Interaction of TLR2 and TLR4 Ligands with the N-terminal Domain of Gp96 Amplifies Innate and Adaptive Immune Responses*

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Activation of dendritic cells by ligands for Toll-like receptors (TLR) is a crucial event in the initiation of innate and adaptive immune responses. Several classes of TLR ligands have been identified that interact with distinct members of the TLR-family. TLR4 ligands include lipopolysaccharide derived from different Gram-negative bacteria and viral proteins. Recent reports have demonstrated the TLR-mediated activation of dendritic cells by heat shock proteins (HSPs). However, doubts were raised as to what extent this effect was due to lipopolysaccharide contaminants of the HSP preparations. We re-examined this issue by testing whether or not HSPs can activate dendritic cells by themselves results in the production of high levels of proinflammatory cytokines, up-regulation of activation markers, and amplification of T cell activation. Our results provide significant new insights into the mechanism of HSP-mediated dendritic cell activation and present a new function of HSPs in the amplification of dendritic cell activation by bacterial products and induction of adaptive immune responses.

HSPs are a family of highly conserved molecules that have the potential to execute several functions. First of all, they act as molecular chaperones involved in the folding of newly synthesized proteins as well as in degradation of misfolded proteins (1, 2). In addition, HSPs have been reported to fulfill immunological functions. These include the ability to associate with peptides derived from intracellular protein degradation and to induce adaptive immune responses against these peptides (3, 4). Based on these features, HSPs have been proposed to be involved in a process called cross-priming, which describes the uptake of antigens by professional APCs, the processing and presentation of these antigens by major histocompatibility complex molecules, and the subsequent activation of naive T cells (5, 6).

A crucial event for this process to take place efficiently is the receptor-mediated interaction of HSPs with professional APCs, first reported in 1999 (7). Only receptor-mediated uptake of HSP-peptide complexes allows peptide representation by major histocompatibility complex molecules (8–10). By now, several receptors on the surface of APCs able to specifically interact with HSPs have been identified. They include the receptor for α2-macroglobulin, CD91 (11, 12), several scavenger receptors such as LOX-1 and CD36 (13, 14), and CD40 ligand (15). In addition, during the incubation of HSP-peptide complexes with bone marrow-derived dendritic cells (BMDCs) for peptide representation, it was observed that this procedure leads to BMDC activation (16–18). This was observed for different members of the HSP family, including Hsp60, Hsp70, Hsp90, and Gp96, and appeared to reflect a peptide-independent feature (19, 20). Evidence from different laboratories suggested that the effect of HSP-mediated activation of professional APCs depends mainly on the presence of functional TLR4, and to a lesser extent, also TLR2 molecules (21–23). This finding immediately raised concerns as to whether or not HSP molecules themselves can act as Toll-like receptor ligands.

PMN, polymorphonuclear neutrophilic granulocytes; GM-CSF, granulocyte-macrophage colony-stimulating factor; FACS, fluorescence-activated cell sorter; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; CTL, cytotoxic T cells; RANTES, regulated on activation, normal T cell expressed and secreted; KC, keratinocyte derived-chemokine; MCP-1, monocyte chemoattractant protein-1.

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§ The abbreviations used are: HSP, heat shock protein; DC, dendritic cells; BMDC, bone marrow-derived dendritic cells; TLR, Toll-like receptor; CFSE, carboxyfluorescein diacetate succimide ester; EU, enzyme units; LPS, lipopolysaccharide; APC, antigen-presenting cell; LAL, limulus amebocyte lysate; FITC, fluorescein isothiocyanate; NTD, N-terminal domain;
toxins are responsible for the effects observed (24). This concern was legitimate because all HSP preparations contained low, but detectable, amounts of endotoxin. Nevertheless, several control experiments, including pretreatment of HSP molecules with polymyxin B, which neutralizes LPS, suggested that HSP molecules and not endotoxins were responsible for the biological activity observed (24). However, modifications of the protocols used for HSP purification were able to dramatically reduce the levels of contaminating endotoxins, and these HSP preparations were not able to activate APCs but retained their chaperone functions as an indication for remaining biological activity (26–28). So we were left with a situation in which endotoxin-free HSPs did not activate professional APCs, but at the same time, concentrations of LPS alone corresponding to those found in activating HSP preparations were also not sufficient to induce activation (29). Inspired by a constant observation during Gp96 purification, in which Gp96-containing fractions always showed 3–5 times higher endotoxin levels as compared with Gp96-free flanking fractions, we hypothesized that Gp96 binds LPS; therefore, LPS could become concentrated in the Gp96-containing fractions. Following this hypothesis, we decided to investigate the possibility that the interaction of Gp96 with LPS augments the biological activity of LPS. The observation that Gp96 molecules can bind LPS in a saturable and competeable manner supported our premise (28). Stimulating DCs with a low concentration of LPS (<1 ng/ml) in the presence or absence of Gp96, we observed a 10–100-fold augmentation of the biological activity of LPS by Gp96 with regard to the production of proinflammatory cytokines, especially IL-12 p70, and the up-regulation of co-stimulatory molecules. When we extended our analysis to the entire spectrum of TLR ligands available to us, we observed that in addition to LPS, Gp96 also dramatically enhanced the biological activity of the TLR2 ligand Pam3Cys. For both ligands, the Gp96 enhanced activation of BMDCs resulted in significant activation of anti-MHC class II, IL-12 p70, and the up-regulation of co-stimulatory molecules. These data provide an explanation for the observed differences in the biological activities of Gp96. More importantly, however, we report here a new function of Gp96 molecules in the amplification of signals induced by TLR2 and four ligands that might be important for an earlier onset of innate immune responses during bacterial infections.

MATERIALS AND METHODS

Reagents—Gp96 purification (Immatics Biotechnologies, Tübingen, Germany) was according to the protocol described (6) with slight modifications (28). In brief, a pellet of the IGELa2 cell line (mouse, B cell myeloma; grown in suspension) was homogenized in hypotonic lysis buffer (30 mM NaHCO3, pH 7.1, containing protease inhibitors) and ultrasonicated. To remove cell debris, the lysate was centrifuged for 1 h at 100,000 × g. The supernatant was sterile-filtered and mixed with sterile phosphate-buffered saline buffer containing potassium and magnesium ions (final concentration 2 mM KCl and 2 mM MgCl2). The cleared lysate was purified by affinity chromatography on Sepharose-coupled concanavalin A (ConA-Sepharose; GE healthcare, Freiburg, Germany). Bound glycoproteins were eluted with phosphate-buffered saline containing 6% (w/v) methyl-α-D-mannopyranoside (Sigma, Taukirchen, Germany), 2 mM MgCl2, and 2 mM KCl. The eluate was loaded on a Mono Q column and further separated by anion exchange chromatography using an ÄKTA explorer fast protein liquid chromatography system (GE Healthcare). Proteins were eluted applying a gradient of increasing NaCl concentration in 5 mM phosphate buffer, pH 7.2. Gp96 elutes in a sharp peak at ~350 mM NaCl. Pooled peak fractions were sterile-filtered (0.22 μm).

To yield the efficient removal of LPS by detergent wash on column, an adapted method described by Reed et al. (28) was applied for the purification of Gp96 used in Fig. 6. All materials were decontaminated prior to use, either by soaking in 70% EtOH, 0.5 m acetic acid or by baking for 4 h at 200 °C. All buffers were made in pyrogen-free water. After loading of the MonoQ column as described above, the column was washed sequentially with 400 ml of TTTE (25 mM Tris-Cl, pH 7.8, 150 mM NaCl, 0.2% (v/v) Tween 20, 0.2% (v/v) Triton X-100, and 10 mM EDTA), 400 ml of 25 mM Tris-Cl, pH 7.8, 150 mM NaCl, and 1% (v/v) Triton X-114, and 100 ml of 25 mM Tris-Cl, pH 7.8, 50 mM NaCl. The purity of Gp96 was >95% as analyzed by Coomassie Blue staining of SDS-PAGE gels. Endotoxin content was determined by limulus amoebocyte lysate assay QCL-1000 (Cambrex, East Rutherford, NJ). The limulus amoebocyte lysate (LAL) assay was performed as determined in the manufacturer’s protocol, which is in accordance with the guidelines published by the U. S. Food and Drug Administration. Bacterial endotoxin catalyzes the activation of a pro-enzyme in the LAL. The Gp96 sample (stock diluted 1:7, 1:49, 1:343) was mixed with the test kit LAL (containing the pro-enzyme) and incubated at 37 °C for 10 min. The synthetic substrate solution was then mixed with the LAL sample, and absorbance was measured at 405 nm. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1–1.0 EU/ml range. As an internal endotoxin standard, we used Salmonella typhimurium LPS (Sigma), which is the same LPS we used for stimulation of the dendritic cells. Endotoxin content of the Gp96 preparations were in a range from 1.7 to 8 EU/mg Gp96 and below 0.5 EU/mg for Gp96 purified using the LPS removal procedure by Reed et al. (28).

Canine GRP94 containing the amino acid residues 22–337 of Gp96 (Gp96.NTD) was purified according to the protocol described by Soldano et al. (53). In short, a Gp96.NTD-6XHis fusion was expressed in Escherichia coli strain BL21. Soluble, monomeric Gp96.NTD was purified by affinity chromatography on Ni2+ agarose (Sigma). To remove contaminating endotoxin, resin-bound NTD was extensively washed in TTTE buffer (25 mM Tris-Cl, pH 7.8, 150 mM NaCl, 0.2% (v/v) Tween 20, 0.2% (v/v) Triton X-114, and 10 mM EDTA) at 4 °C. Following endotoxin removal, Gp96.NTD was eluted by the addition of imidazole buffer, and imidazole was subsequently removed by dialysis against endotoxin-free phosphate-buffered saline. Endotoxin was determined by limulus amoebocyte lysate assay (QCL-1000, Cambrex). The endotoxin content of Gp96.NTD was below 0.5 EU/ml. On SDS-PAGE, Gp96.NTD runs at about 36 kDa; the predicted molecular mass is 32 kDa.

TLR4 agonist LPS (S. typhimurium) was from Sigma. TLR2 agonist palmitoyl-3-Cys-Ser-(Lys)3 (Pam3Cys) was from EMC microcollections (Tübingen, Germany). TLR3 was stimulated...
Stimulation of Cells—After 6 days of GM-CSF-mediated differentiation, mouse BMDCs, human PMN, or the macrophage-like cells RAW264.7, respectively, were seeded out in 96-well culture plates for activation. Cells were incubated with 20 μg/ml Gp96, 10 μg/ml N-terminal domain Gp96 in triplicate wells. To enhance the binding of TLR ligands to Gp96, a preincubation was performed for 1 h at 37 °C before adding them to the cultures. After 16–20 h of activation, supernatants were collected for analyses. Cells were fed and used for FACS analysis 48 h after stimulation.

Detection of Cytokines—Cytokines were measured in the cell culture supernatant by standard ELISA protocols or by the Luminex detection system. The Multicytokine Beadle kits were purchased from Biomerin, Hamburg, Germany and produced by Upstate Biotechnology (Charlottesville, VA). They were used according to the manufacturer’s protocol. IL-8 production by human PMN was assessed by ELISA (R&D Systems, Wiesbaden, Germany). For analyses of mouse cytokines by ELISA, all antibodies and recombinant standards were from Pharmingen. The capture antibody was bound onto the Maxisorb (NUNC, Roskilde, Denmark) assay plate overnight at 4 °C. The amount of biotinylated detection antibody was determined using streptavidin-conjugated horseradish peroxidase, an enzyme that interacts with the 3,3′,5,5′-tetrachlorobenzidine liquid substrate (Sigma). After stopping of the reaction with 2 mM H2O2, the assay was read out at an absorbance of 450 nm using the SpectraFluorPlus reader from Tecan (Crailsheim, Germany).

Stimulation of T Cell Receptor Transgenic CD8+ T Cells in Vitro—Day 6 GM-CSF differentiated immature BMDCs were activated with 25 μg/ml Gp96, 1 ng/ml LPS, 10 ng/ml Pam3Cys, Gp96/LPS, Gp96/Pam3Cys in 24-well plates for 12 h. Preincubation of stimuli was as described above; the concentration was equal to the single stimuli. 100 pm GSP peptide (H2-D6-restricted Ad5-E1a234–243) was loaded onto the DCs for 1 h at 37 °C and then washed off cautiously but extensively. T cell receptor transgenic T cells from the spleen of a St35 were labeled with 2.5 μM carboxyfluorescein diacetate succimide ester (CFSE; Molecular Probes, Eugene, OR) for 4 min at 37 °C in the dark. The DC:T cell co-culture was performed in 48-well plates at a ratio of 1:60. After 2 and 3 days, the cells were analyzed by flow cytometry. Expression of surface activation markers (CD25, CD62L, CD44) was analyzed on viable (propidium iodide-negative) and CD8-positive cells. In a separate staining reaction from the identical sample, intracellular IFN-γ production was determined. In both assays, the T cells were CFSE-labeled to monitor cell division. To measure the cytokine intracellularly, the cells were restimulated with 1 μM peptide plus 1 μg/ml brefeldin A (Sigma) to prevent secretion for 4 h. After CD8-PerCP staining of the surface, the cells were washed and incubated in Cytofix/Cytoperm solution (Pharmingen) for 30 min. Washing and intracellular staining (IFN-γ-APC) steps thereafter were in 0.1% Saponin (Sigma)/0.5% bovine serum albumin (Roth) buffer.

FACS Analysis—We performed all flow cytometric analyses with a FACSCan flow cytometer and FACS Diva software (Pharmingen).

with Poly(I:C) (Amersham Biosciences, Freiburg, Germany), TLR7 with R-848 from InvivoGen (Toulouse, France), and TLR9 with the phosphoethanolamine-conjugated oligonucleotide CpG 1668 from TIB Molbiol (Berlin, Germany). Recombinant human LPS-binding protein and the recombinant soluble form of human CD14 were purchased from R&D Systems (Minneapolis, MN). The peptide SGPSNTPPEI was synthesized by N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry and kindly provided by Stefan Stevanovic (Tübingen, Germany). The following monoclonal antibodies were used for analyses by flow cytometry. Anti-mouse CD8-APC-Cy7, CD8-PerCP, CD25-PE-Cy7, CD44-APC, CD11c-APC, CD14-FITC, CD80-FITC, CD86-PE, and CD40-FITC were obtained from Pharmingen. Anti-mouse IFN-γ-APC was from Immunotools (Friesoythe, Germany).

Mice—C57BL/6 and BALB/c mice were obtained from the local animal facility of the University of Mainz. TLR2-/- mice, generated by Tularik, Inc. (South San Francisco, CA), were provided by Hermann Wagner (Technical University, Munich, Germany). TLR4-defective BALB/c mice, which express the LPSα allele from the LPS hyporesponsive strain C3H/HeJ (30), were provided by Chris Galanos (Max Planck Institute for Immunobiology, Freiburg, Germany). St35 mice (C57BL/6 background) are transgenic for a T cell receptor recognizing the peptide SGPSNTPPEI (SGP) from the adenovirus Ad5 E1a protein (amino acids 234–243), presented on H2-D6. They were cross-bred with B6.SJL-ptpcrac/BoCrTac(KO)RAG2 N10 (RAG2-/-, Ly5.1) (Taconic, Germany). The following monoclonal antibodies were used for analyses by flow cytometry. Anti-mouse CD8-APC-Cy7, CD8-PerCP, CD25-APC, CD3-PE, CD40-APC, CD44-APC, CD11c-APC, CD14-FITC, CD80-FITC, CD86-PE, and CD40-FITC were obtained from Pharmingen. Anti-mouse IFN-γ-APC was from Immunotools (Friesoythe, Germany).

Purification of Human PMN—Human polymorphonuclear neutrophilic granulocytes (PMN) were purified from heparinized peripheral blood from healthy volunteer donors as described previously using Polymorphrep™ (Nycomed, Oslo, Norway) (29). Contaminating red blood cells were removed by a hypotonic lysis step (150 mM ammonium chloride, 1 mM potassium bicarbonate, 0.1 mM ethylene diamine tetra acetate (all from Sigma) in distilled water, pH 7.3). Culture medium was Iscove’s modified Dulbecco’s medium supplemented with 3% fetal calf serum. Purity of cells was assessed by cytofluorometry, yielding 95–98% of CD66b-positive PMN.

Cell Culture—Mouse immature DCs were generated from bone marrow according to standard protocols (21, 31). Minor modification included full replacement of culture medium containing GM-CSF (200 units/ml) on day 2 (removal of non-adherent cells); on day 4, about 75% of medium/GM-CSF (200 units/ml) was replaced. Culture medium was Iscove’s modified Dulbecco’s medium (Cambrex, East Rutherford, NJ) supplemented with 5% fetal calf serum. On day 6, DCs were considered immature by FACS analysis (CD11c+, CD14+, CD40low, CD80low, CD86low). The macrophage-like cells RAW264.7 were kept in RPMI 1640 (Cambrex) supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. For harvest, the medium was removed, and cells were incubated for 5–10 min at 4 °C in ice-cold Versene (1:5000; Invitrogen).
Gp96 Amplifies TLR Ligand-mediated Activation

RESULTS

Gp96 has been demonstrated to directly activate BMDC cell function (12, 18). In more recent studies, it was reported that this activity was lost following rigorous reduction of contaminating LPS levels (28). Enigmatically, LPS concentrations corresponding to those found in activating HSP preparations were also not sufficient for BMDC activation (29). One possible explanation for these apparently conflicting observations is that LPS and Gp96 associate with one another and that this interaction acts synergistically to enhance the BMDC response to LPS. As Gp96 has been demonstrated to function as an LPS-binding protein (28), we determined whether such interactions influence the cellular response to LPS.

Titration experiments were performed to establish the range of Gp96 and LPS concentrations necessary to elicit production of the proinflammatory cytokines IL-6 and IL-12 to allow the detection of a Gp96-mediated amplification. As shown in Fig. 1, titrated amounts of Gp96 mediated activation of mouse BMDCs in a concentration-dependent manner at high concentrations (>50 μg/ml), shown by the production of IL-6 and the chemokines RANTES and KC (A) as well as by the production of low amounts of IL-10 (B). This was also true for the production of MCP-1 and RANTES by the macrophage cell line RAW309 and for the release of IL-8 by human neutrophils (data not shown). At lower concentrations (<25 μg/ml), however, the activating effects of Gp96 on either cell type were negligible.

We performed similar titrations for LPS, focusing our further experiments on the effects on BMDCs since this cell type is crucially involved in bridging innate and adaptive immune responses. Here, maximal activation was still observed at LPS concentrations of 1 ng/ml (Fig. 1C). Further reducing the amount of LPS resulted in reduced levels of IL-6 production, and at about 0.25 ng/ml, no IL-6 production was detected anymore. The same was observed for the production of IL-12 p40.

Now, using LPS and Gp96 at concentrations unable to induce DC activation alone (0.25 ng/ml for LPS and 25 μg/ml for Gp96, respectively), an increase in IL-6 and IL-12 p40 production was detectable when both stimuli were combined (Fig. 2A). However, after preincubation of LPS with Gp96 for 1 h at 37 °C, a significant enhancement of the IL-6 or IL-12 production became evident (A, LPS + Gp96 preincubated). This was accompanied by the up-regulation of co-stimulatory molecules CD86 and CD40 (Fig. 2B) and also by the release of RANTES and KC, whereas the production of IL-10 was not enhanced.

To investigate this observation in more detail, we preincubated titrated amounts of LPS with constant amounts of Gp96 and stimulated BMDCs. As depicted in Fig. 3, we observed a concentration-dependent IL-6 production that was now already detectable at 50 pg/ml of LPS in the presence of Gp96 (A). The same behavior was found for the production of IL-12 p40 (B) and IL-12 p70 (C). Here, a considerable enhancement was even visible at an LPS concentration of 1 ng/ml. Interestingly, the enhancing effect of Gp96 was also observed for the chemokines RANTES and KC but not for the production of IL-10.

Having observed the amplification of LPS-mediated DC activation, we determined whether Gp96 could augment the response to other TLR ligands. Therefore, we incubated BMDCs with different TLR ligands at concentrations that induce only a weak activation of BMDCs as indicated by the production of low amounts of proinflammatory cytokines and expression of co-stimulatory molecules. The experiment was performed in the presence or absence of constant amounts of Gp96. As depicted in Fig. 4, among the different TLR ligands tested, only the TLR2 agonist Pam3Cys mediated a significant enhancement of the production of IL-12 p40 (A) and IL-12 p70 (B) in the presence of Gp96 (indicated by brackets). The same
was found for IL-6 (data not shown). These results were paralleled by the increase in the expression of CD86 (C) and CD40 (data not shown). As observed for the augmentation of the LPS stimulus (Fig. 2A), preincubation of Pam3Cys with Gp96 was required for the optimal induction of cytokine secretion (data not shown).

The activation of BMDCs by Pam3Cys depends on the expression of TLR2 (33). Since Gp96 was shown previously to mediate its effects mainly via TLR4 (21), we decided to analyze whether or not TLR4 contributes to the enhancement of the Pam3Cys-mediated BMDC activation in the presence of Gp96. For this purpose, we stimulated BMDCs from wild-type C57BL/6 and TLR2−/− mice with Pam3Cys or LPS as a control stimulus in the presence or absence of Gp96. Shown in Fig. 5, BMDCs from wild-type mice responded with enhanced cytokine production to Pam3Cys or LPS stimulation in the presence of Gp96 (A), but TLR2−/−-derived BMDCs were only able to respond to LPS (B). The reciprocal observation was made if BMDCs from TLR4-deficient BALB/cHeJ mice (D) were compared with BMDCs from BALB/c mice (C). Here, the presence of Gp96 did not allow BMDCs from these mice to respond to LPS, whereas the Pam3Cys-mediated stimulation was again enhanced. Similar results were obtained for the production of IL-12 (data not shown). These results clearly demonstrate that low dose Gp96 does not mediate BMDC activation on its own and does not interfere with the specificity of the TLR-ligand interaction.

Subsequently, we determined whether the presence of Pam3Cys interferes with the Gp96-mediated augmentation of the LPS stimulation. We therefore added increasing concentrations of Pam3Cys to preformed LPS-Gp96 complexes for 1 h at
37 °C and analyzed the activation of BMDCs derived from TLR2−/− mice, which are unable to respond to Pam3Cys. As before, Gp96 purified according to the modified purification protocol to further reduce endotoxin levels below 0.5 EU/mg or low concentrations of LPS alone were not able to induce BMDC activation (Fig. 6A). Again, preincubation of Gp96 and LPS induced a strong BMDC activation, which declined with further incubations of increasing concentrations of Pam3Cys. The addition of Pam3Cys at 1 μg/ml completely abolished BMDC activation but only if preincubated with pre-existing LPS-Gp96 complexes for 1 h. If the same amount of Pam3Cys was added just before BMDC stimulation without further incubation, no reduction could be observed (Fig. 6A, Pam3Cys co-inc.). To determine whether the entire Gp96 molecule or the N-terminal domain (Gp96.NTD) previously shown to possess immunostimulatory capacities (51) was responsible for the observed effects, we incubated low amounts of LPS with different concentrations of Gp96, Gp96.NTD, or human serum albumin as a control protein and stimulated BMDCs. As shown in Fig. 6B, both Gp96 and Gp96.NTD but not the control protein human serum albumin were able to augment the LPS-induced IL-6 secretion by BMDCs from C57/BL6 mice in a dose-dependent manner. As observed before, without preincubation, no augmentation of the LPS signaling was detected (data not shown).

Next, we decided to investigate whether or not the Gp96-mediated amplification of the TLR2 and TLR4 stimuli translates into augmented adaptive immune responses. These experiments were inspired by the significant enhancement of IL-12 p70 production observed in the experiments shown in Figs. 3C and 4B. Therefore, naive T cells from St35 mice expressing a transgenic T cell receptor specific for an adenoviral, E1A-derived CTL epitope were labeled with CFSE and stimulated with peptide-loaded congenic BMDCs activated with LPS or Pam3Cys at low concentrations in the presence or absence of Gp96. As shown in Fig. 7A, LPS- or Pam3Cys-mediated BMDC activation in the presence of Gp96 resulted in a significant enhancement of CTL proliferation as visible by the dilution of the CFSE dye and in an up to 3-fold enhanced CTL activation as indicated by the production of IFN-γ. As to be expected, CTL
proliferation and IFN-γ production were accompanied by the up-regulation of CD25 and CD44 molecules and the down-regulation of CD62L (Fig. 7B). Again, CTL stimulated with BMDCs activated by PamCys or LPS in the presence of Gp96 showed the strongest change in surface marker expression. CTL activation by non-activated BMDCs was identical to Gp96-, LPS-, or Pam3Cys-stimulated BMDCs, and CTL did not respond to stimulation with a control peptide (data not shown).

**DISCUSSION**

HSP-mediated activation of cells of the innate immune system has been described by many groups, and it was postulated that this family of molecules belongs to endogenous immunostimulators that signal distress and cell damage (3, 4, 34, 35). However, this effect has been challenged by the finding that most HSP preparations used previously were contaminated with low amounts of endotoxins (36, 37).

The experiments presented here now might be able to settle this controversial issue on the function of HSPs in the activation of innate immune responses via certain TLRs. As demonstrated previously, we find that high amounts of Gp96 are able to induce activation of innate immune cells, such as BMDCs, macrophages, or neutrophils (Fig. 1) (28, 29).

However, preincubation of Gp96 at non-stimulating concentrations with the TLR agonists Pam3Cys or LPS at concentrations also unable to induce BMDC activation alone resulted in substantial activation as determined by the analysis of cytokine production and expression of co-stimulatory molecules. On average, we observe a 10-fold enhanced cytokine production, and about 10–200-fold less LPS ligand is required for the induction of comparable BMDC activation levels (Figs. 3 and 4). In addition to the production of proinflammatory cytokines IL-6 and IL-12 p40/70, we also observe a significant enhancement in the production of the chemokines RANTES and KC when Gp96 was complexed with LPS or Pam3Cys but no generation of IL-10. Interestingly, Gp96 on its own induced the production of RANTES, KC, and low amounts of IL-10 at higher concentrations (Fig. 1, A and B). To achieve the optimal enhancement, both LPS and Pam3Cys need to be preincubated with Gp96 (Figs. 2B and 6A and data not shown).

In addition, the interaction of endotoxins with HSPs does not only amplify their biological activity but might also interfere with their neutralization by polymyxin B as suggested (36) and therefore explain the inability of polymyxin B to completely neutralize the effects of different HSP preparations (21, 22, 25). With respect to the Gp96-augmented LPS activation of dendritic cells, our finding is not entirely unexpected since a specific interaction with Gp96 has been demonstrated recently (28). However, an augmentation on the LPS-mediated activation of the NF-κB pathway was not observed in that report. This can be explained by the fact that LPS and Gp96 were not preincubated, and in addition, by a less sensitive readout because LPS at 1 μg/ml or more had to be used for optimal stimulation. How Gp96 is involved in the augmentation of the LPS-dependent stimulation of dendritic cells in detail remains to be determined. However, the fact that Gp96 has been shown to interact directly with TLR4 molecules (32, 38) suggests that a direct interaction takes place and initiates TLR signaling.

The augmentation of the Pam3Cys-mediated BMDC activation was somewhat unexpected because an interaction with Gp96 has not been described. However, Gp96 has also been shown to interact with TLR2 molecules inside the endoplasmic reticulum (32), and both LPS and Pam3Cys molecules share extensive lipid components. Therefore, we speculate that these structures are responsible for the interaction with Gp96 and thus allow the amplification of their biological activity. This speculation is supported by the finding that the activities of other TLR ligands such as poly(I:C), R-848, or CpG DNA lacking lipid tails are not enhanced (Fig. 4). Furthermore, we observe that the presence of Pam3Cys interferes with the Gp96-mediated augmentation of LPS (Fig. 6A). Whether this is due to a competition for the identical binding site on Gp96 or to the induction of conformational changes by Pam3Cys binding at a different site, which might then interfere with the LPS-Gp96 interaction, remains to be determined.
The binding of LPS to proteins has been described before (39–42), and in the case of hemoglobin, an augmentation in the production of cytokines was observed (39–41, 43). What makes the augmentation by Gp96 special from an immunological point of view is the fact that HSPs, including Gp96, are released during necrotic cell death (17). The physiological relevance for the Gp96-mediated augmentation of LPS and Pam3Cys signaling might be the enhancement of activation signals for the innate immune system in the early phase of an infection. In addition, the strongly enhanced production of IL-12 p70 will also support and influence the induction of adaptive immune responses (Figs. 2C and 7) and provides an explanation for the observed induction of a Th1 cytokine profile by Gp96 (44) and possibly also Hsp60 (25). These features strongly resemble the activities of the recently identified Hsp70L1 protein (45).

In addition to the studies in which purified Gp96 with potential endotoxin contaminations was used for the activation of innate immune cells, several reports describe immuno-stimulatory effects of Gp96 expressed on cell surfaces or secreted in vivo (46–48). Here, endotoxin contaminations can be excluded, but nevertheless, Gp96-mediated activation of immune responses is observed. In this context, it was interesting to observe that the N-terminal domain of Gp96, which retained the immuno-stimulatory capacity in vivo (48), was also able to augment LPS-induced BMDC activation to similar levels as the intact molecule (Fig. 6B). Since we and others observe that purified HSPs with barely detectable or very low levels of endotoxin contaminations do not possess immuno-stimulatory capacities at low concentrations, the in vivo effect of HSPs might depend on the presence of endogenous immune stimulators, which are amplified in their biological function by the interaction with HSPs. Uric acid (49) or heparan sulfate, which induces DC activation via TLR4 (50), might be examples for this.

Our experiments provide the first example for the efficient cooperation of pathogen-associated molecular patterns with damage-associated molecular patterns in the induction of innate and adaptive immune responses. This cooperation has been suggested by Wallin et al. (24), who proposed an interaction of stress-associated endogenous and microbial products in the activation of professional APCs. Our experiments indeed

FIGURE 7. Gp96-amplified LPS and Pam3Cys activation of BMDC results in enhanced CTL activation. Naive T cells from St35 mice were labeled with 2.5 μM CFSE and co-cultured with DC activated by the indicated stimuli at a 60:1 ratio. DC activation was performed using 20 μg/ml Gp96, 500 pg/ml LPS, 10 ng/ml Pam3Cys, 20 μg/ml Gp96 preincubated with 500 pg/ml LPS, or 20 μg/ml Gp96 preincubated with 10 ng/ml Pam3Cys (Pam3Cys:Gp96). After 48 h, cells were either harvested for surface staining of viable cells or incubated with peptide and brefeldin A for intracellular cytokine staining. Dot plots show CD8+ T cells. In the case of surface staining, dead cells were excluded by propidium iodide staining. The numbers represent the percentage of cells in the indicated region. The graph shows one of two independent experiments.
provide the perfect example for a cooperation in immune surveillance by components of the extended self-non-self model (51) and the danger model (52).

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