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Heat Shock Proteins gp96 and hsp70 Activate the Release of Nitric Oxide by APCs¹

Naveed N. Panjwani,* Lana Popova,* and Pramod K. Srivastava^{2†}

NO is a cytotoxic and immunomodulatory cytokine produced by macrophages and dendritic cells. We show that stimulation of murine and human macrophages with the heat shock proteins gp96 and hsp70 results in induction of inducible NO synthase and the production of NO. The release of NO by monocytes exposed to hsp60 has been documented previously. Immature, but not mature, dendritic cells respond in the same manner. The activity of heat shock proteins is relatively unaffected by an antagonist of LPS, and is abrogated by heat denaturation. Macrophages have been shown previously to produce NO in response to stimulation with IFN- γ ; stimulation of macrophages with mixtures of IFN- γ and gp96 or hsp70 leads to a synergistic production of NO. The present observations extend the roles of these heat shock proteins in innate immune responses to another potent and highly conserved function of APC. *The Journal of Immunology*, 2002, 168: 2997–3003.

Heat shock proteins (HSP)³ gp96, hsp90, and hsp70 elicit protective immune responses against challenge with the antigenic context from which they are isolated (1–4; reviewed in Ref. 5). This immunogenicity results from the receptor-mediated uptake (6–8) and processing and re-presentation of HSP-associated peptides by MHC I molecules of APCs to cognate T cells (9–11). HSP-APC interaction also results in the activation of a proinflammatory program of gene expression, culminating in the production of cytokines TNF- α , IL-12, IL-1 β , and GM-CSF and the up-regulation of Ag-presenting and costimulatory molecules such as MHC II and CD86 (12–15). This latter phenomenon is independent of antigenic context. The highly abundant HSPs are released upon necrotic, but not apoptotic, cell death, and the proinflammatory activity of HSPs may constitute a mechanism for the immune system to perceive necrotic events, putting it in a state of molecular alert to be able to respond to simultaneous antigenic context (12).

In the present study, we show that interaction of HSPs gp96 and hsp70 with APCs results in a potent and highly conserved immunological phenomenon, i.e., production of NO by APCs. The release of NO by monocytes exposed to hsp60 has been documented previously (15). HSPs gp96 and hsp70 stimulate murine and human macrophage and dendritic cells (DC) to induce the expression of inducible NO synthase (iNOS) and the consequent production of NO. This aspect of HSP-APC interaction is yet another example of the increasingly emergent pattern in which HSPs function as the mammalian body's internal agents for activation of APCs.

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³ Abbreviations used in this paper: HSP, heat shock protein; DC, dendritic cell; BMDC, bone marrow-derived DC; iNOS, inducible NO synthase; N-MMA, *N*-monomethyl arginine.

Materials and Methods

Cell lines, reagents, and media

The RAW264.7 cell line (ATCC TIB-71) and U937 cells (ATCC CRL 1593.2) were obtained from American Type Culture Collection (Manassas, VA). Cell lines were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (HyClone, Logan, UT), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies) at 37°C in 5% CO₂. *N*-monomethyl arginine (N-MMA), *Escherichia coli* 0128 strain LPS, BSA, and PMA were purchased from Sigma-Aldrich (St. Louis, MO). Murine recombinant IFN- γ and human recombinant IFN- γ were purchased from Calbiochem (La Jolla, CA) and Pierce-Endogen (Woburn, MA), respectively. U937 monocyte cells were differentiated into macrophage stage cells by treatment with PMA at 25 ng/ml for 72 h at 37°C in 5% CO₂. Macrophage stage cells were harvested by trypsinization and washed to remove PMA before assay. Differentiation was confirmed by adherence phenotype and up-regulation of CD36 differentiation marker (16) using FA6.152 anti-human CD36 mAb (Immunotech, Marseilles, France).

Purification of HSPs, testing for LPS and FITC conjugation

gp96 and hsp70 were prepared from pooled livers and kidneys of C57BL/6 mice as previously described (17). All glassware used in HSP preparation was previously depyrogenated. The LPS content of preparations was assayed using the *Limulus* ameocyte lysate assay kit (BioWhittaker, Walkersville, MD). gp96, hsp70, and histone (type III-SS; Sigma-Aldrich) were conjugated with FITC using the FluoroTag kit (Sigma) according to the manufacturer's protocol, using a 20:1 FITC monomer:protein molar ratio.

Preparation of bone marrow dendritic cells (BMDC)

BMDC were generated as previously described (18). Briefly, femurs from 8- to 12-wk-old female C57BL/6 mice were flushed, and the bone marrow cells recovered were incubated with 20 ng/ml GM-CSF (Pierce-Endogen) for 6 days, followed by transfer of loosely adherent cells to fresh plates and recovery of nonadherent cells on day 7. This population was highly enriched for CD11c⁺ cells as determined by FACS analysis. Mature DC were prepared by the addition of 100 ng/ml LPS to the freshly transferred day 6 culture described above, followed by recovery of nonadherent cells on day 7. LPS was removed by washing mature DCs before assay.

Preparation of primary peritoneal macrophages

Female C57BL/6 mice (8–12 wk old) were injected i.p. with 0.2 ml Pristane (Sigma-Aldrich). Peritoneal exudate cells were recovered 5 days postinjection by peritoneal lavage with DMEM. Peritoneal macrophages were purified by adherence to plastic for 4 h before assay.

Assay for NO

RAW264.7, macrophage or monocyte stage U937 cells, or adherence-purified peritoneal macrophages were incubated at 1×10^5 cells/well in 96-well plates along with increasing quantities of gp96, hsp70, or control

stimuli as indicated in DMEM (Life Technologies) supplemented with 10% FCS (HyClone), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Life Technologies) for 24 h at 37°C in 5% CO_2 . Cell-free supernatants were recovered after incubation, and NO production was analyzed as NO_3^- and NO_2^- concentrations using an enzymatic colorimetric NO assay (Oxford Biomedical, Oxford, MI) according to the manufacturer's protocol.

Immunoblotting for iNOS

Adherence-purified peritoneal macrophages were stimulated with gp96 and control stimuli as indicated for 24 h at 37°C in 5% CO_2 . Stimulated cells were lysed in 1% Triton X-100 containing lysis buffer supplemented with 200 μM PMSF, 5 μM leupeptin, and 10 μM E64 protease inhibitors. Lysates were separated by SDS-PAGE, followed by immunoblotting with rabbit anti-mouse iNOS polyclonal Ab (Calbiochem). The blot was stripped and reprobed with an anti-mouse β -actin mAb to ascertain equal loading of all lanes.

Results

Murine and human macrophage release NO upon stimulation with HSPs

Homogenous preparations of the HSPs gp96 and hsp70 were purified from the livers and kidneys of C57BL/6 mice under near-GMP conditions. RAW264.7 cells (*d* haplotype) of murine macrophage origin were incubated with increasing quantities of gp96 or hsp70, or with LPS as a positive control. NO was produced in response to stimulation with either HSP in a dose-dependent manner (Fig. 1A) and upon stimulation with LPS (Fig. 1B). The generation of NO via iNOS has been shown previously to be inhibited

by the L-arginine analog N-MMA (19). The production of NO induced by gp96, hsp70, or LPS was also inhibited by N-MMA (Fig. 1A). NO generation was not elicited by an irrelevant protein such as BSA at comparable protein concentrations.

The possibility that LPS contamination of HSP preparations was responsible for the activity was addressed in three independent ways (Fig. 1C). All HSP preparations were tested for LPS content by *Limulus* amoebocyte lysate assay and were found to have no detectable LPS up to the sensitivity limits of the assay (≤ 0.01 endotoxin U/ml). Heat denaturation of HSPs abolished the ability of HSP to elicit NO production (85% inhibition); heat denaturation of LPS had no effect on its ability to elicit NO production. RSLP, a component derived from *Rhodopseudomonas spheroids* (20), has been shown to be a competitive inhibitor of LPS. RSLP significantly inhibited LPS-elicited NO production (75% inhibition), but did not have a significant effect on gp96-induced NO release (7% inhibition; Fig. 1C). RSLP did cause significant, but limited, inhibition of hsp70-elicited NO production. Approximately 50% activity of hsp70 was retained in the presence of RSLP; this was not the case with LPS. Also, in this particular assay the inhibition by RSLP was tested at a high hsp70 concentration; at lower concentrations, RSLP inhibited the activity of hsp70 more modestly (data are not shown, as they were parts of other experiments). The abrogation of the activity of hsp70, but not LPS, by boiling also substantiates the lack of contribution of LPS in the activity of hsp70.

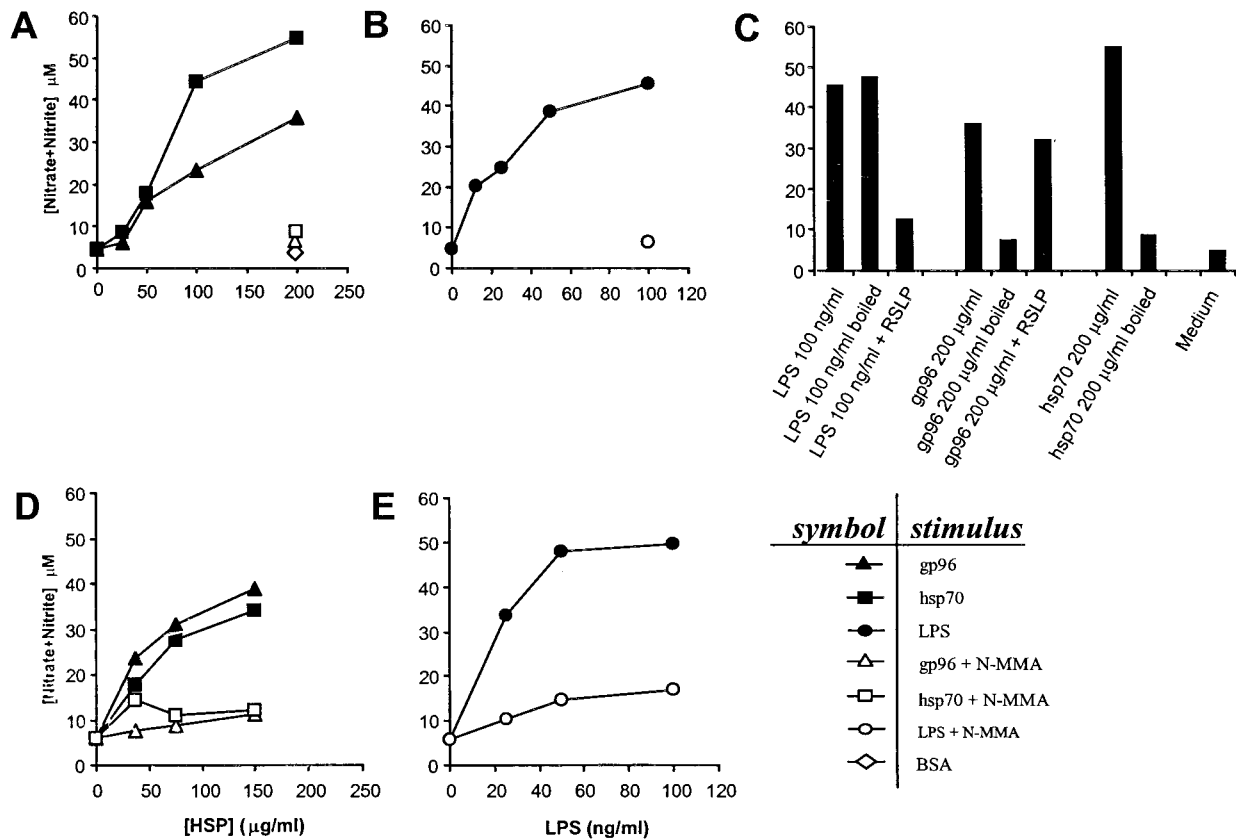


FIGURE 1. Murine macrophage cell lines and primary murine peritoneal macrophages release NO upon stimulation with HSPs. RAW264.7 were exposed to the indicated and titrated quantities of gp96 and hsp70 (A) or LPS (B) for 24 h in the presence and the absence of 2 μM N-MMA. Supernatants were recovered, and NO production over the dose range, as measured by the concentrations of nitrate and nitrite ions, was plotted. C, RAW264.7 cells were stimulated with the indicated quantities of native or heat-denatured gp96, hsp70, or LPS or with native gp96, hsp70, or LPS in the presence or the absence of 1 $\mu\text{g}/\text{ml}$ RSLP. NO was measured as described in A and B. D and E, Output of NO by adherence-purified primary peritoneal macrophages incubated for 24 h with increasing doses of gp96, hsp70, or LPS in the absence or the presence of 2 μM N-MMA. Heat-denatured gp96 or hsp70 was ineffective in mediating NO production, while heat-treated LPS was unaffected in its ability (data not shown). The data shown for all experiments are representative of four or more independent experiments.

The ability of primary macrophages to release NO in response to HSPs was evaluated. Pristane-induced, adherence-purified, primary mouse peritoneal macrophages were incubated with increasing quantities of the HSPs gp96 and hsp70. As with the mouse macrophage cell line RAW264.7, a dose-dependent, N-MMA-sensitive induction of NO output was observed with gp96 or hsp70 (Fig. 1D). LPS also induced NO release from primary cells (Fig. 1E).

Human myeloid U937 cells were tested for their ability to generate NO in response to HSP stimulation. The nonadherent human monocyte cell line U937 can be differentiated to an adherent macrophage-like state by treatment with PMA (21), with a concomitant increase in the expression of HLA-DR, CD36, and the macrophage marker CD11b (16, 22, 23). U937 monocyte cells were induced to

differentiate into the macrophage stage by addition of PMA, and differentiation was monitored by increased expression of CD36 (Fig. 2A). Equal numbers of PMA-treated U937 macrophages or untreated U937 monocytes were incubated with increasing quantities of gp96, hsp70, or LPS. gp96 and hsp70 induced a dose-dependent increase in NO output in both macrophage and monocyte stage U937 cells (Fig. 2B); however, the production of NO was markedly greater from macrophage stage cells in the case of both HSPs. In contrast, the macrophage and monocytic stages of U937 responded equally effectively to LPS (Fig. 2C).

The observations in Fig. 2B gave rise to the possibility that the differences in response of macrophage stage and monocytic stage U937 myeloid cells to HSP stimulation result from quantitative differences in cell surface binding of HSPs by the two cell types.

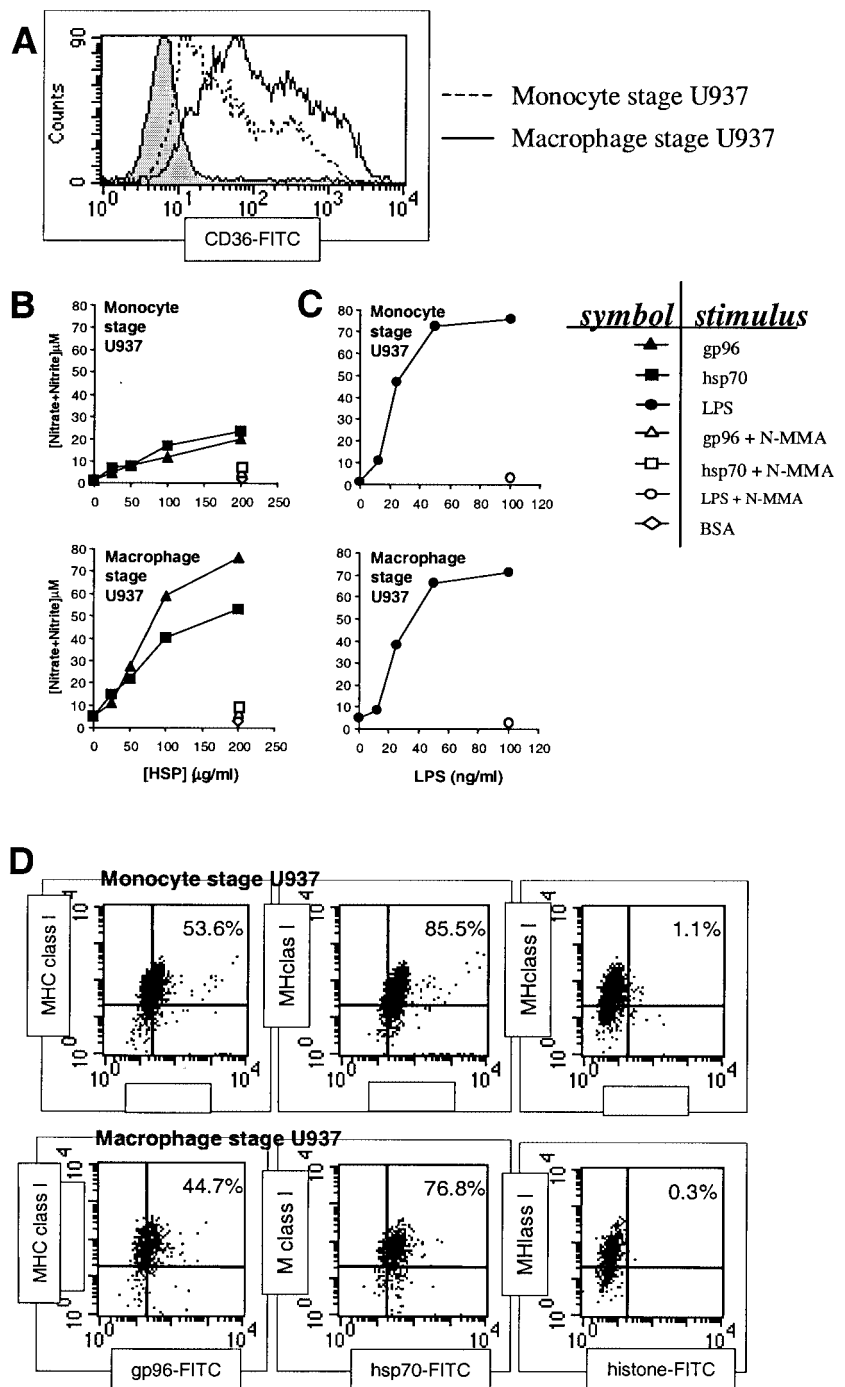


FIGURE 2. Human myeloid cell lines release NO in monocyte and myeloid differentiation states. *A*, Differentiation of U937 cells upon PMA treatment was confirmed by staining for the CD36 differentiation Ag. The solid line corresponds to expression on PMA-treated cells, and the dashed line corresponds to expression on untreated cells. *B* and *C*, U937 monocyte cells or PMA-differentiated U937 macrophage cells were exposed to increasing quantities of gp96, hsp70, or LPS. Supernatants were recovered and assayed for NO production over the dose range, as measured by the concentrations of nitrate and nitrite ions. As observed with RAW264.7 cells, HSP- and LPS-induced NO production by U937 monocyte and macrophage stage cells was sensitive to N-MMA treatment. Heat treatment of HSPs, but not LPS, resulted in a loss of induction of NO production by U937 cells, indicating that LPS contamination was not contributing to HSP activation of NO (data not shown). In addition, the inclusion of the LPS antagonist RSLP inhibited the ability of LPS, but not gp96, to stimulate NO from cells at both stages of U937 cells (data not shown). *D*, The effect of maturation of U937 cells on HSP binding was assessed by incubating monocyte and macrophage stage cells with 100 μ g/ml FITC-conjugated gp96, hsp70, or histone. FACS analysis revealed no increase in binding of either HSP to macrophage stage cells relative to monocyte stage cells. Cells were costained with anti-MHC class I Abs as a nonvariant marker between the two differentiation stages.

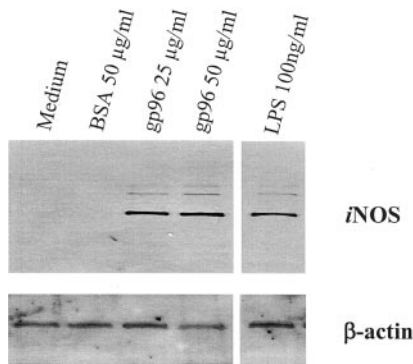


FIGURE 3. Induction of expression of iNOS protein in primary peritoneal macrophages by gp96. Adherence-purified peritoneal macrophages were incubated with medium alone or the indicated concentrations of BSA, gp96, or LPS for 24 h. Treated cells were harvested, lysed, and subjected to SDS-PAGE and immunoblotting using a rabbit anti-mouse iNOS polyclonal Ab (see *Materials and Methods*). Staining resulting from stripping and reprobing of the blot for β -actin is shown as a loading control.

However, FACS analysis of gp96-FITC binding showed no increase in the ability of macrophage stage cells to bind gp96 or hsp70 relative to monocyte stage cells (Fig. 2D), suggesting that differences in downstream signaling events between the two cell types might instead be responsible.

gp96-APC interaction induces iNOS

HSP-stimulated induction of the enzyme responsible for NO production by mouse macrophages was studied at the protein level. Primary mouse macrophages were incubated for 24 h with LPS, BSA, or gp96 at the concentrations indicated or in medium alone. Treated cells were lysed and subjected to SDS-PAGE, immunoblotting, and probing with an anti-mouse iNOS polyclonal Ab (see *Materials and Methods*). Induction of iNOS protein was observed in cells stimulated with LPS or gp96, but not in those stimulated with comparable quantities of BSA or in cells incubated with medium alone (Fig. 3). Immunoblotting of the same extracts, performed with a mAb to β -actin, confirmed that cell lysates from equivalent numbers of cells were applied to each lane.

Differential NO response by matured and nonmatured DCs upon stimulation with HSPs

The ability of BMDCs to produce NO in response to stimulation with gp96 was tested. Nonmatured BMDCs as well as BMDCs that had been exposed to LPS as a maturation signal and thus expressed high levels of MHC II (Fig. 4A) were used in such assays. In nonmatured BMDCs, gp96, but not BSA, stimulated NO release in a dose-dependent, N-MMA-sensitive fashion (Fig. 4B). The LPS-matured BMDCs, on the other hand, showed a high level of N-MMA-sensitive NO release even without stimulation with gp96. Exposing them to gp96 did not further enhance this level of NO release (Fig. 4B). Similar to the data shown in Fig. 1C, heat-

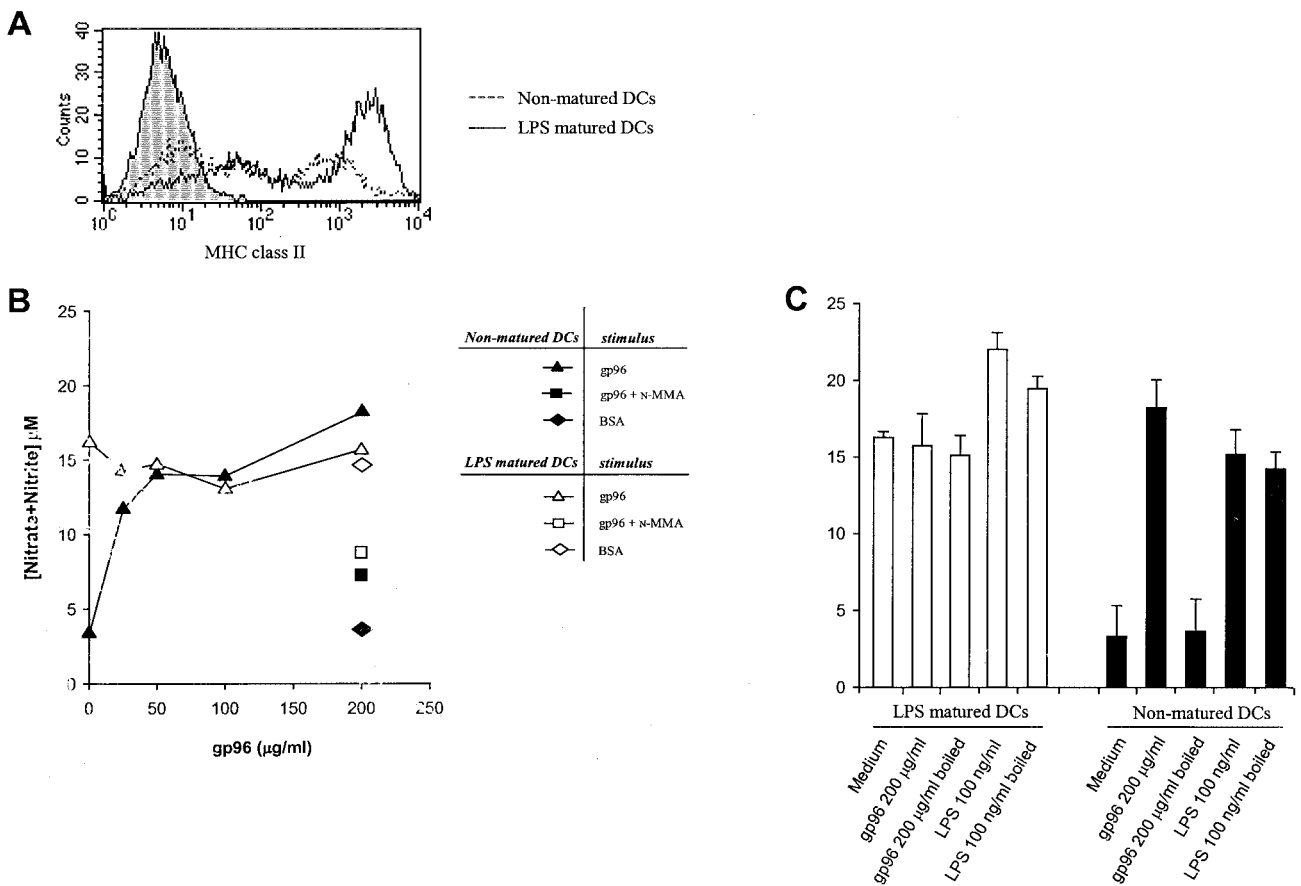


FIGURE 4. Release of NO by HSP-stimulated dendritic cells. *A*, Up-regulation of expression of MHC II on ex vivo generated BMDCs caused to mature by exposure to LPS. *B*, Production of NO by nonmatured and LPS-matured DC incubated with increasing quantities of HSP gp96 in the presence or the absence of 2 μ M N-MMA. BSA is used as a negative control for HSPs. *C*, NO release by LPS-matured and nonmatured BMDC stimulated by native or heat-treated gp96 or native or heat-treated LPS as indicated. Error bars indicate the SD derived from triplicate determinations.

denatured gp96 did not stimulate nonmatured or LPS-matured BMDCs to produce NO; heat-treated LPS was still active in the same assay (Fig. 4C).

Synergistic effect of HSPs and IFN- γ on NO release by APCs

IFN- γ is known to be an inducer of NO production in macrophages and synergizes with cytokine stimuli such as TNF- α and CD40 ligand in this function (24, 25). The effect of HSPs on the release of NO by primary macrophage in concert with IFN- γ was examined (Fig. 5). It was observed that 1) activation of mouse peritoneal macrophages with gp96, hsp70, or IFN- γ resulted in the release of NO; 2) stimulation with gp96 and IFN- γ caused a level of NO release that exceeds the sum of the levels of NO released by either agent alone (Fig. 5A); 3) stimulation with hsp70 and IFN- γ caused a level of NO release that exceeds the sum of the levels of NO released by either agent alone (Fig. 5B); and 4) stimulation with gp96 and hsp70 caused a level of NO release that was synergistic to the sum of the levels of NO released by either agent alone (Fig. 5C). Thus, the two HSPs tested not only synergize with IFN- γ , but also with each other in the stimulation of macrophage NO release. A similar effect was observed with LPS and IFN- γ , as has been reported previously (19) (Fig. 5D).

Discussion

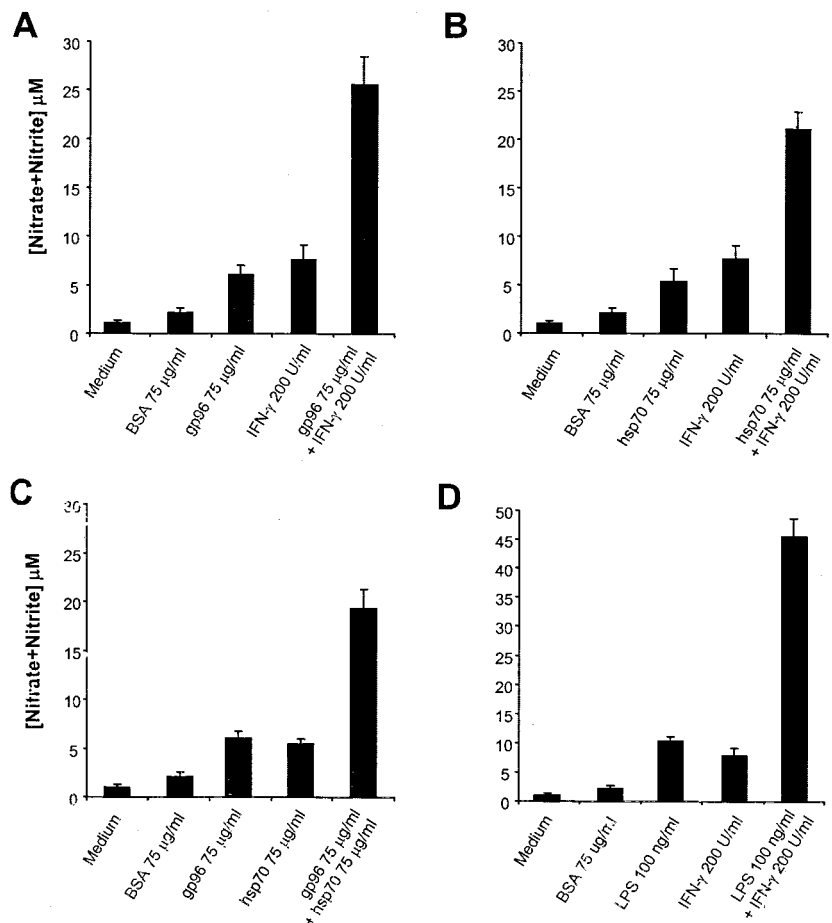
NO is produced by activated macrophages through the enzymatic action of iNOS on L-arginine. The induction of iNOS can be detected in macrophages recovered from individuals undergoing active infection. In vitro, iNOS induction occurs in response to stimuli such as IFN- γ , endotoxin, TNF- α (19), and ligation of CD40 (25, 26). DCs produce NO in response to similar stimuli (27, 28).

In addition to production of TNF- α , production of NO has been shown to be a major tumoricidal mechanism of activated macrophages in vitro (29–31) and in vivo (32, 33). A number of tumor immunotherapeutic protocols can be ablated by pharmacological interference with NO production (34, 35). Macrophage NO production has also been shown to be critical for the control of microbial pathogens such as *Leishmania major* (36, 37) and *Staphylococcus aureus* (38) and viral pathogens such as HSV-1 (39).

NO production is also an immunomodulator of Th cell function. This control is exercised through direct cytostatic and proapoptotic effects of NO, which appear selective for Th1 cells (40, 41), as well as regulation of the agonist (p35)/antagonist (p40) forms (42) of macrophage IL-12 (43–46) and T cell cytokines (47). Regulation of IL-12 agonism also affects Th1 function preferentially, since IL-12 is an inducer of the expression of the Th1 effector cytokine IFN- γ . Such immunomodulatory activity may serve to limit potentially dangerous local cellular immune responses.

We show here that the interaction of the HSPs gp96 and hsp70 with a variety of murine and human APCs and APC cell lines results in the induction of iNOS and the release of NO. HSP-APC interaction has been shown previously to result in the secretion of an array of inflammatory cytokines by macrophages and maturation of DCs (12–15). The results shown here extend that paradigm and add to it in three novel ways. First, they identify NO secretion as a novel consequence of interaction of gp96 and hsp70 with APCs. Interaction of human hsp60 with human monocytes, leading to NO release, has been reported previously (15). Second, our results identify the differentiation status of APCs as being determinative of the NO response upon encounter with HSPs. In the case of monocyte-macrophage differentiation, the macrophage are more

FIGURE 5. Synergy between IFN- γ and gp96, hsp70, and LPS on NO release by primary macrophages. Primary peritoneal macrophages (2×10^5 cells/well) were stimulated with HSPs alone, IFN- γ alone, or combinations of the two (A and B), with a combination of gp96 and hsp70 (C), or with LPS, IFN- γ , or their combination (D). Albumin was used in all experiments as a negative control. Production of NO was measured as described in Fig. 1 legend. Error bars, SD derived from triplicate determinations.



responsive to gp96 or hsp70 than the monocytes. This is not due to quantitative differences in the engagement of a gp96 or hsp70 receptor by either cell type, as both cell types bind comparable quantities of the HSPs. The differences may derive from other factors, such as activation status and signaling components of the two cell types. It is also noteworthy that the macrophage and monocytes respond differentially to HSPs, but not to LPS, suggesting that HSPs and LPS signal through different receptors. Third, our results show that the nonmatured and mature DCs differ markedly in NO production. Nonmatured DCs release little, if any, NO under unstimulated conditions, whereas LPS-matured DCs produce high levels of NO under the same conditions. Gp96 induces nonmatured, but not LPS-matured, DCs to liberate NO.

The production of NO by HSP-activated APC is likely to have a consequence for the innate control of tumors and infectious diseases. APCs patrolling interstitial spaces where necrosis of hypoxic or nutrient-starved tumor cores or lysis of infected cells by cytopathic pathogens have occurred would encounter released HSPs. In addition to providing antigenic information to initiate an effector T cell response against the antigenic context provided, control of the target tumor or infected tissue in this case could be further potentiated by the cytotoxic contribution of NO-producing macrophages that have arisen in proximity to CTL-targeted, HSP-releasing necrotic tissue.

NO release by HSP-activated APC may also provide a layer of immunomodulation of Th cells by necrosis-released HSP. Whereas lower levels of NO are cytoprotective (by inactivation of proapoptotic proteases and up-regulation of Bcl-2) (48–50), higher levels are cytotoxic to T cells, particularly Th1 cells (40, 41, 51). In the case of HSP released from damaged tissue, the concurrent danger of a deleterious immune response being mounted to self-Ags could be prevented by the coinduction of high levels of NO by IFN- γ and HSP, leading to the death of T cells. The context of the IFN- γ produced by infiltrating, autoreactive Th1 cells would therefore be modified away from its usual effector functions by the presence of released HSP and would instead result in NO-mediated Th1 suppression by alteration of the IL-12 p35/p40 balance and direct selective cytotoxic elimination of Th1 cells. This may constitute an ongoing innate mechanism for the avoidance of T cell autoreactivity and autoimmune disease. The involvement of NO in autoimmune disease remains unclear. Interference with NO production in vivo in various models of autoimmune disease has resulted in amelioration or exacerbation of disease (reviewed in Ref. 52). It is possible that the former is a result of prevention of destruction of normal tissues by the cytotoxic actions of NO, whereas the latter may be the result of lifting of immunomodulatory control of potentially destructive T cell activity.

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