cultures at responder (T lymphocytes):stimulator (mDC) ratios of 10:1 or 20:1 (Fig. 4A). Next, we evaluated T cell proliferation in CFSE assays in response to the mitogen Con A (1 μg/ml). The proliferating cell population (gated in M1) was identified by a decrease in CFSE intensity (CFSE low). Untreated T cells stimulated with Con A showed increased cell proliferation (22% on day 3, 41% on day 4) as compared with the control unstimulated T cells (3% on day 3, 7% on day 4) (Fig. 4B). In contrast, Con A-induced T cell proliferation was significantly decreased in the Hsp90 inhibitor–treated T cell cultures (0% on day 3, 3% on day 4). Thus, these results demonstrate that both MHC–TCR-mediated (allogeneic mDC stimulation) and non-MHC–TCR-mediated (Con A stimulation) CD3⁺ T cell proliferation was significantly decreased by Hsp90 inhibition. We further examined the effects of Hsp90 inhibition on CD4⁺ or CD8⁺ T cell subset proliferation using the CFSE assay. Proliferation of CD4⁺ T cells and CD8⁺ T cells (gated in Q1) was significantly reduced in Hsp90-inhibited T cell cultures as compared with the control T cells after stimulation with Con A (Fig. 4C). Overall, Hsp90 inhibition reduced CD4⁺ T cell proliferation from 28 to 0% and CD8⁺ T cell proliferation from 13 to 0%, as measured on day 3 of culture. Therefore, these results
demonstrate that the proliferation of CD4⁺ T cells and CD8⁺ T cells in response to mitogen stimulation was significantly reduced by Hsp90 inhibition.

Geldanamycin abrogates IFN-γ production and cellular activation of CD4⁺ or CD8⁺ T cells

The impact of Hsp90 inhibition on T lymphocyte function was further investigated by measuring IFN-γ production and cellular activation. In contrast to the previous results presented in Fig. 4, enriched CD3⁺ T cells were initially activated with the superantigen SEB (1 µg/ml) and then treated with Hsp90 inhibitor to measure the effects of Hsp90 inhibition on activated T cells. Our results demonstrate that Hsp90 inhibition induced a significant decrease in IFN-γ production and cell activation in both CD4⁺ T lymphocytes (untreated versus Hsp90 inhibitor treated; 2.55% versus 0.48% IFN-γ⁺/CD69⁺ cells) and CD8⁺ T lymphocytes (untreated versus Hsp90 inhibitor treated; 4.08% versus 0.39% IFN-γ⁺/CD69⁺ cells) (Fig. 5). These results offer evidence that Hsp90 inhibition interrupts cytokine production and cellular activation in both activated CD4⁺ and CD8⁺ T lymphocyte subsets.

Geldanamycin regulates mRNA expression in T lymphocytes

Next, we evaluated mRNA expression profiles of key molecules in purified human CD4⁺ or CD8⁺ T lymphocytes treated with Hsp90 inhibitor (1 µM for 24 h) using HG-U133A gene arrays. In contrast to the consistent and significant downregulation of all the Ags tested at the protein level, the impact of Hsp90 inhibition on mRNA expression was varied, but limited among the Ags for both CD4⁺ and CD8⁺ T lymphocytes. Compared with untreated cells, Hsp90 inhibitor–treated CD4⁺ T lymphocytes showed only modest upregulation of only CD3ε (fold change: 1.44) and CD28 (fold change: 1.72) mRNA expression (Fig. 6A). Hsp90 inhibition also has the limited effect on mRNA expression in CD8⁺ T lymphocytes (Fig. 6B), including downregulation of CD3γ (fold change: −1.21) and upregulation of CD3ξ (fold change: 1.38) and CD28 (fold change: 1.98), but insignificant changes in other genes, including CD8 in CD8⁺ T lymphocytes.

FIGURE 7. Hsp90 inhibition downregulates key activating receptors on human NK cells. Control untreated (©) or Hsp90 inhibitor–treated (●) NK cells were analyzed for cell surface expression of key activating receptors by flow cytometry. Hsp90 inhibition resulted in a significant downregulation in the cell surface expression of CD2, CD11a, CD94, NKp30, NKp44, NKp46, KARp50.3, and KIRp70 receptors on NK cells. Results are shown as MFI (mean ± SE) of three separate experiments using purified NK cells obtained from different individual donors.

Geldanamycin downregulates expression of activating receptors on NK cells

NK cells isolated from three normal individuals were evaluated by flow cytometry for the expression of activating receptors following Hsp90 inhibition with geldanamycin (1 µM for 24 h). Hsp90 inhibition resulted in decreased expression of the NK cell-activating receptors, including CD2, CD11a, CD94, NKp30, NKp44, NKp46, KARp50.3, and KIRp70 (Fig. 7). Among these receptors, CD2, CD11a, CD94, NKp30, and NKp44 showed a significant (p < 0.05) downregulation in their MFI on NK cells after Hsp90 inhibition as compared with the untreated control cells.

Geldanamycin decreases the cytotoxic activity of NK cells

Finally, we evaluated the effect of Hsp90 inhibitor on the cytotoxic activity of NK cells against cancer cell lines. Untreated control NK cells demonstrated effective lysis of NK-sensitive multiple myeloma cell lines MM1S (11–48%) and ARP (0–64%) at various E:T cell ratios. However, the cytotoxic activity of NK cells was significantly decreased following Hsp90 inhibition against both MM cell lines, including MM1S (2–10%) and ARP (0–5%) (Fig. 8). Thus, these results demonstrate that Hsp90 inhibition induced a dramatic decrease in cytotoxic activity of NK cells against multiple myeloma cells, which correlated with a reduction in the expression of activating receptors on NK cells.

Geldanamycin modifies mRNA expression of activation Ags in NK cells

We further evaluated mRNA expression profiles of the key activation molecules in purified NK cells following Hsp90 inhibition. In contrast to the consistent downregulation of cell surface Ags at the protein level seen by flow cytometry in NK cells after Hsp90 inhibition, we observed variable modifications in mRNA expression (Fig. 9). Specifically, Hsp90 inhibitor–treated cells showed a downregulation in mRNA expression for CD2 (fold change: −2.36) and CD94 (fold change: −2.23), although a minor upregulation of mRNA expression was observed for CD11a (fold change: 1.23) and NKG2D (fold change: 1.24), as compared with untreated

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individual donors. Three separate experiments using purified NK cells obtained from different cytotoxicity was calculated, as described in Materials and Methods. Hsp90 inhibitor–treated NK cells showed a significant reduction in cytotoxic activity against both MM1S and ARP multiple myeloma cell lines as compared with untreated NK cells. The values represent the mean ± SE of three separate experiments using purified NK cells obtained from different individual donors.

NK cell control. The majority of these Ags had no significant change in their mRNA expression.

Discussion
Chaperones are ubiquitous and highly conserved proteins that passively prevent aggregation of damaged proteins or use active ATP-driven conformational processes to unfold their target proteins (30–32). Chaperones are also involved in quality control of the MHC–class I complex, T and B cell receptors, and many other key proteins required for immune signaling (33–37). Among the chaperone proteins, Hsp90 has emerged as an exciting target for cancer therapy due to its critical function in the conformation, stability, activity, regulation, and cellular localization of onco- genic client proteins that contribute to tumor cell survival. Numerous Hsp90 client proteins, including transmembrane tyrosine kinases, MET, and insulin-like growth factor–1 receptor, and mutated signaling proteins regulate multiple signal transduction pathways in human malignancies (38–42). Therefore, targeting Hsp90 offers the unique prospect of simultaneously inhibiting multiple signaling pathways required for the development and maintenance of malignant cancer cells. In addition, Hsp90 is known to be overexpressed in many types of cancer; moreover, Hsp90 isolated from tumor cells has a 20–200 times higher binding affinity for their inhibitors than does Hsp90 isolated from normal cells (43–45). This difference might be due to the fact that tumor cells, as compared with normal cells, exhibit a stressed phenotype, which is associated with an enhanced dependency on the cytoprotective action of Hsp90, providing further rationale to develop Hsp90 inhibitor drugs to treat cancer patients.

The majority of Hsp90 inhibitors to date act by docking in the N-terminal nucleotide binding site, thereby inhibiting the intrinsic ATPase activity and blocking the formation of the mature complex. Many of these Hsp90 inhibitors cause selective degradation of important signaling proteins involved in cell proliferation, cell cycle regulation, and apoptosis in a wide range of tumor models (46, 47). Previously, we have demonstrated in human tumors in vitro and in murine models that Hsp90 inhibitors result in the destabilization and eventual degradation of Hsp90 client proteins, thereby leading to apoptotic cell death (48).

The objective of current studies was to evaluate the effects of Hsp90 inhibition on the survival, activation, proliferation, and functional activities of human immune effector cells, including T lymphocytes and NK cells, and to estimate its potential impact on clinical outcomes in cancer patients treated with the specific inhibitor. Previous reports by other investigators have shown that geldanamycin at a concentration of either 1.78 μM (34, 35) or 3.6 μM (36) decreased T cell proliferation, IL-2R expression, and IL-2 secretion through blockage kinase-mediated signaling events (i.e., lck, Raf-1, ERK-2). Additionally, in a previous study using a panel of both drug-sensitive and drug-resistant MM cell lines, our group has demonstrated that the optimal dose and duration of exposure for anti-MM activity was 1 μM geldanamycin for 24 h (48). In these studies, we demonstrate that geldanamycin at a lower concentration and duration (1 μM, 24 h) than previously reported decreased T cell function and provided new information regarding blockage of NK cell function. In contrast to malignant cells, the Hsp90 inhibitor did not induce significant apoptosis or reduce viability of normal T lymphocytes (Fig. 1) under the same dose and treatment duration, which is consistent with our previous observations on inhibition of DC function (49). These observations are also consistent with others showing that Hsp90 extracted from malignant cells has significantly higher ATPase activity and binding affinity to ATP compared with various normal cells, which can lead to a significantly higher lysis of malignant cells than normal cells (43, 50–52). In addition, these studies further demonstrate that Hsp90 inhibition significantly decreases expression of key cell surface Ags on normal human T lymphocytes or NK cells. The downregulation of these critical markers was irreversible, because removal of Hsp90 inhibitor from the cells followed by long-term incubation in fresh media did not result in the recovery of Ag expression. Besides surface Ags, intracellular protein expression was also decreased in T lymphocytes following Hsp90 inhibition. These results are consistent with our previous study that demonstrated an irreversible downregulation of key cell surface Ags (CD40, CD80, CD83, CD86, HLA-A, -B, -C, and HLA-DP, -DQ, -DR) and intracellular proteins (CD40, CD83) on human DC after Hsp90 inhibition (49).

We hypothesize that the decrease in protein expression following Hsp90 inhibition may be mediated by several potential mechanisms, including modulation of transcription levels (2, 53), blockage of protein degradation required for their conformational maturation...
Hsp90-treated (presented as an arbitrary unit for untreated (C) and Hsp90-treated (●) NK cells.

Results demonstrate that Hsp90 inhibitor induced only a very modest modification of transcription, is varied among the different Ags, and is distinct from the consistent Ag downregulation we observed at the protein level. Thus, we postulate that effect of Hsp90 inhibitor on human T lymphocytes and NK cells may be on change mainly in protein folding and conformational changes, resulting in its lack of cell surface expression, anchoring, or eventual proteasomal degradation.

Lck is a member of the Src family of nonreceptor tyrosine kinases and is expressed primarily in T lymphocytes and thymocytes. The protein is predominantly associated with the cytosolic side of the plasma membrane (56, 57), a localization consistent with the importance of Lck in early signaling events through the TCR (58). Bijlmakers and Marsh (59) demonstrated that Lck associates with intracellular CD4 early after synthesis and with cell surface CD4 at later times. This group as well as other investigators demonstrated the essential role of Hsp90 for the synthesis and subsequent membrane association of Src-kinase p56Lck, thus, failure of this regulation would lead to a significant degradation of CD4 Ag (60–62). Our observations are consistent with these previous studies, by demonstrating a dramatic decrease of CD4 protein expression after Hsp90 inhibition, at both the cell surface as well as intracellular levels. Furthermore, our functional study showed that the downregulation of critical Ags on T cells by Hsp90 inhibition resulted in decreased cell activation and proliferation in response to either an alloantigen or mitogen. T cells in cancer patients can be somewhat activated due to the higher level of cytokines or chemokines released from different types of cells. To estimate the potential effect of Hsp90 inhibition in cancer patients, we first activated T lymphocytes with the superantigen SEB, followed by Hsp90 inhibition, and monitored the effects on the T cell functional activities (Fig. 5). In the analyses, we observed a downregulation of surface Ag expression and a decrease in activated T cell function, as demonstrated by reduced IFN-γ production and CD69 expression after Hsp90 inhibition (2–4% of IFN-γ+/CD69+ in T lymphocytes), which was consistent with a previous report using normal donors’ T cells (63). Finally, our observation using human T cells was consistent with other reports demonstrating abrogation of a mitogen-induced mouse splenocyte proliferation upon exposure to geldanamycin (36).

Unlike T lymphocytes, which are involved in adaptive immunity, NK cells are a key component of innate immunity and are involved not only in the elimination of tumor cells, but also in the regulation of the immune response through secretion of cytokines and chemokines that can activate other important cellular components. Development of the antitumor activities of NK cells is controlled by multiple mechanisms, including direct cytotoxic activity against tumor cells (64, 65). Because activating NK cell receptors render tumor cells potentially susceptible to NK cell attack (66, 67), we investigated the effects of Hsp90 inhibition on the expression of activating receptors as well as their functional activity. Our studies show that Hsp90 inhibition leads to downregulation of CD2, CD11a, CD94, NKp30, NKp44, NKp46, KARp50.3, and KIRp70 activating receptors on NK cells. Corresponding to the reduction of their expression, a significant inhibition of cytotoxic activity was detected in the Hsp90 inhibitor–treated NK cells against NK-sensitive tumor cell lines. These results are consistent with our previous investigation, which demonstrated that Hsp90-mediated protein processing and folding are critical for the expression of critical proteins and function of DC (49).

In summary, our data offer a unique insight on the potential of Hsp90 inhibitor to modulate immune function. We demonstrate that Hsp90 inhibition could lead to a significant modulation of effector cells, including T lymphocytes and NK cells, with important clinical implication. On the basis of these findings, we highlight the concern that Hsp90 inhibition may result in immune suppression and suggest the importance of close monitoring of immune cells for their functional activities in patients being treated with a Hsp90 inhibitor. Finally, our results provide the rationale to investigate the effects of a Hsp90 inhibitor in preventing graft versus host disease as well as treating autoimmune diseases.
Disclosures

The authors have no financial conflicts of interest.

References

Corrections


The fifth author’s name was published incorrectly. The correct name is Rao Prabhala.

This has been corrected in the online version of the article, which now differs from the print version as originally published.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1390005