gp96-Independent Inhibition of Endotoxin Response By Chaperone-Based Peptide Inhibitors

CHAPERONE-BASED INHIBITORS AGAINST LPS

Shuang Wu¹, Krystal Dole², Feng Hong¹, Abu Shadat M Noman¹, Jennifer Issacs², Bei Liu¹ and Zihai Li¹*

¹Department of Microbiology & Immunology, ²Department of Cell and Molecular Pharmacology & Experimental Therapeutics, Medical University of South Carolina, Charleston, SC 29425.

*Address correspondence to: Zihai Li, M.D., Ph.D., Department of Microbiology & Immunology, Hollings Cancer Center, Medical University of South Carolina (MUSC), Charleston, SC 29425, phone: (843)-792-1034; E-mail: zihai@musc.edu

Background:
gp96 is a master chaperone for TLRs. A gp96-derived peptide (PIER) inhibits LPS response. The underlying mechanism is unclear.

Results:
PIER inhibits LPS binding and signaling, independently of gp96. It targets HSP90 and has anti-cancer activity.

Conclusion:
PIERs inhibit LPS responsiveness and tumor growth in a gp96-independent manner.

Significance:
This study shall facilitate the development of HSP90-based inhibitors to treat inflammation and cancer.

Abstract
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 TLR-chaperone gp96, an endoplasmic reticulum paralogue of HSP90, in fact blocks HSP90-LPS interaction. It inhibits the binding of LPS to the cell surface of both wild type and gp96 null cells, thereby abrogates cellular response to LPS, but not to other TLR ligands. We also generated a series of peptide derivatives (named as peptide inhibitors of endotoxin responsiveness, or PIERs) from the N-terminal helix structure of HSP90 and demonstrated their effectiveness in blocking LPS activity. PIER inhibition of LPS signaling is partially reversed by CD14 expression. Moreover, we find that a cell-permeable PIER abrogates HSP90 function and causes 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.
Introduction
Heat-shock protein 90 (HSP90) is a family of molecular chaperones that are highly conserved in bacteria and eukaryotes (1). The cytoplasmic form of HSP90 includes HSP90AA1 and HSP90AB1, known also as HSPC1 and HSPC3 respectively (2). There exists several paralogs of HSP90 in other cellular compartments, including the endoplasmic reticulum (ER) gp96 (also known as grp94, HSPC4) and mitochondria TRAP-1 (HSPC5). Cytosolic HSP90 chaperones a large number of clients, and it is essential for organism survival as demonstrated with yeast. As a member of the GHKL (Gyrase, HSP90, Histidine Kinases and MutL) super family, HSP90 adopts a “V” shaped parallel homodimer structure (3) comprising of the N-terminal ATPase domain, the relative flexible charged middle domain, followed by the C-terminal dimerization domain. The ATPase activity is conserved among all its orthologs and paralogs and is critical for its role as a molecular chaperone. The ATP hydrolysis cycle of HSP90 is coupled with the binding and releasing of clients and is critical for its chaperon function.

LPS or endotoxin is one of the most potent microbial products that can stimulate robust host inflammatory responses (4). Study of a spontaneous LPS-resistant C3H/HeJ mouse strain led to the discovery of TLR4 as the receptor for LPS (5). The dimer of TLR4 forms a complex with two MD-2 molecules (6). The binding of LPS by MD-2 brings the two protomers of TLR4 together, which triggers conformational changes in the cytoplasmic TIR domains of TLR4 to activate downstream signaling. TLR4 is the only TLRs that utilize all four known adaptors: MyD88, TRIF, TRAM and TIRAP (7,8). The signaling of TLR4 is regulated spatially and temporarily. LPS binds to TLR4/MD-2 complex on the cell surface, which then engages TIRAP and MyD88 to initiate the proinflammatory pathway. LPS-bound TLR4/MD-2 complex can also be endocytosed and transported into the endolysosome compartment (9). It is in the endolysosome that TIR domains of TLR4 binds to TRAM and TRIF, which initiates the late NFkB signaling. The event in endolysosome also triggers signaling of IRF pathways, leading to the production of type I IFNs (10).

Two other molecules are known to play important roles in LPS binding: LPS-binding protein (LBP) and CD14. LBP is a soluble protein that helps to extract LPS from Gram-negative bacterial cell walls (11). Mostly expressed as a membrane bound form on cells of myeloid lineages, CD14 is a glycosylphosphatidylinositol (GPI)-linked membrane protein (12). Without a signaling tail, CD14 functions as a co-receptor by transferring LPS from LBP 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 a 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 ER paralog of HSP90, gp96 is the master chaperone for TLRs (18-20). With the exception of TLR3, all the rest of TLRs are exclusively dependent on gp96 for folding and functional expression, despite the abundance of other HSPs in the ER (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). gp96 therefore appears to be an attractive drug target for inflammation, sepsis and autoimmune disease. Utilizing in silico methods, a recent report designed a peptide inhibitor of gp96 by targeting the N-terminal helix-loop-helix sequence, and 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 preB cells 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 pumo-murine CD14 (InvivoGen).

*Peptide*

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 shown as below:

PIER1: NH2-LNVSRETLQHKLLKVRKKTLDIMIKKIADDKY-COOH
PIER2: NH2-LNVSRETLQHKLLKVRKLPKTLDMIKKIADDKY-COOH
PIER3: NH2-LNISREMLQQSKILKVRKNIVKCCLELFSELAEDKEN-COOH
PIER4 (Tat-PIER1): GRKKRRQRRRPQ-PIER1

*Reagents*

Biotin-LPS was obtained from InvivoGen. Purified human HSP90 was purchased from Enzo Life Sciences. Streptavidin-APC secondary antibody was purchased from eBioscience. The non-permeable geldanamycin (NPGA), known also as DMAG-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 hours followed by stimulation with LPS for 24 hours 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).

*NFκB GFP reporter assay*

As described previously (18), all cells are derived from E4.126 parental cell line which contains 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 hr before FACS instrumentation. To determine the roles of surface HSP90, 1X10^5 full-length gp96 expressing E4.126 cells were pretreated with 20 μM NPGA, a cell-inpermeant HSP90 inhibitor for 1 h, and then stimulated with LPS (200 ng/ml) or P/I (PMA: 50 ng/ml; ionomycin 1 μg/ml) for 5 h, followed by flow cytometric analysis.

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

**Flow cytometry**
All staining protocol, flow cytometry instrumentation as well as data analysis were performed essentially as described without significant modifications (20,26). For cell surface staining, single cell suspension of live cells was obtained and washed in FACS buffer twice. FcR blocking with or without serum was performed depending on individual primary antibody used for staining. Primary and secondary antibody staining was performed stepwise, with FACS buffer washing in between. Propidium iodide (PI) was added right before FACS instrumentation in order 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. Cell permeabilization was done with 0.25% saponin in FACS buffer. The same buffer was used in subsequent steps including blocking, washing and Ab 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 the 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 minutes. The mixture was then incubated with streptavidin agarose beads at 4°C over night. Beads were then washed with Tris lysis buffer and subjected to boiling in SDS loading buffer for 5 min prior to resolving on SDS-PAGE. The intensity of western blot bands was quantified by ImageJ software.

**LPS binding assay to cell surface**
Biotin-LPS stock (500 μg/ml) was diluted in complete RPMI medium to the desired working concentration. About 0.2 million cells were resuspended in 50 μl medium per well in a 96-well plate. Another 50 μl 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 minutes. After the incubation, cells were harvested from the well into cold PBS, washed with PBS and FACS buffer, followed by staining in streptavidin-APC Ab at 4°C for 30 minutes. Samples were then thoroughly washed with FACS buffer and analyzed on a FACS Calibur.

**RESULTS**
**PIER peptide inhibits NFκB response to LPS but not to other TLR ligands**
We synthesized a 27-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 as PIER1. We tested PIER1 activity using a murine preB cell line that stably expresses NFκB-GFP reporter. When stimulated with LPS for 16-18 hrs, untreated preB cell line had a dose-dependent induction of NFκB-GFP (Fig. 1A). Consistent with the Kliger study, we found that concurrent treatment of the cells with 5 μM PIER1 without preincubation significantly suppressed NFκB-GFP in response to LPS.
gp96 is the master chaperone for TLRs including TLR4, TLR2 and TLR9 (19,21). Inhibition of gp96 affects the de novo TLR biogenesis but it is not expected to affect the preexisting mature TLRs, which argues against gp96 being the target of PIER1. To further address this possibility, we also examined the effect of PIER1 on TLR2 and TLR9 signaling using Pam₃CSK₄ and CpG respectively. The folding and assembly of both TLR2 and TLR9 is dependent on gp96 in the ER lumen before transporting to cell surface and endolysosome respectively. If PIER1-mediated suppression of LPS responsiveness were via inhibiting 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. We conclude therefore that PIER1 inhibit LPS responsiveness in a gp96-independent manner.

PIER1 inhibits the LPS response by disrupting the binding of LPS to cell surface

We next examined the effect of PIER1 on LPS binding to cell surface. Given the fact that PIER1 inhibition does not require pre-incubation of cells before adding LPS, we hypothesize that the inhibitory effect of PIER1 is at the upstream level of LPS response, i.e. the binding of LPS to the TLR4-MD2 complex. This consideration is also consistent with the fact that PIER1 is not expected to enter cells readily.

We performed standard LPS binding assay with biotinylated LPS, followed by streptavidin-conjugated FITC to detect bound LPS on cell surface (Fig. 2). Strikingly, we found that LPS was able to bind to both WT and gp96 null preB cells, which was significantly inhibited by PIER1. Since gp96 mutant preB cells do not express cell surface gp96, TLR4/MD2 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 direct binding assay by incubating purified HSP90 with Biotin-LPS in the presence or absence of PIER1. After a 30-minute incubation period, we pulled down the HSP90-LPS complex with streptavidin-beads, followed by analysis on the SDS-PAGE. 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 study, the design of PIER1 peptide is based on a potential helix-helix interaction between PIER1 and the target sequence in the HSP, which 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, Leu and Lys, 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 preB 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 non-permeable HSP90 inhibitor DMAG-oxide (27), also known as NPGA (28). Cells were pre-treated with NPGA or vehicle control, followed by stimulation with either LPS or the combination of PMA and ionomycin (PI). We found that NPGA specifically inhibits NFκB activity in response to LPS but not to PI.

Cell-permeable PIER1 inhibits the 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, GRKRRQRRRQP. 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).

Over expression of CD14 partially abolishes the inhibitory effect of PIER1
Previous studies suggest that HSP90/HSP70 complex on cell surface contributes preferentially to CD14-independent TLR signaling (15). In our present work, we found that cells without CD14 expression such as preB cells and THP-1 cells were more sensitive to PIER1 compared to CD14+ RAW264.7 cells (Fig. 7). Our data is 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 preB cells (Fig. 8). When stimulated with a range of concentration 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 cell surface in the absence of TLR4 or CD14.

Our results are novel in several aspects. 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 inhibits LPS binding to both WT and gp96 null cells. In addition, direct binding of LPS to HSP90 is 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 anti-cancer property. Third, since PIER1 does not enter cells readily, our study is
consistent with the notion that HSP90, a cytosolic HSPs, 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 non-permeable 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, most likely, the analogous region in the HSP90, residues 39-77 (FLRELISNASLDALKIRLISLTDENALSGNEELTVK IK), is 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 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+ preB 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 maybe more important in protecting the host and in generating subsequent adaptive T 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 when demand for 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-MD2 complex is a possibility. However, it is unclear if such a transfer occurs on the plasma membrane, or if it happens in other 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-demethoxy-geldanamycin (17-AAG) 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 anti-cancer activity via inhibiting 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 interests to determine if more potent PIER inhibitors can be developed to specifically targeting surface-bound HSP90 for attenuating both inflammation and cancer invasion as a new generation of cancer therapeutics.

Acknowledgements
This work was supported in part by National Institutes of Health grant AI070603 and AI077283 (Z.L.), and by a pilot research funding from Hollings Cancer Center’s Cancer Center Support Grant P30 CA138313 at the Medical University of South Carolina. We thank past and present members of the Li laboratory for insightful discussions and generous technical advice. We are grateful to Drs. Adam Adler and Pramod Srivastava for helpful input.
References


27. Tsutsumi, S., Scroggins, B., Koga, F., Lee, M. J., Trepel, J., Felts, S., Carreras, C., and


Figure 1. PIER1 specifically inhibits responses to LPS but not to Pam3CSK4 or CpG. A, PIER1 inhibits LPS responses in THP-1 cells. THP-1 cells were matured with PMA prior to LPS stimulation in the presence or absence of PIER1. Supernatant was harvested at 24 hr and examined for TNFα by ELISA. B, PIER1 inhibits LPS responses in preB cells. preB cells stably expressing NFκB GFP reporter were stimulated by LPS for 16-18 hrs. GFP expression was examined by flow cytometry. MFI was normalized to percentage of maximum 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.
Figure 2. PIER1 inhibits the binding of LPS to cell surface independently of gp96. WT or gp96 mutant (KO) preB cells were incubated with Biotin-LPS in the presence (red) or absence (blue) of PIER1, followed by flow cytometry for LPS-binding cells with strepavidin-APC. Data are presented as both dot plots and histograms. Numbers indicate the percentage of LPS-binding cells. Multiple experiments were performed with similar results.
**Figure 3.** Dose-dependent inhibition of LPS binding by PIER1. LPS binding was performed as in Fig. 2 for both WT (A) and gp96 mutant preB cells (B) in the presence of increasing concentration of PIER1 or control peptide. Percentage of LPS-binding cells was plotted. Two experiments were performed with similar results.
Figure 4. PIER1 inhibits the interaction between LPS and HSP90 in vitro. Purified HSP90 was incubated with Biotin-LPS with or without PIER1 peptide (5 μM) prior to incubating with streptavidin-beads. Beads were then washed and boiled. The elutes were resolved on SDS-PAGE followed by immunoblotting for HSP90. The density of HSP90 bands were determined by ImageJ software and normalized to the second lane.
Figure 5. Inhibition of LPS responsiveness by HSP90-based inhibitors. A, Sequence alignment of PIER peptides. B, WT preB cells were stimulated with LPS in the presence of various PIER peptides at 5 μM for 16-18 hrs. MFI of NFκB-GFP was normalized as in Fig. 1B. Experiments were repeated for at least three times with similar results. *p<0.05. C, WT gp96-expressing preB cells were pretreated with 20 μM NPGA for 1h, and then stimulated with LPS (200 ng/ml) or PI (PMA: 50 ng/ml; ionomycin 1 μg/ml) for 5 h, followed by flow cytometric analysis for GFP (open histogram). The shaded histograms represent GFP of unstimulated cells.
Figure 6. Cell-permeable PIER1 has anti-cancer effect and induces degradation of HSP90 client proteins. A, Killing curve of breast cancer cell SKBR3 by Tat-PIER1 and control peptide after 48 hrs. B, SKBR3 cells were treated with PIER peptides for 12 hrs, followed by Western blot analysis of HSP90 client proteins.
Figure 7. CD14\textsuperscript{high} RAW264.7 cell is less sensitive to PIER1-mediated inhibition of LPS binding and cytokine production. A, TNF\textalpha intracellular stain in response to LPS or PI in the presence or absence of PIER1. Number represents percentage of TNF\textalpha producing cells. B, RAW264.7 cells were incubated with Biotin-LPS in the presence or absence of PIER1, followed by flow cytometry for LPS-binding cells with streptavidin (SA)-APC. Number represents percentage of LPS-binding cells.
Figure 8. Enforced expression of CD14 partially abolished the inhibition of PIER1 peptide. A, CD14 expression (open histogram) on preB cells by flow cytometry. Filled grey histogram represents staining with isotype control Ab. B, and C, WT (B) or CD14 transduced preB cells (C) were stimulated with LPS in the absence or presence of PIER1 peptide (5 μM) for 16-18 hrs. NFκB GFP was detected by FACS. Absolute value of MFI was shown. One representative data from multiple experiments is shown.