One essential immunoregulatory function of heat shock protein (HSP) is activation of the innate immune system. We investigated the activation of human monocytes and monocytederived dendritic cells (DC) by recombinant human HSP60, human inducible HSP72, and preparations of human gp96 and HSP70 under stringent conditions, in the absence of serum and with highly purified monocytes. HSP60 induced human DC maturation and activated human DC to secrete proinflammatory cytokines. HSP72 induced DC maturation to a lesser extent, but activated human monocytes and immature DC as efficiently as HSP60 to release proinflammatory cytokines. The independence of the effects of HSP60 and HSP72 from endotoxin or another copurifying bacterial component was shown by the resistance of these effects to polymyxin B, their sensitivity to heat treatment, the inactivity of endotoxin controls at concentrations up to 100-fold above the endotoxin contents of the HSP, and the inactivity of a recombinant control protein. Preparations of HSP70, which consisted mainly of the constitutively expressed HSP73, induced only marginal cytokine release from monocytes. The gp96 preparations did not have significant effects on human monocytes and monocytederived DC, indicating that these human APC populations were not susceptible to gp96 signaling under the stringent conditions applied in this study. The biological activities of gp96 and HSP70 preparations were confirmed by their peptide binding activity. These findings show that HSP can differ considerably in the capacity to activate monocyte-derived APC under certain conditions and underline the potential of HSP60 and HSP72 as activation signals for the innate immune system. The Journal of Immunology, 2002, 169: 6141–6148.

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Materials and Methods

Heat shock proteins

Recombinant human HSP60, HSP72, and recombinant bovine HSP73-ATPase fragment were obtained from StressGen (Victoria, Canada). HSP70 was purified from human spleen or liver as described for the HSP70 preparation from murine liver (15). Briefly, a 100,000 g supernatant was prepared from tissue homogenate and applied to Blue Sepharose CL-6B (Amersham Pharmacia, Upplala, Sweden) to remove albumin. The pre-cleared lysate was applied to ADP-agarose affinity columns (Sigma, Taufkirchen, Germany). The ADP-binding protein fraction was eluted with 3 mM ADP and after buffer exchange applied to DEAE-Sepharose anion exchange columns (Amersham Pharmacia), which were equilibrated with 20 mM NaCl buffer in 20 mM sodium phosphate (pH 7.0). HSP70 was eluted with 150 mM NaCl in 20 mM sodium phosphate.

gp96 was purified from a human EBV-transformed B cell line, SK-EBV-29, as described originally for murine gp96 (16) with slight modifications as described later by the same group (17). Briefly, cells were homogenized in hypotonic buffer (30 mM NaHCO3 and 0.2 mM PMSF, pH 7.1) by Dounce homogenization (Kontes, Vineland, N.J.), and a 100,000 g supernatant was obtained. Proteins were fractionated by 50–70% ammonium sulfate precipitation and then applied to a Con A-Sepharose column (Amersham Pharmacia, Berlin, Germany). The ADP-binding protein fraction was eluted with 3 mM ADP and after buffer exchange applied to DEAE-Sepharose anion exchange columns (Amersham Pharmacia) equilibrated with 300 mM NaCl in 5 mM sodium phosphate buffer (pH 7.0). Finally, gp96 was eluted by 700 mM NaCl in 5 mM sodium phosphate buffer. The buffer of HSP70 and gp96 preparations was exchanged to PBS by ultrafiltration (Biomax-30kDa and Biomax-50kDa; Millipore, Bedford, MA), thereby achieving final protein concentrations of 2 mg/ml.

Protein concentrations were determined by Bradford assays photometrically. The purity of the protein preparations was controlled by SDS-PAGE and silver staining of gels. The identity of the proteins was controlled by immunoblot analysis with rat anti-gp94, rat anti-HSP73 (constitutive form of HSP70), mouse anti-HSP72 (inducible form of HSP70), and mouse anti-HSP96/HSP73 (StressGen). The HSP72 concentration in HSP70 preparations was determined by sandwich ELISA (HSP70 enzyme immunossay kit, StressGen).

The biological activities of gp96 and HSP70 preparations were confirmed by testing the peptide binding activity of both HSP as recently described (17). For these peptide binding studies the tyrosinase peptide Ag YMDGTMGQV, a human melanoma Ag (18), was tested for binding to HSP as previously described (17). Briefly, gp96 or HSP70 at 300 µg/ml was incubated with the radioiodinated peptide at a molar ratio of 1:100 in sodium phosphate buffer containing 0.7 M NaCl for 30 min at 50°C. This was followed by incubation at 21°C for 30 min. HSP-peptide complexes were visualized by SDS-PAGE, protein staining, and autoradiography.

The endotoxin, present in HSP or LS stock solutions (Escherichia coli 0111:B4 LPS; Sigma), was determined with the quantitative chromogenic Limulus amebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD) according to the manufacturer’s instructions. The mean endotoxin concentrations of HSP stock solutions were calculated from an E. coli 0111:B4 LPS standard curve and expressed in endotoxin units (EU) per milliliter. From these, the endotoxin concentrations of dilutions from these stock solutions were calculated as EU per milliliter. The endotoxin activities in relation to the amount of protein in the HSP stock solutions were low, with 0.006–0.14 EU/µg protein for HSP60 or HSP72 and 0.0002–0.015 EU/µg protein for HSP70 and gp96 preparations. The EU activity of LPS was 4.58 EU/µg LPS.

Monocytes and DC

PBMC were derived by Ficoll-Hypaque density gradient centrifugation from buffy coats of healthy blood donors obtained from the blood bank of University Hospital of Mainz. CD14-positive monocytes were enriched to 95% purity by immunomagnetic elimination of T cells, NK cells, B cells, DC, and basophils by a cocktail of CD3, CD7, CD19, CD45RA, CD56, and anti-IgE Abs (MACS Monocyte isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany).

DC were generated as previously described (13, 14). Briefly, immature DC were generated by application of high-purity monocytes to X-VIVO-15 medium (BioWhittaker) supplemented with 800 U/ml GM-CSF and 1000 U/ml IL-4 for 4 days, replacing 50% of the culture medium every other day. If indicated, 10% human AB serum was included in the medium. Mature DC were generated by transferring immature DC to fresh GM-CSF and IL-4 supplemented X-VIVO-15 medium, with the addition of IL-1β (10 ng/ml), IL-6 (1000 U/ml), TNF-α (10 ng/ml), and PGE2 (1 µg/ml) for 2 days.

Induction of cytokine release from monocytes and DC by HSP

Monocytes (65,000/well) or DC (50,000/well) were stimulated with titrated amounts of HSP and LPS as an endotoxin background control for the HSP in X-VIVO-15 in the presence or the absence of 10% human AB serum in duplicate wells of 96-well, round-bottom plates for 24 h. Concentrations of IL-6, TNF-α, IL-12p40, IL-12p70, and IL-10 were determined in culture supernatants by standard sandwich ELISA techniques (BD Pharmingen, Heidelberg, Germany). In blocking experiments HSP or LPS was heated (95°C, 30 min), or cells were preincubated for 1 h with 10 µg/ml polymyxin B sulfate (Sigma) or 10 µg/ml anti-CD14 (Beckman Coulter, Krefeld, Germany).

Culture of monocytes and DC with HSP

Monocytes were cultured in X-VIVO-15 medium with HSP or with LPS as an endotoxin background control at the same endotoxin concentration (EU per milliliter) as the corresponding dilution of HSP. The cultures were supplemented every other day with half the amount of HSP or LPS as that used at the start of the culture. After 6 days the cultures were analyzed by flow cytometry.

Maturation of DC was tested by culturing immature DC in X-VIVO-15 medium conditioned with GM-CSF and IL-4 with or without additional supplements: HSP, LPS, or the cytokine mixture of IL-1β, IL-6, TNF-α, and PGE2, described above, as a positive control. After 2 days DC cultures were analyzed by flow cytometry or applied to allogeneic MLR experiments.

Flow cytometry

Viable leukocytes were gated by excluding cells stained with 7-aminoactinomycin D (Via-Probe; BD Pharmingen) and including cells stained with anti-CD45-FITC (Beckman Coulter, Krefeld, Germany). Specific cell surface staining was performed using PE-conjugated Ab in saturating amounts from mouse IgG1 isotype control; anti-CD14, -CD1a, -CD83, and -CD80 Abs (Beckman Coulter); anti-CD86 Ab (Dianova, Hamburg, Germany); and anti-mannose receptor Ab (BD Pharmingen). The relative median fluorescence intensity was calculated as the quotient of the median fluorescence intensities of the respective Ab and the IgG1 isotype control.

Allogeneic MLR experiments

Immature DC were incubated with HSP, with LPS containing the same endotoxin activity as the corresponding HSP preparation, or without additional supplements in GM-CSF- and IL-4-conditioned X-VIVO-15. After 48 h the DC were washed and added to 96-well, flat-bottom plates at concentrations of 2,500, 5,000, and 10,000/well in triplicate. Immunomagnetically purified CD4-positive allogeneic T cells (MACS CD4 Microbeads; Miltenyi Biotech) were added at a concentration of 0.2 × 104/well and were cocultured with the DC for 4 days in X-VIVO-15 medium without serum. [3H]Thymidine (1 µCi/well) was added, and after 16 h [3H]thymidine incorporation was determined.

Statistical analysis

Statistical analysis of the data was performed with StatView (Abacus Concepts, Berkley, CA), employing descriptive statistics and nonparametric Mann-Whitney tests to determine the significance of differences between data.

Results

Characterization of HSP

The following HSP were tested for their capability to induce cytokine release from highly purified human monocytes: recombinant human HSP60, recombinant inducible HSP72, recombinant bovine HSP70 ATPase fragment, gp96 preparations from a human mycoplasma-free EBV-transformed B cell line, and HSP70 preparations from human liver and spleen. HSP70 preparations from the EBV-transformed B cell line variably contained a low m.w. contamination and accordingly were not used as a source of HSP70 in this study. The high purity of gp96 and HSP70 preparations was checked by SDS-PAGE, silver staining of gels, and immunoblotting (Fig. 1). The protein purification methods were employed exactly as described by Srivastava and co-workers (15–17); they were highly reproducible with consistent protein yields and without signs of protein denaturation-like protein aggregation.
or insolubility in aqueous buffers. All HSP70 preparations consisted mainly of constitutively expressed HSP73 and contained only minor amounts of inducible HSP72, as demonstrated by immunoblotting with specific mAb (Fig. 1). This was confirmed by HSP72-ELISA, which showed that the HSP70 preparations contained, on the average, 3.8% HSP72 (SD 1.2). The biological activities of gp96 and HSP70 preparations were confirmed by peptide binding activity of both HSP as described recently (17, 19), and the biological activity of gp96 is demonstrated in Fig. 2.

Induction of proinflammatory cytokine release from human monocytes and DC by HSP

Recombinant HSP60 and HSP72 had the highest activity in the induction of IL-6 release from highly purified human monocytes under serum-free conditions (Fig. 3). The concentrations at which both HSP induced significant IL-6 release from monocytes varied between individual tests within a range of 0.01–0.5 µg/ml, when different charges of HSP and different monocyte donors were employed. The recombinant bovine HSP73 ATPase fragment served as a specificity control for recombinant HSP60 and HSP72 and did not induce any significant release of IL-6 from monocytes at concentrations up to 10 µg/ml (data not shown). HSP70 preparations started to induce cytokine release only at concentrations of 10–30 µg/ml. gp96 did not induce any cytokine release up to concentrations of 270 µg/ml. The presence of human serum increased the cytokine release induced by HSP, except for gp96 (data not shown). Since the endotoxin concentration expressed as EU per milliliter of protein had been determined for each HSP stock solution, the endotoxin concentration of each HSP dilution could be calculated and used for a comparative analysis. HSP60 and HSP72 induced IL-6 release at dilutions of <1% of the minimal endotoxin concentration of LPS causing IL-6 release. Results from one representative experiment of four are shown.
release of TNF-α and IL-12p40 with similar concentration-response relationships as those observed for IL-6 release (Fig. 4, A and C). In contrast, HSP did not induce significant IL-10 release from monocytes (Fig. 4B), which indicates a predominantly proinflammatory cytokine response of monocytes to HSP60 and HSP72. Neither LPS nor HSP induced release of IL-12p70 from monocytes. The inactivity of gp96 in these tests was not due to toxicity of the gp96 preparations, because gp96 in concentrations up to 270 μg/ml did not block LPS-induced monocyte stimulation (data not shown). Also, gp96 preparations from other sources, a melanoma cell line and stomach cancer tissue, did not induce any cytokine release by human monocytes at concentrations up to 270 μg/ml (data not shown).

The activities of HSP60 and HSP72 to stimulate monocytes could be further differentiated from endotoxin effects because they were completely blocked by heat treatment of the HSP (30 min, 95°C), but were only marginally blocked by the addition of polymyxin B (Fig. 5, A and B), while the activity of LPS in parallel experiments was resistant to heat, but was completely blocked by polymyxin B (Fig. 5C). Anti-CD14 mAb blocked the effects of HSP and LPS as well.

When monocyte-derived immature DC were tested for their response to HSP, only HSP60 and HSP72 induced the release of IL-6 and TNF-α and IL-12p40 (Fig. 6) starting at concentrations between 1–3 μg/ml. IL-10 release, and IL-12p70 release was not detected (data not shown). The activity was 300-fold for HSP72 and 10^5-fold for HSP60 above the endotoxin background (data not shown). In contrast to their immature precursors, mature DC were not responsive to any of the tested HSP (data not shown).

Influence of HSP on differentiation of monocytes into immature DC

Highly purified human monocytes were cultured in X-VIVO-15 medium alone or with one of the following supplements: 10 μg/ml of HSP60 or HSP72, 400 μg/ml of gp96 or HSP70, LPS at the different background endotoxin concentrations of each HSP, or GM-CSF/IL-4 as a positive control for the generation of immature DC. After 6 days cells were evaluated for the expression of surface markers by flow cytometry (Fig. 7). As expected, the cultures incubated with GM-CSF, and IL-4 showed the phenotypic characteristics of immature DC with up-regulation of the mannose receptor, indicating endocytic activity, low expression of the costimulatory signal molecules CD80 and CD86, and loss of CD14
creases in CD83, CD80, and CD86 expression. HSP60 at 10 kine mixture induced DC maturation, as shown by the major in- the DC maturation marker CD83 (Fig. 8). As expected, the cyto-
sion of the costimulatory signal molecules CD80 and CD86 and of (13). After 48 h cells were harvested and analyzed for the expres-
were generated from highly puri

expression. CD83 expression was not detected (data not shown). HSP60 and HSP72 induced a slight up-regulation of CD14 compared with medium and endotoxin background controls and a slight decrease in the expression of the mannose receptor compared with endotoxin background controls. In addition, HSP60 induced a slight increase in CD80 expression compared with the endotoxin background control. Preparations of HSP70 and gp96 did not induce significant changes in marker expression compared with their endotoxin background controls. The presence of 10% human serum in the cultures had no significant effect on these results (data not shown). As a result it can be stated that none of the HSP induced differentiation of human monocytes into immature DC.

Influence of HSP on maturation of monocyte-derived DC

Immature DC were cultured in X-VIVO-15 medium containing GM-CSF and IL-4 with or without further supplements: HSP as used in the previous experiments, LPS at the different background endotoxin concentrations of each HSP and at higher concentrations of 10, 100, and 1000 ng/ml, or a cytokine mixture consisting of IL-1β, IL-6, TNF-α, and PGE2, which leads to DC maturation (13). After 48 h cells were harvested and analyzed for the expression of the costimulatory signal molecules CD80 and CD86 and of the DC maturation marker CD83 (Fig. 8). As expected, the cyto-
kine mixture induced DC maturation, as shown by the major in-
creases in CD83, CD80, and CD86 expression. HSP60 at 10 μg/ml almost induced a similar DC maturation, although there was some variation between individual experiments (Fig. 8). HSP72 at 10 μg/ml as well as HSP70 preparations at 400 μg/ml induced a minor up-regulation of CD80, CD86, and CD83 compared with their endotoxin background control. gp96 preparations at 400 μg/ml did not induce any DC maturation. DC maturation caused by 100 ng/ml of LPS was less pronounced than that for HSP60 and the cytokine mixture (Fig. 8). LPS at 100 ng/ml had a 6000-fold higher endotoxin activity than HSP60 in this experiment, which clearly shows the independence of HSP60-induced DC maturation from endotoxin. Additionally, polymyxin B at 10 μg/ml did not block this HSP60 effect (data not shown). The recombinant bovine HSP73 ATPase fragment served as a specificity control for recom-
binant HSP60 and HSP72 and did not induce maturation of DC at concentrations up to 10 μg/ml (data not shown).

The capacities of the different HSP-stimulated DC to induce proliferation of cocultured allogeneic CD4-positive T cells were compared (Fig. 9). Immature DC that had been incubated with LPS at concentrations equivalent to the low endotoxin activities of the HSP preparations induced, on the average, 15-fold T cell prolif- eration above background. This was not significantly different from the proliferation induced by immature DC without additional stimuli (data not shown). However, immature DC incubated with HSP60 and HSP72 in contrast to DC incubated with LPS at back-
ground concentrations enhanced this T cell proliferation signi-
ificantly (2-fold for HSP60 (p < 0.0003) and 1.4-fold for HSP72 (p < 0.015)). DC incubated with gp96 and HSP70 preparations did not show an enhanced T cell stimulatory capacity compared with control DC (Fig. 9).

Discussion

It is shown for the first time that human HSP60 represents a pow-
ful signal for maturation of human monocyte-derived DC. Fur-
thermore, we could demonstrate that recombinant human HSP60 as well as recombinant human inducible HSP72 induce the release of proinflammatory cytokines from immature monocyte-derived CD14-negative DC. HSP72 showed only slight effects as far as maturation of DC is concerned. However, this does not exclude that HSP72 can induce more mature DC under different experimental conditions, as reported when this manuscript was in prepara-
tion (12). In this recent report plastic-adherent PBMC containing 30% non-monocyte-derived cells were used as starting cultures for DC generation, and DC maturation was performed in presence of human serum, both factors presumably supporting DC matura-
tion. This is underscored by the more mature phenotype of the GM-CSF/IL-4 cultured DC in this study, which partially expressed CD83 and relatively high CD86 in contrast to the more immature phenotype of the GM-CSF/IL-4 cultured DC in our analysis (Figs. 6 and 7).

In contrast to the observed DC maturation mediated by HSP60, none of the tested HSP induced differentiation of human monocytes to immature DC. We confirmed that human HSP60 and in-
ducible HSP72 induce the release of proinflammatory cytokines from human monocytes (5–7). Human serum increased the cyto-
kine release of monocytes caused by HSP60 and HSP72, which might be due to the presence of soluble CD14 in serum or of other as yet undefined serum components. As shown in recent studies and here, CD14 plays an important role in cytokine release by monocytes induced by HSP60 and HSP70 (5–7). However, the observed effects of HSP60 and HSP72 on CD14-negative dendritic cells under serum-free conditions make it obvious that HSP60 and HSP72 must also have CD14-independent signal functions. Com-
parison of the effects of the recombinant HSP72 and HSP70 protein preparations, which contained mainly constitutively expressed HSP73 with minor amounts of HSP72, indicate that the danger
signal function of HSP70 molecules relies mainly on inducible HSP72. This is in accordance with the finding of Kupper et al. (12) referred to above; only the inducible HSP72, but not the constitutively expressed HSP73, induced DC maturation.

TNF-α, IL-6, and IL-12p40 were released by monocytes and immature DC in response to HSP60 and HSP72, whereas release of IL-10 and IL-12p70 was not observed. TNF-α and IL-6 are pleiotropic proinflammatory cytokines, both supporting T cell responses. TNF-α as well as IL-6 support DC maturation (13). IL-6 supports T cell proliferation and differentiation (20). Transcription of the IL-12p40 promoter is a prerequisite of release of the bioactive IL-12p70 heterodimer, the key cytokine for priming a Th1 response (21). The observation that IL-12p70 was not released by monocytes and DC in response to HSP is in accordance with previous reports that two signals, a Toll-like receptor (TLR)-mediated signal such as LPS together with IFN-γ priming or CD40 ligation, are needed for DC to release IL12p70 (22, 23). HSP60 provides TLR2- and TLR4-mediated signals to innate immune cells (24), but obviously does not provide the second signal needed for IL-12p70 release. Since we did not observe release of IL-10 by human monocytes and DC in response to HSP, the cytokine response to HSP seems to be mainly proinflammatory, in accordance with previous reports (5, 24).

We ruled out by extensive controls that endotoxin contamination is responsible for the effects of HSP60 and HSP72. First, every experiment was performed with an LPS control either treated with or without LPS at a concentration corresponding to the endotoxin concentration of the respective HSP. HSP60 and HSP72 activated monocytes and DC at protein concentrations that had at least 100-fold lower endotoxin concentrations than the endotoxin concentrations of LPS dilutions necessary for inducing a similar APC activation. Secondly, monocyte activation by HSP60 and HSP72 could be blocked by heat treatment of the HSP, but not by polymyxin B, while the opposite results were obtained when monocytes were activated by LPS. Thirdly, DC maturation induced by HSP60 could not be blocked by polymyxin B. It is obvious that the most active HSP in our study, HSP60 and HSP72, were recombinant proteins, whereas the less active or inactive HSP, HSP70 and gp96, had been purified from tissues or cells biochemically. Therefore, we included a recombinant protein, bovine HSP73-ATPase fragment, as a specificity control to exclude a copurifying stimulating agent in the recombinant protein stock solutions. This recombinant control protein neither induced cytokine release from monocytes nor led to DC maturation.

The observed inactivity of gp96 to stimulate highly purified human monocytes and monocyte-derived DC was unexpected in view of recent reports describing cytokine release of murine peritoneal exudate cells and maturation of murine bone marrow-derived DC in response to murine gp96 (8, 25) and cytokine release and maturation of human DC in response to murine gp96 (11, 26). However, our results do not contradict these reports, but suggest that the capability of gp96 to activate monocytes and DC is not a stable characteristic, but may depend on specific conditions. For example, the maturation of murine DC was much more pronounced in response to cell surface-targeted gp96 (25) than for soluble gp96. The latter induced up-regulation only of CD86, not of CD80, at a high concentration of 400 µg/ml (8). Different experimental conditions may be responsible for the different results of two recent reports from one group concerning the response of human monocytes and DC to gp96 (11, 26). First, in these reports murine gp96 instead of human gp96 as in our work was employed for testing human DC. Despite the fact that gp96 is a highly conserved molecule, there is no complete homology between murine and human gp96 (27). Secondly, we used exactly the same method.

FIGURE 7. HSP do not induce differentiation of human monocytes into immature DC. Human monocytes were cultured with HSP or LPS as an endotoxin background control as described in Materials and Methods. HSP were used at the following concentrations: 10 µg/ml HSP60, 10 µg/ml HSP72, 400 µg/ml HSP70, and 400 µg/ml gp96. As a positive control, immature DC were generated in GM-CSF- and IL-4-conditioned medium. As a negative control, monocytes were cultured in medium without cytokine or HSP supplements. After 6 days the expression of surface markers CD14, CD80, CD86, and mannose receptor (ManR) was determined by flow cytometry. The left column shows that the cultures with GM-CSF and IL-4 (bold line histograms) have the phenotypic characteristics of immature DC compared with the medium control (dotted line histograms). The other columns show that none of the cultures stimulated with HSP (bold line histograms) or with LPS (dotted line histograms) have characteristics of immature DC. The numbers indicate the relative median fluorescence intensity. The upper numbers result from the bold line histograms, and the lower numbers result from the dotted line histograms. One representative experiment of three is represented.
for gp96 purification as that described recently by Srivastava and co-workers (17) employing a sequential ammonium sulfate precipitation as a preclearing step before Con A chromatography and DEAE anion exchange chromatography, whereas in the recent report a method without sequential ammonium sulfate precipitation was cited (28). In our experience, the preclearing step is necessary to clearly separate human gp96 from a 110-kDa protein, even if anion exchange chromatography with a linear NaCl gradient is used as the last gp96 purification step (M. Heike, unpublished observations). Thirdly, in the recent reports plastic-adherent PBMC were used as a start-up population for the DC cultures (11), which contain monocytes and variable contaminations of other mononuclear cells, for example, DC, as discussed above (12, 29). Finally, in the previous report DC cultures were performed with FCS (11), which can contain undefined factors influencing DC maturation (29) in contrast to the serum-free conditions in the DC maturation experiments reported here. We can rule out biological inactivity of gp96 as a reason for the failure to activate monocytes and DC because gp96 as well as HSP70 preparations showed significant peptide binding activity as described previously (17). Additionally, in a parallel study complexes of gp96 and HSP70 with peptide Ags stimulated peptide-specific CTL clones in the presence of APC (M. Heike, manuscript in preparation).

Our results give some additional information about the mechanism involved in HSP60-induced monocyte and DC activation.

FIGURE 8. HSP60 and, to a lesser extent, HSP72 induce maturation of human monocyte-derived DC. Immature DC were cultured with HSP at the concentrations described in Fig. 7 or with LPS as an endotoxin background control and at higher concentrations. As a positive control for DC maturation, immature DC were incubated with a cytokine mixture of IL-1β, IL-6, TNF-α, and PGE2. As a negative control, immature DC were cultured in GM-CSF- and IL-4-conditioned medium without additional supplements. After 2 days the expression of surface markers CD80, CD86, and CD83 was determined by flow cytometry. The left column shows that the cultures stimulated with the cytokine mixture have the phenotypic characteristics of mature DC (bold line histograms) compared with the medium control (dotted line histograms). The right column shows the results from cultures stimulated with 10, 100, and 1000 ng/ml LPS (histograms from left to right). The other columns show the results from the cultures stimulated with HSP (bold line histograms) compared with the endotoxin background controls (dotted line histograms). HSP60 was the only HSP that induced DC maturation comparable to that induced by the cytokine mixture (Expt. 1), although the results showed some variation between individual experiments (Expt. 2). DC maturation by 100 ng/ml LPS was less pronounced than for HSP60 and the cytokine mixture. LPS (100 ng/ml) corresponded to 460 EU/ml endotoxin activity, while HSP60 at 10 μg/ml only had an endotoxin activity of 0.07 EU/ml. The numbers in the histograms indicate the relative median fluorescence intensity. The bold numbers result from the bold line histograms, the intermediate italic numbers in the right column result from the lined histogram, and the lower numbers result from the dotted line histograms. Results from two experiments of three are presented.

FIGURE 9. Enhanced capability of HSP60- and HSP72-stimulated DC to induce alloreactive CD4-positive T cell proliferation. Immature DC that had been incubated for 48 h with HSP or with LPS as an endotoxin background control for the HSP were cocultured with CD4-positive alloreactive T cells for 4 days. T cell proliferation was then determined by [3H]thymidine incorporation. Results are presented as the mean [3H]thymidine incorporation ± SD.

for gp96 purification as that described recently by Srivastava and co-workers (17) employing a sequential ammonium sulfate precipitation as a preclearing step before Con A chromatography and DEAE anion exchange chromatography, whereas in the recent report a method without sequential ammonium sulfate precipitation was cited (28). In our experience, the preclearing step is necessary to clearly separate human gp96 from a 110-kDa protein, even if anion exchange chromatography with a linear NaCl gradient is used as the last gp96 purification step (M. Heike, unpublished observations). Thirdly, in the recent reports plastic-adherent PBMC were used as a start-up population for the DC cultures (11), which contain monocytes and variable contaminations of other mononuclear cells, for example, DC, as discussed above (12, 29). Finally, in the previous report DC cultures were performed with FCS (11), which can contain undefined factors influencing DC maturation (29) in contrast to the serum-free conditions in the DC maturation experiments reported here. We can rule out biological inactivity of gp96 as a reason for the failure to activate monocytes and DC because gp96 as well as HSP70 preparations showed significant peptide binding activity as described previously (17). Additionally, in a parallel study complexes of gp96 and HSP70 with peptide Ags stimulated peptide-specific CTL clones in the presence of APC (M. Heike, manuscript in preparation).

Our results give some additional information about the mechanism involved in HSP60-induced monocyte and DC activation.
The activation of macrophages or DC by HSP60 and gp96 has to be preceded by endocytosis via a specific receptor and is mediated by TLR2 and TLR4 signaling (24, 26). The endocytosis receptor of gp96 and other HSP, HSP60 not included, has been identified as the α2-macroglobulin receptor CD91 (19), which is expressed by human monocytes and DC (30). Obviously, our results on the difference between gp96 and HSP60 in their ability to activate monocytes and DC support the finding of Habich et al. (31) that the receptor for HSP60 is distinct from the gp96 receptor.

It should be emphasized that our experiments and conclusions are restricted to the effects of HSP on highly purified human monocytes and monocytederived DC under serum-free conditions. It remains to be investigated whether other human APC populations, such as freshly isolated myeloid and plasmacytoid blood DC or DC derived from CD34-positive blood or bone marrow progenitor cells, show other response patterns to HSP. Nevertheless, our results extend previous work by showing that HSP can differ considerably in their capacities to activate monocyte-derived APC under certain conditions and underline the potential of HSP60 and HSP72 as activation signals for the innate immune system. Together with the recently demonstrated receptor-mediated uptake of HSP by APC (19, 32, 33), this function renders HSP to powerful natural adjuvants with important implications for the development of vaccines against tumor and infectious diseases.

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