HSP90 chaperones a large number of proteins, and it plays essential roles in multiple signaling pathways to maintain protein homeostasis in the cytosol. In addition, HSP90 has been implicated in mediating recognition of lipopolysaccharide (LPS). However, no pharmacologic agents have been developed to interrogate this pathway. Herein we demonstrate that a peptide-based inhibitor that was previously reported to inhibit the master Toll-like receptor-chaperone gp96, an endoplasmic reticulum paralog of HSP90, in fact blocks HSP90-LPS interaction. It inhibited the binding of LPS to the cell surface of both wild type and gp96-null cells and thereby abrogated the cellular response to LPS but not to other Toll-like receptor ligands. We also generated a series of peptide derivatives (named peptide inhibitors of endotoxin responsiveness (PIERs)) from the N-terminal helix structure of HSP90 and demonstrated their effectiveness in blocking LPS activity. PIER inhibition of LPS signaling was partially reversed by CD14 expression. Moreover, we found that a cell-permeable PIER abrogated HSP90 function and caused degradation of multiple known HSP90 client proteins in cancer cells. Thus, targeting HSP90 is a promising modality for treatment of both LPS-mediated pathology and cancer.

Significance: This study facilitates the development of HSP90-based inhibitors to treat inflammation and cancer.
glycosylphosphatidylinositol-linked membrane protein (12). Without a signaling tail, CD14 functions as a co-receptor by transferring LPS from LPS-binding protein to TLR4-MD-2 complex. However, additional molecules other than CD14 might act as co-receptors for TLR4. The earliest evidence for the involvement of HSP90 came from the LPS-like molecule Taxol, which induces TNFα in mouse macrophages (13). It was found that HSP70 and HSP90 were the two major Taxol-binding proteins. The role of HSP70 and HSP90 was further suggested by their direct binding to LPS and the indirect evidence of interaction between HSP70-HSP90 and LPS through fluorescence resonance energy transfer studies (14–16). Later on, it was shown that HSP70 and HSP90 function in a complex that also includes CXCR4 and growth differentiation factor 5 (17).

As an endoplasmic reticulum paralog of HSP90, gp96 is the master chaperone for TLRs (18–20). With the exception of TLR3, the rest of the TLRs are exclusively dependent on gp96 for folding and functional expression despite the abundance of other HSPs in the endoplasmic reticulum (21). Overexpression of gp96 causes lupus-like diseases in a manner that is dependent on TLR4 (22, 23). On the other hand, deletion of gp96 from the macrophage compartment leads to LPS resistance (19). Therefore, gp96 appears to be an attractive drug target for inflammation, sepsis, and autoimmune disease. In a recent study utilizing in silico methods, a peptide inhibitor of gp96 was designed by targeting the N-terminal helix-loop-helix sequence, and the authors demonstrated that this peptide could effectively inhibit LPS responses both in vitro and in vivo (24). The proposed mechanism of action was that this peptide mimics the sequence of the helix, therefore disrupting the helix-helix interaction and the chaperoning function of gp96. However, based on the crystal structure of gp96 (25), this N-terminal helix structure is unlikely to be a substrate-binding site. In addition, the inhibitory effect of this peptide has a very rapid kinetics, arguing against its roles in inhibiting gp96-mediated TLR folding as the mechanism of its action. In the current study, we demonstrate that N-terminal helix-based peptides from both HSP90 and gp96 are able to inhibit LPS binding to HSP90 and to attenuate LPS-mediated NFκB signaling in a manner that is independent of their activity against gp96.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Plasmids**—THP-1 and SKBR3 cell lines were obtained from ATCC. Wild type or gp96 KO mutant pre-B cell lines were kind gifts from B. Seed and were described previously (18). All culture conditions have been reported before (26). MigR1-mCD14TLR4HA was cloned into MigR1 vector from pUNO-murine CD14 (InvivoGen).

**Peptides**—All peptides were synthesized by NEO Group to more than 98% purity as verified by HPLC and mass spectrometry. All peptides were dissolved in sterile PBS. Sequences of peptides are as follows: PIER1, NH2-LNVSRTELQQHKLKK-VIRKKLVKTLDMKIAADDKY-COOH; PIER2, NH2-LNVSRTELQQHKLKKVLKPKTLDMKIAADDKY-COOH; PIER3, NH2-LNISREMLQQSKILVKRINWKCKCELFDSELA-EDKEN-COOH; and PIER4 (TAT-PIER1), GRKKRRQRRP-RQ-PIER1.

**Reagents**—Biotin-LPS was obtained from InvivoGen. Purified human HSP90 was purchased from Enzo Life Sciences. Streptavidin-allophycocyanin secondary antibody was purchased from eBioscience. The non-permeable geldanamycin (NPGA), also known as dimethylaminoethylamino-17-demethoxygeldanamycin-N-oxide (27), was synthesized by Zuping Xia (Pharmaceutical Sciences, Medical University of South Carolina) and was described previously (28).

**TNFα ELISA**—THP-1 cells were pretreated with phorbol 12-myristate 13-acetate (PMA) at 20 ng/ml for 48 h followed by stimulation with LPS for 24 h in the presence or absence of PIER. The supernatant was then collected, and TNFα was measured by an ELISA kit according to the manufacturer’s specifications (BD Biosciences).

**NFκB-GFP Reporter Assay**—As described previously (18), all cells were derived from that E4.126 parental cell line, which contains an NFκB-driven GFP reporter. Cells were stimulated with Pam3CSK4 (10 μg/ml), LPS (10 μg/ml), CpG ODN1826 (5 μM), PMA (100 ng/ml), and ionomycin (2 μg/ml) for 16–18 h before FACS instrumentation.

To determine the roles of surface HSP90, 1 × 10⁶ full-length gp96-expressing E4.126 cells were pretreated with 20 μM NPGA, a cell-impermeant HSP90 inhibitor, for 1 h and then stimulated with LPS (200 ng/ml) or with both PMA (50 ng/ml) and ionomycin (1 μg/ml) for 5 h followed by flow cytometric analysis.

**Western Blot and Antibodies**—Antibodies were purchased from Cell Signaling Technology unless otherwise specified. Essentially all procedures were performed as described (26) without significant changes.

**Flow Cytometry**—All staining protocols, flow cytometry instrumentation, and data analysis were performed essentially as described (20, 26) without significant modifications. For cell surface staining, a single cell suspension of live cells was obtained and washed in FACS buffer twice. Fc receptor blocking with or without serum was performed depending on the individual primary antibody used for staining. Primary and secondary antibody staining was performed stepwise with FACS buffer washing in between. Propidium iodide was added right before FACS instrumentation to gate out dead cells. For intracellular cytokine staining, cells were stimulated in the presence of 10 μg/ml brefeldin A before harvesting and washing with FACS buffer. Cells permeabilization was done with 0.25% saponin in FACS buffer. The same buffer was used in subsequent steps including blocking, washing, and antibody staining. The last washing step before FACS instrumentation was done with FACS buffer alone without detergent. Stained cells were acquired on a FACS Calibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**HSP90 in Vitro Binding Assay**—Purified HSP90 (1 μg) was incubated on ice in the presence of biotin-LPS (2 μg/ml) and/or PIER1 peptide (5 μM) at 4 °C for 30 min. The mixture was then incubated with streptavidin-agarose beads at 4 °C overnight. Beads were then washed with Tris lysis buffer and subjected to boiling in SDS loading buffer for 5 min prior to resolving by SDS-PAGE. The intensity of Western blot bands was quantified by ImageJ software.
Assay of LPS Binding to Cell Surface—Biotin-LPS stock (500 μg/ml) was diluted in complete RPMI 1640 medium to the desired working concentration. About 0.2 million cells were resuspended in 50 μl of medium/well in a 96-well plate. Another 50 μl of biotin-LPS suspension was added into the same well. The plate was then placed on a gentle shaker and rocked at 37 °C for 30 min. After the incubation, cells were harvested from the well into cold PBS, washed with PBS and FACS buffer, and stained with streptavidin-allophycocyanin antibody at 4 °C for 30 min. Samples were then thoroughly washed with FACS buffer and analyzed on a FACSCalibur.

RESULTS

PIER Peptide Inhibits NFκB Response to LPS but Not to Other TLR Ligands—We synthesized a 37-mer peptide corresponding to residues 444–480 of gp96 based on the study by Kliger et al. (24). For consistency in this study, we named this peptide PIER1. We tested PIER1 activity using a murine pre-B cell line that stably expresses the NFκB-GFP reporter. When stimulated with LPS for 16–18 h, the untreated pre-B cell line had a dose-dependent induction of NFκB-GFP (Fig. 1A). Consistent with the Kliger et al. (24) study, we found that concurrent treatment of the cells with 5 μM PIER1 without preincubation significantly suppressed NFκB-GFP expression (after stimulation with PMA and ionomycin). *, p < 0.01 C, same as B except Pam3CSK4 was used as the stimulus. D, same as B and C except CpG was used as the stimulus. Experiments were repeated more than three times with similar results. Error bars represent standard error of the mean. AU, absorbance units.

PIER1 Inhibits LPS Response by Disrupting Binding of LPS to Cell Surface—We next examined the effect of PIER1 on LPS binding to the cell surface. Given the fact that PIER1 inhibition does not require preincubation of cells before adding LPS, we hypothesized that the inhibitory effect of PIER1 is at the upstream level of the LPS response, i.e. the binding of LPS to the TLR4-MD-2 complex. This consideration is also consistent with the fact that PIER1 is not expected to enter cells readily.

We performed a standard LPS binding assay with biotinylated LPS followed by streptavidin-conjugated FITC to detect bound LPS on the cell surface (Fig. 2). Strikingly, we found that PIER1-mediated suppression of LPS responsiveness was via inhibition of the chaperone function of gp96, we would also expect significant inhibition of TLR2 and TLR9 function. Contrary to this prediction, we found that PIER1 had no activity against NFκB activation induced by TLR2 or TLR9 ligands. Therefore, we conclude that PIER1 inhibit LPS responsiveness in a gp96-independent manner.

PIER Peptide Binds to HSP90—We next focused on the possibility of PIER1 to inhibit LPS binding to HSP90 as previous studies have suggested that cell surface HSP90 is another LPS-binding protein for TLR4 signaling (14–17). Additionally, a sequence alignment between HSP90 and gp96 of both human and mouse origin demonstrated that the target sequence of

Assay of LPS Binding to Cell Surface—Biotin-LPS stock (500 μg/ml) was diluted in complete RPMI 1640 medium to the desired working concentration. About 0.2 million cells were resuspended in 50 μl of medium/well in a 96-well plate. Another 50 μl of biotin-LPS suspension was added into the same well. The plate was then placed on a gentle shaker and rocked at 37 °C for 30 min. After the incubation, cells were harvested from the well into cold PBS, washed with PBS and FACS buffer, and stained with streptavidin-allophycocyanin antibody at 4 °C for 30 min. Samples were then thoroughly washed with FACS buffer and analyzed on a FACSCalibur.

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We performed a standard LPS binding assay with biotinylated LPS followed by streptavidin-conjugated FITC to detect bound LPS on the cell surface (Fig. 2). Strikingly, we found that LPS was able to bind to both WT and gp96-null pre-B cells, and this binding was significantly inhibited by PIER1 (Fig. 3). Because gp96 mutant pre-B cells do not express cell surface gp96, TLR4-MD-2, or CD14 (18–20), we conclude that the surface binding by LPS must be mediated by other LPS-binding proteins such as HSP90 but not gp96.

PIER Peptide Binds to HSP90—We next focused on the possibility of PIER1 to inhibit LPS binding to HSP90 as previous studies have suggested that cell surface HSP90 is another LPS-binding protein for TLR4 signaling (14–17). Additionally, a sequence alignment between HSP90 and gp96 of both human and mouse origin demonstrated that the target sequence of
PIER1 peptide is highly conserved, especially in the first half of the HSP90 sequence. We performed a direct binding assay by incubating purified HSP90 with biotin-LPS in the presence or absence of PIER1. After a 30-min incubation period, we pulled down the HSP90/LPS complex with streptavidin beads followed by SDS-PAGE analysis. We found that HSP90 could directly bind to LPS, and this binding was significantly inhibited by 5 μM PIER1 (Fig. 4).

As proposed by the Kliger et al. (24) study, the design of PIER1 peptide is based on a potential helix-helix interaction between PIER1 and the target sequence in the HSP that requires a conformation-dependent interaction. To examine this possibility, we designed another peptide, PIER2, by substituting the middle two residues in the helix of PIER1, Val and Arg, with two Pro residues. We hypothesized that the inflexible backbone of two Pro residues will kink the helix-loop-helix structure of PIER1 peptide, therefore abolishing its inhibitory effect. In addition, we designed PIER3 peptide based on the target region of HSP90 (Fig. 5A).

We tested the efficacy of these two peptides in the same murine pre-B cell line as described earlier. As predicted, PIER3 was equally effective in inhibiting LPS-mediated NFκB-GFP activation. Introduction of Pro to PIER1 (PIER2) completely abolished the inhibitory effect of PIER1 (Fig. 5B). These results suggest that PIER inhibits LPS responsiveness by blocking LPS binding in a conformation-dependent manner.

To further determine the roles of cell surface HSP90 in LPS signaling, we took advantage of a well-characterized cell-impermeable HSP90 inhibitor, dimethylaminomethylamino-17-demethoxygeldanamycin-4-O-oxide (27), also known as NPGA (28). Cells were pretreated with NPGA or vehicle control followed by stimulation with either LPS or a combination of PMA and ionomycin. We found that NPGA specifically inhibits NFκB activity in response to LPS but not to PMA and ionomycin.

### Cell-permeable PIER1 Inhibits Chaperone Function of HSP90

If PIER1 indeed inhibits HSP90 function on the cell surface, we would expect it to have a negative impact on the HSP90 clientele inside the cell. To address this possibility, we generated a
cell-permeable PIER1 by fusing PEIR1 with a TAT peptide, GRKKRRQRRRQP. We found that TAT-PIER1 dose-dependently killed a breast cancer cell line, SKBR3. Importantly, by Western blot, we found that TAT-PIER1 treatment led to degradation of a variety of well known HSP90 clients, including Her2/neu, AKT, CDK2, and p53 (Fig. 6).

Overexpression of CD14 Partially Abolishes Inhibitory Effect of PIER1—Previous studies suggest that HSP90-HSP70 complex on the cell surface contributes preferentially to CD14-independent TLR signaling (15). In our present work, we found that cells without CD14 expression such as pre-B cells and THP-1 cells were more sensitive to PIER1 compared with CD14+ RAW264.7 cells (Fig. 7). Our data are thus consistent with the notion that HSP90 plays more important roles for LPS recognition in cells that do not express CD14 such as epithelial cells, B cells, and hepatocytes. If so, ectopic expression of CD14 should make cells more resistant to PIER1 inhibition. To address this hypothesis, we stably expressed murine CD14 in pre-B cells (Fig. 8). When stimulated with a range of concentrations of LPS, CD14-expressing cells were less sensitive than WT cells to PIER1-mediated NFκB inhibition, particularly at the higher concentration of LPS. We thus conclude that PIER preferentially inhibits CD14-independent LPS recognition on the cell surface.

**DISCUSSION**

Our study has demonstrated that peptide-based inhibitors targeting an N-terminal helix structure of both gp96 and HSP90 can effectively inhibit NFκB responses to LPS but not to other TLR ligands. Further study suggested that these peptides disrupt LPS binding to the cell surface in the absence of TLR4 or CD14.

Our results are novel in several respects. First, we have provided evidence that a peptide-based inhibitor that was previously reported to inhibit gp96 (24) is in fact most likely targeting HSP90. PIER1 inhibited LPS binding to both WT and gp96-null cells. In addition, direct binding of LPS to HSP90 was compromised in the presence of PIER1. More importantly, we demonstrated that PIER1 only inhibits TLR4 function but not that of other TLRs such as TLR2 and TLR9, which is incompatible with...
the claim that PIER1 suppress the function of gp96. Second, we demonstrated that a cell-permeable PIER1 is a novel inhibitor of cytosolic HSP90 and has anticancer activity. Third, because PIER1 does not enter cells readily, our study is consistent with the notion that HSP90, a cytosolic HSP, can indeed be expressed on the cell surface to serve as another important molecule in mediating LPS recognition. This point is further supported by our finding that a cell-impermeable HSP90 inhibitor, NPGA, was able to potently inhibit LPS responsiveness. PIER1 was designed originally to target residues 100–137 of gp96. Based on the consideration above, the analogous region in HSP90, residues 39–77 (FLRELNASDALKDRIKYESLTDPSKLDGKELKIDII), is most likely the target of PIER1. This region is more than 80% identical to the gp96 sequence and appears to bind favorably to PIER1 as well as a corresponding region of the PIER1 sequence on HSP90, PIER3. Fourth, our study also reinforced the notion that the cell surface HSP90-mediated LPS recognition is a dominant pathway in cells that do not co-express CD14. In the case of RAW264.7 or CD14⁺ pre-B cells, the inhibitory effect of PIER1 became marginal. In this context, it is noteworthy that PIER1 has been shown to attenuate sepsis in vivo (15). Together with this finding, we thus speculate that CD14 non-expressors such as B cells, T cells, and non-hematopoietic parenchymal cells are the most important cellular types to mediate endotoxin shock. CD14⁺ cells may be more important in protecting the host and in generating subsequent adaptive immune responses in the presence of the subclinical dose of LPS.

HSP90 is an abundant cytosolic chaperone. However, cell surface expression has been frequently described, particularly in cancer cells where demand for the chaperone is high (29, 30).

Cell surface expression of HSP90 has been implicated in cancer invasion (31), autoimmunity (32), bacteria adhesion (33), and LPS signaling (34). However, the mechanism by which cell surface HSP90 exerts its function in these biological processes remains unclear. In the case of LPS recognition, a direct transfer model in which HSP90-associated LPS is directly transferred from HSP90 in the lipid raft to TLR4/H18528 MD-2 complex is a possibility. However, it is unclear whether such a transfer occurs on the plasma membrane or whether it happens in another endocytic compartment.

Multiple HSP90 inhibitors have been reported to inhibit inflammation and TLR4 responses. For example, EC144, a synthetic HSP90 inhibitor, was shown to block LPS-induced TLR4 signaling in macrophages by inhibiting activation of ERK1/2, MEK1/2, JNK, and p38 MAPK (35). SNX-7081, another small molecule inhibitor of HSP90, can inhibit NFκB in vitro and attenuate a mouse model of rheumatoid arthritis (36). Inhibition of HSP90 by 17-allylamino-17-demethoxygeldanamycin was successful in the treatment of endotoxin-induced uveitis (37). However, these studies utilized HSP90 inhibitors that effectively penetrate the cell membrane, resulting in the global inhibition of HSP90 function. Thus, the inhibition of LPS responsiveness by these inhibitors is exerted not at the ligand binding level on the cell surface but at the level of downstream signaling given the known roles of HSP90 in chaperoning many...
critical kinases in the TLR pathway. In this regard, PIER is a novel class of HSP90 inhibitors that could be used specifically to probe the function of cell surface HSP90 in sepsis as well as in oncogenesis. It is well known that HSP90 clients include molecules in multiple signaling pathways that are crucial for cancer (1, 38–42). Indeed, we found that a cell-permeable PIER has anticancer activity via inhibition of multiple HSP90 clients. Thus, the knowledge we learned from the PIER inhibitors could potentially be applied to develop inhibitors to block and uncover other aspects of HSP90 function. Given the increased appreciation of inflammation in oncogenesis (43), it will be of interest to determine whether more potent PIER inhibitors can be developed to specifically target surface-bound HSP90 for attenuation of both inflammation and cancer invasion as a new generation of cancer therapeutics.

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