Mechanisms of Signal Transduction:

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Human Heat Shock Protein 70 Enhances Tumor Antigen Presentation through Complex Formation and Intracellular Antigen Delivery without Innate Immune Signaling*[S]

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Heat shock proteins (HSPs) have shown promise for the optimization of protein-based vaccines because they can transfer exogenous antigens to dendritic cells and at the same time induce their maturation. Great care must be exercised in interpretating HSP-driven studies, as by-products linked to the recombinant generation of these proteins have been shown to mediate immunological effects. We generated highly purified human recombinant Hsp70 and demonstrated that it strongly enhances the cross-presentation of exogenous antigens resulting in better antigen-specific T cell stimulation. Augmentation of T cell stimulation was a direct function of the degree of complex formation between Hsp70 and peptides and correlated with improved antigen delivery to endosomal compartments. The Hsp70 activity was independent of TAP proteins and was not inhibited by exotoxin A or endosomal acidification. Consequently, Hsp70 enhanced cross-presentation of various antigenic sequences, even when they required different post-uptake processing and trafficking, as exemplified by the tumor antigens tyrosinase and Melan-A/MART-1. Furthermore, Hsp70 enhanced cross-presentation by different antigen-presenting cells (APCs), including dendritic cells and B cells. Importantly, enhanced cross-presentation and antigen-specific T cell activation were observed in the absence of innate signals transmitted by Hsp70. As Hsp70 supports the cross-presentation of different antigens and APCs and is inert to APC function, it may show efficacy in various settings of immune modulation, including induction of antigen-specific immunity or tolerance.

Cytotoxic CD8 T cells have an essential role in cellular immunity in that they destroy infected or malignantly transformed cells. They are activated by the recognition of complexes of major histocompatibility complex (MHC)[4] class I and antigenic peptides present on the surface of antigen-presenting cells (APC). Conventionally, the antigenic peptides presented by MHC class I are derived from endogenous cytosolic antigens. In specialized situations, MHC class I molecules additionally present peptides derived from exogenous antigens. This noncanonical MHC class I presentation, which is referred to as cross-presentation, requires that the exogenous antigen is internalized by APCs, subsequently enzymatically processed into peptides, and channeled into the MHC class I loading pathway (1, 2). Cross-presentation is crucial for the generation of CD8 T cell responses against antigens that are not endogenously produced by APCs, such as tumor antigens and pathogen-derived proteins. In an applied setting, cross-presentation is the required pathway for the generation of protein-based vaccines that are intended to stimulate antigen-specific CD8 responses. Critical parameters that define the efficacy of a vaccine are the amount of delivered antigen and the context in which the antigen is presented to the T cells. As the physiological capacity of APCs to cross-present antigen is generally low (3), there is significant interest to develop reagents that enhance the targeting of exogenous antigens to the cross-presentation pathway.

HSPs are a family of intracellular proteins, which act as molecular chaperones with essential functions in folding and

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[4] The abbreviations used are: MHC, major histocompatibility complex; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; HSP, heat shock protein family; Hsp, individual heat shock protein family member; Hsp70, stress-inducible member of the 70-kD family; LCL, lymphoblastoid cell line; rhu, recombinant human; MART-1, melanoma antigen recognized by T cells-1; TAP, transporter associated with antigen presentation; pMHC, peptide·MHC complex; pep70, peptide sequence binding to Hsp70; Tf, transferrin; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; DMA, dimethylamiloride; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; FACS, fluorescence-activated cell sorter; GM-CSF, granulocyte-macrophage colony-stimulating factor; MFI, median fluorescence intensity; IFN, interferon; IL, interleukin; FCS, fetal calf serum; PBS, phosphate-buffered saline; ER, endoplasmic reticulum.

intracellular translocation of other proteins (4, 5). An extracellular localization following release by necrotic cells or secretion in response to cellular stress has been reported for a number of family members, including Hsp60 and Hsp70 (6–9). These extracellular HSPs, in particular Hsp70, are thought to mediate immune activation in that they transfer their chaperoned protein-cargo to APCs for cross-presentation (10–14) and concomitantly alert the immune system to danger by inducing APC maturation and cytokine secretion (15–18). This dual activity (19, 20) implied that Hsp70 fulfills the two central requirements of a tumor vaccination tool, antigen delivery to APC and innate immune activation. Recently, however, controversy developed regarding several immunological properties of HSPs, in particular the ability of HSPs to mature DCs, thus questioning their utilization in vaccination settings. Most relevant were the findings that recombinant HSPs (Hsp70 and Hsp60) expressed in *Escherichia coli* contained LPS and flagellin as by-products, which cause DC maturation and cytokine and chemokine secretion (21–27).

In this study we clarify the role of human Hsp70 in cross-presentation and the dependence on innate signaling. We purified recombinant human (rhu) Hsp70 to homogeneity and carefully monitored for by-products. In addition, we devised a novel experimental protocol that encompasses exposure of APCs to identical concentrations of tumor antigen-derived peptides either alone or complexed to rhuHsp70. This setup allowed us to evaluate whether rhuHsp70-bound antigen is cross-presented, and more importantly, we could quantify the enhancement mediated through the chaperone.

Cross-presentation is a complex process that requires enzymatic processing of the exogenous antigen and its trafficking through different intracellular compartments (1, 28, 29). Dependent on the characteristics of the exogenous antigen and the functional capacity of the involved APC, different steps may be utilized. As these requirements cannot be predicted, the agent best suited as a delivery vehicle for cross-presentation should be independent of these specific processes. To fully assess the capacity of rhuHsp70 in cross-presentation, we therefore used two antigenic sequences with distinct processing requirements and two types of APC, DCs and B cell lines, with different cross-presentation capacity (1, 30).

Our results demonstrate that rhuHsp70 significantly enhances cross-presentation of exogenous antigens by means of complex formation and augmenting antigen uptake. rhuHsp70 enhanced cross-presentation of different APCs and different antigenic sequences and thus can be utilized in a variety of settings. Innate immune activation by rhuHsp70 was not observed and more importantly was not required for antigen-specific T cell activation. The possibilities resulting from the absence of innate activation are discussed.

MATERIALS AND METHODS

Reagents—ADP, ATP, γ-ATP, ionomycin, dimethylamiloride (DMA), chloroquine, exotoxin A, *E. coli*-derived LPS (strain 0111:B4), and reagents for calcium signaling, Indo-I AM, Pluronic F-127, and Me_2SO, were purchased from Sigma. All buffers and solutions are prepared using *aqua ad injectabilia* (Braun Melsungen, Germany).

Proteins and Synthetic Peptides—Full-length protein of Melan-A/MART-1 and BSA were purchased from ProSpec (Rehovot, Israel) and Sigma, respectively. T cell epitopes were synthesized either as nonameric peptides or as hybrids containing the nonameric HLA-A2-binding motif, the octameric sequence HWDFAWPW (here named pep70), which is known to bind with high affinity to the endoplasmic reticulum resident Hsp70 homolog (BiP) (31, 32), and a GSG linker (single letter amino acid code) (Table 1). The HWDFAWPW sequence was originally (31, 32) named BiP to describe its high affinity binding to the BiP protein. In this study we use the denomination pep70 (for peptide sequence binding to Hsp70) to describe its binding property to the Hsp70 protein. N-terminal biotinylation ("b-") was done to allow peptide detection using streptavidin. Synthetic peptides were obtained from Biosyntan GmbH (Berlin, Germany), University of Munich, Gene Center (Munich, Germany), or the Technical University of Munich (Munich, Germany).

Recombinant Human Hsp70, Purification and Analysis—rhuHsp70 (GenBank™ accession number P08107) was prepared from *E. coli* (strain WKG 191ΔDnaK, kindly provided by M. P. Mayer) according to a modified protocol of Buchberger *et al.* (33). *E. coli* were grown at 30 °C in LB medium (Invitrogen) containing 100 μg/ml ampicillin (Sigma) and induced overnight with 0.1% (w/v) L-arabinose (Sigma). Cell lysis were performed in 50 mM Tris, pH 7.6, containing 1 mM phenylmethylsulfonyl fluoride, 0.4 mg/ml lysozyme, 1 μg/ml DNase I (Sigma), and protease inhibitor mixture tablets (1 tablet/50 ml of lysis buffer) (Roche Diagnostics). After sonification (Branson Sonifier 250, Danbury, CT) the cell extract was centrifuged (40,000 × *g*, 45 min at 4 °C). Hsp70 was purified by anion exchange chromatography using DEAE-Sepharose (GE Healthcare) followed by ATP-affinity chromatography using ATP-agarose (Sigma). Buffer was exchanged by Sephadex G-25 columns, PD10 (GE Healthcare), and dialysis against HKM buffer (25 mM HEPES, 150 mM KCl, and 5 mM $MgCl_2$). Endotoxin was depleted using Detoxi-Gel™ endotoxin removing gel (Pierce). Quantitation of endotoxin was performed using the *Limulus amoebocyte* lysate assay (QCL-1000, Cambrex Bio Science, Walkersville, MD). The resulting endotoxin content was below 0.5 EU/mg rhuHsp70. The folding status of highly purified rhuHsp70 was confirmed by circular dichroism spectroscopy.

Protein was quantified by the Bradford assay (protein assay, Bio-Rad), and purity was determined by SDS-PAGE and silver staining (34) with a detection sensitivity between 2 and 5 ng/protein per band (not shown).

The ATPase activity was determined from the amount of [^{32}P]ADP produced from [α-^{32}P]ATP as described (35, 36). The reaction mixture, consisting of HKM buffer, 250 μM ATP, 0.1 Ci of [α-^{32}P]ATP (GE Healthcare), and 1 μM rhuHsp70, was incubated at 30 °C. After separation on thin layer chromatography, the amount of radioactive ADP and ATP at 10, 20, 40, 60, 90, 120, 150, and 180 min was quantified using Packard Instant Imager (Canberra Packard, UK) and used to calculate the rate of ATP hydrolysis (37). The intrinsic ATPase rates of different rhuHsp70 preparations were between 4 and 10 × 10^{-4} s^{-1}, which is in the published range (35).

rhuHsp70·b-pep70·peptide Complex Formation for Cross-presentation Assays—rhuHsp70 at different concentrations (0.21–7 μM) were incubated in 96-well cell culture plates with b-pep70-MART-1 peptide (490 nM) or b-pep70-tyrosinase peptide (70 μM) in 30 μl of HKM buffer at room temperature for 4 h. Where indicated, the mixture contained 4.2 μM γ-ATP. To evaluate cross-presentation, DCs and T cells were added to the rhuHsp70·peptide mixture yielding a total volume of 210 μl and resulting in a 1:7 dilution of proteins and peptides. The concentrations stated in this study correspond to the final concentrations present in the cross-presentation assay.

The K_d value of b-pep70-MART-1 peptide complexed to rhuHsp70 was determined by fluorescence titration[5] and was 0.093 μM ± 0.002. The fraction of peptide in complex with rhuHsp70 was calculated using the quadratic binding Equation,

$$\alpha = \frac{P_0 + L_0 + K_d - \sqrt{(P_0 + L_0 + K_d)^2 - 4P_0 \cdot L_0}}{2P_0} \quad \text{(Eq. 1)}$$

where P_0 is the total concentration of pep70-peptide; L_0 is the total concentration of rhuHsp70; and K_d is the dissociation constant.

Cell Culture—Human B-lymphoblastoid cell lines (B-LCL), L721.45 (positive for transporter associated with antigen presentation (TAP)), and L721.174 (negative for TAP) (38) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% FCS (Invitrogen). The HLA-A*0201-restricted tyrosinase peptide $Tyr^{368-376}$ (YMNGTMSQV)-specific cytotoxic T cell clone TyrF8 (39, kindly provided by Dr. P. Schrier, Dept. of Clinical Oncology, Leiden University Hospital, NL) and the HLA-A2-restricted Melan-A/MART-1 peptide (AAGIGLTV)-specific cytotoxic T cell clone A42 (40, 41, kindly provided by M. C. Panelli, National Institute of Health, Bethesda) were cultured as described (42). For cross-presentation assays, both CTL clones were used between day 8 and 14 after the last stimulation.

Dendritic Cell Culture—Dendritic cells (DCs) were derived from monocytes. Briefly, peripheral blood mononuclear cells were isolated from heparinized venous blood of healthy volunteers by density gradient centrifugation over Pancoll (PAN Biotech GmbH, Aidenbach, Germany), and monocytes were positively isolated using micromagnetic beads coated with antibody to CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD14^+$ cells were plated in 6-well plates at a concentration of 5×10^6/4-ml cells per well in AIM-V medium (Invitrogen). rhuGM-CSF (800 units/ml) (Leukine Berlex, Richmond, CA) and rhuIL-4 (500 units/ml) (Cell Genix, Freiburg, Germany) were added to the monocyte cultures at days 0, 3, and 6. Nonmature IL4/GM-CSF differentiated DCs were used between day 6 and day 8. They expressed CD209, CD11c, HLA-DR, CD40, CD32, low levels of CD80 and CD86 and were negative for CD83 and CD38, confirming their nonmature differentiation state. The institutional review board of the Ludwig-Maximilans-University approved these studies. Informed consent was provided according to the Declaration of Helsinki.

[5] M. J. Pandya, manuscript in preparation.

Cytokine Quantitation—Nonmature DCs were incubated with rhuHsp70, LPS, or without stimuli for 48 h at 37 °C. Cytokines in the supernatants were measured using the Bio-Plex cytokine assay (Bio-Rad), and concentrations were calculated using the Bio-Plex Manager™ software program.

Flow Cytometry for Surface Markers and b-pep70-peptide Localization—Surface expression of molecules was determined by flow cytometry. 0.1×10^6 cells (DCs or B-LCL) were stained with specific antibodies (anti-human CD80, CD86, CD83, CD91 (all from BD Biosciences)) and anti-human CD38 (Immunotech, Hamburg, Germany) or IgG control antibodies (BD Biosciences) diluted in PBS containing 2% FCS. For HLA-A2 staining, the antibody HB54 (43) and isotype control anti-mouse immunoglobulin were used. After 30 min of incubation on ice, cells were washed and fixed with 1% paraformaldehyde. Immunofluorescence analyses employed the FACSCalibur and CellQuest software (BD Biosciences).

To determine the distribution of b-pep70-peptides, nonmature DCs or L721.45 were incubated for 60 or 120 min, respectively, with the biotin-peptide or an identical amount of biotin-peptide that was complexed to rhuHsp70 for 12 h at room temperature prior to the addition of cells. Cells were washed, and the amount of biotin-peptide bound to the cell surface or found inside the cells was quantified by staining with streptavidin-FITC. Surface-bound peptide was quantitated by directly staining cells with streptavidin-FITC (DakoCytomation, Hamburg, Germany) in ice-cold PBS containing 0.1% NaN_3 and 2% FCS. To determine the amount of peptide inside the cells, streptavidin-FITC was given to cells after fixation and permeabilization using the intracellular staining buffer set (eBiosciences, San Diego). The mean fluorescence of FITC was measured with FACSCalibur and CellQuest software (BD Biosciences).

Macropinocytosis Assay—Nonmature DCs (day 6) (2×10^6 cells in 5 ml of AIM-V medium containing 400 units/ml rhuIL-4 and 800 units/ml rhuGM-CSF) were stimulated with 0.3 μM rhuHsp70, 250 μM ADP, or 1 μg/ml LPS. As control, the same volume of medium was added. After 24 h of incubation at 37 °C, cells were washed and resuspended in AIM-V medium at a concentration of 0.5×10^6/ml. 1 mg/ml BSA-FITC (Sigma) was added to the cells and incubated for 90 min at 37 °C or on ice. In another setting, unstimulated DCs were incubated with 1 mg/ml BSA-FITC together with the stimulating agent (as above) for 2 h at 37 °C or on ice. Thereafter, cells were washed intensively with PBS containing 2% FCS and 0.01% NaN_3, fixed with 1% paraformaldehyde, and analyzed by flow cytometry, employing FACSCalibur and CellQuest software. Surface binding was determined by subtracting the fluorescence values of cells incubated on ice from those values obtained for cells incubated at 37 °C as described (44).

Intracellular Calcium Analysis—For calcium analysis, a modified protocol according to Grundler *et al.* was used (45). Nonmature DCs, B cells, or CTL clones (1×10^6 cells/ml) were loaded with the fluorescent calcium indicator Indo-1 AM (5 μg/1×10^6 cells) in AIM-V medium for 30 min at 37 °C. Indo-1 AM stock solution was prepared in Me_2SO with 10% pluronic F-127. Loaded cells were washed and resuspended in medium at 0.5×10^6 cells/1.5 ml. Cell aggregates were

removed by filtration through a cell strainer (100 μm, Nalgene). Changes in intracellular calcium were measured with MoFlo (DakoCytomation) using Summit software (DakoCytomation). The stimulating agents, ionomycin or ADP at different concentrations, were added 1 min after the cells were measured without stimulation for background establishment. Cell populations gated according to FSC/SSC scatter and single cell events, determined by means of pulse width of the FSC signal, were selected. Violet (405 nm) *versus* green (530 nm) fluorescence was recorded over 7 min. The ratio 405/530 nm is proportional to the intracellular ionized calcium concentration. As control, the buffers without stimulating agents were measured and did not result in a calcium signal. Data analysis was done with Summit and the graphic software Sigma Plot. Results are shown as changes in the ratio of the mean fluorescence over time.

Cross-presentation Assay—b-pep70-MART-1- or b-pep70-tyrosinase peptides in HKM buffer at indicated concentrations were added to wells of 96-well cell culture plates. The complex formation reaction was performed by adding rhuHsp70 in HKM buffer or a corresponding volume of HKM buffer without rhuHsp70 as control. After 4 h of incubation at room temperature, nonmature DCs or B-LCL (15,000 in 90 μl AIM-V medium) with or without chloroquine (final concentration of 10 μg/ml) or exotoxin A (final concentration of 10 μg/ml) were added to each well. To allow uptake, cells were incubated at 37 °C for 1 h. Thereafter, TyrF8- or A42-CTLs (4,000 cells/90 μl) in RPMI medium containing 10% FCS, 10% human serum, and rIL-2 (50 units/ml, Proleukine, Cetus Corp. Emeryville, CA) were added to each well. After 24 h at 37 °C, supernatants were harvested and the content of IFN-γ was measured by ELISA (OptEIA™, Pharmingen). Control samples, containing all components except the peptide, were used to determine IFN-γ background, which was subtracted from the experimental sample.

Fluorescence Labeling of Proteins—Purified rhuHsp70 (own preparation) or BSA (Sigma) were incubated with Cy™5 bisfunctional reactive dye (GE Healthcare) in carbonate/bicarbonate buffer (0.1 M; pH 9.5) for 3 h at room temperature. Free unconjugated Cy5 was removed by passing the mixture over a gel filtration column (Sephadex G-25; GE Healthcare). Labeled proteins were centrifuged at 100,000 × *g* before use to remove any particulate matter.

Quantitation of Endocytosis by Flow Cytometry—100,000 DCs were incubated with 100 μl of AIM-V medium containing 50 μg/ml of either Cy5-BSA, A647-transferrin (Molecular Probes, Eugene, OR), or Cy5-rhuHsp70. Where indicated, DCs were pretreated for 10 min with medium containing the fluid phase macropinocytosis inhibitor DMA (1 mM dissolved in Me_2SO) (46–48) or Me_2SO alone before addition of the fluorescent protein. Protein uptake was allowed to proceed for 30 min at 37 °C or at 4 °C as control. After 30 min, the tubes were placed on ice, and internalization was stopped immediately by washing of the cells with ice-cold PBS containing 2% FCS, 0.1% NaN_3. Cells were kept on ice and immediately analyzed by flow cytometry. The analysis gate was set on live cells, and the median fluorescence intensity (MFI) was determined using CellQuest Pro™ software. The MFI of 4 °C samples was subtracted. The percentage of uptake inhibition by DMA was calculated according to the formula: % inhibition = 100 × MFI (protein alone)/MFI (protein alone) − MFI(protein + DMA). Me_2SO alone had no effect on the protein uptake, excluding unspecific side effects that might have occurred in the DMA-treated samples.

Confocal Microscopy—DCs or B-LCL cells were incubated with 50 μg/ml FITC-labeled BSA (Sigma), Alexa568-labeled transferrin (Molecular Probes), and either Cy5-labeled rhuHsp70, Cy5-labeled BSA, or A647-labeled transferrin (Molecular Probes) for 30 min at 37 °C. After washing, cells were allowed to settle on poly-L-lysine-coated glass slides for 15 min, fixed with 4% paraformaldehyde in PBS, and mounted with Vectashield (Vector Laboratories). Fluorescence images were captured with a Leica TCS SP2 confocal system, equipped with lasers exciting at 488 and 543 nm (Ar/Kr), 633 nm (HeNe), and 405 nm (diode laser) on a Leica DM IRBE microscope stand with HCX PL APO 63 × 1.40 NA oil immersion objective lens (Leica Microsystems, Heidelberg, Germany). Cells were examined with pinhole 1.0 Airy units, 1024 × 1024 pixel image format, six frame averaging, and a TD488/568/633 dichroic beam splitter. To avoid possible cross-talk of the various fluorochromes, the width of the detection channels and filter settings were carefully controlled, and furthermore, images for FITC, A568, Cy5 or A647, and 4,6-diamidino-2-phenylindole were acquired using the sequential image recording method. For evaluation of colocalization, single *z*-planes were analyzed with Leica confocal software LCSLite (Leica Microsystems, Heidelberg, Germany) and ImageJ 1.37 (Wright Cell Imaging Facility, Toronto, Canada). For image presentation, size and contrast were adjusted with Photoshop 7.0 software (Adobe Software, Palo Alto, CA).

Single Molecule Tracing—20,000 DCs in 400 μl of AIM-V medium were seeded in 8-well chambered coverglass slides (Lab-Tek Nunc Inc., Naperville, IL) and allowed to settle for at least 30 min at 37 °C. Cy5-Hsp70 (2 μg in 200 μl of PBS) was added immediately before imaging. Images, and sequences of images, were recorded with a wide field imaging setup as described (49). Briefly, the laser beam used for the excitation of the molecules was expanded and focused onto the back-focal plane of a microscope objective (Nikon Plan Apo 100 ×/1.4 oil on a Nikon eclipse TE200) microscope stand. The fluorescence was collected by the same objective, separated from backscattered laser light with a combination of filters (dichroic mirror 640 nm cutoff and bandpass BP730/140 AHF), and imaged onto a CCD detector. The lateral resolution of this setup was almost at the diffraction limit or about 300 nm. Images were analyzed with ImageJ 1.37 (Wright Cell Imaging Facility, Toronto, Canada). The trajectories of fluorescent signals of Hsp70 were created with ImageJ 1.37 using the manual tracking plugin.

Statistical Analysis—Bars are the mean of data, with error bars representing the mean deviation (M.D.), which is the mean of the absolute deviation of the data's mean. Statistically analysis was done with S plus software (Insightful Corp., Seattle, Washington). For calculation of significance the Wilcoxon rank sum test was employed.

TABLE 1

Peptides used in this study

pep70 corresponds to the amino acid sequence HWDFAWPW, which is colinearly synthesized with the T cell epitope sequences (MART-1 or tyrosinase) in order to facilitate binding to Hsp70 (31, 32); b- indicates N-terminal biotinylation.

Peptide	Amino acid sequence (single letter code)
b-pep70-MART-1 peptide	Biotin-GSGHWDFAWPWGSGLAGIGILTV
Melan-A/MART-1 peptide (aa27–35)	LAGIGILTV
b-pep70-tyrosinase peptide	Biotin-GSGHWDFAWPWGSGYMNGTMSQV
Tyrosinase peptide (aa368–376)	YMNGTMSQV

RESULTS

rhuHsp70 Enhances Cross-presentation of Exogenous Tumor Antigen-derived Peptides by DCs and B Cells through High Affinity Hsp70·Peptide Complex Formation—To critically evaluate the capacity of human Hsp70 as an immune stimulatory agent, we purified rhuHsp70 protein to homogeneity and carefully removed nonproteinaceous products, such as endotoxin and nucleotides.

The role of rhuHsp70 in cross-presentation was determined using a novel experimental setup that encompasses exposure of APCs to identical concentrations of tumor antigen-derived peptide either alone, complexed to rhuHsp70, or added to rhuHsp70 without preincubation. In the latter setting only little complex formation occurs because of the slow binding kinetics at room temperature (50). We selected two tumor antigen-derived peptides, Melan-A/MART-1 (amino acids 26–35) and tyrosinase (amino acids 368–376), which are the ligands for HLA-A2 restricted T cell clones, A42-MART-1 and TyrF8, respectively (42). The antigenic peptides were synthesized as hybrid peptides with the hydrophobic octapeptide sequence HWDFAWPW, here named pep70 (for peptide sequence binding to Hsp70), known to bind to Hsp70 with high affinity (peptide nomenclature and sequences in Table 1). The efficacy of cross-presentation by DCs or B cells of these epitopes either applied without or with rhuHsp70 was assessed by the capacity of DCs to stimulate IFN-γ release by antigen-specific T cell clones. Antigen-specific T cells secrete IFN-γ when activated by antigen-peptide·MHC complexes (pMHC), and the amount of secreted IFN-γ correlates with the intensity of T cell activation, which correlates with the amount of presented antigen.

As demonstrated in Fig. 1*A*, some A42-T cell activation was achieved by DCs incubated with b-pep70-MART-1 peptide (70 nM) alone. This level was used as the reference value of exogenous b-pep70-MART-1 cross-presentation in the following experiments. If the b-pep70-MART-1 peptide was preincubated with rhuHsp70 for 4 h before DCs were applied, the IFN-γ response of A42-T cells to the same peptide concentration (70 nM) was significantly higher, indicating that more of the exogenous peptide had formed HLA-A2 peptide ligands for T cell recognition. rhuHsp70 in the absence of peptide did not stimulate IFN-γ release of T cells at any concentration (Fig. 1*A*). DCs incubated with peptide or rhuHsp70·peptide complexes had identical phenotypes (CD209, CD40, CD80, CD86, MHC class II, CD32, and CD83, Fig. 1*F*, and data not shown). rhuHsp70 itself also did not alter DC phenotypes (see Fig. 5*A*). The efficacy of cross-presentation of DCs and subsequent T cell stimulation showed a direct correlation to the amount of rhuHsp70 that was available within the complex formation mixture (Fig. 1*B*). Using the quadratic binding equation and the K_d value for the interaction of rhuHsp70 with b-pep70-MART peptide (determined by fluorescence titration of b-pep70-MART-1 peptide to rhuHsp70), the percentage of peptide complexed to rhuHsp70 was calculated and compared with the T cell response. As shown in Fig. 1*C*, increasing the rhuHsp70 concentration resulted in a higher percentage of b-pep70-MART peptide complexed to rhuHsp70, which directly correlated to the intensity of the T cell response (Fig. 1, *B* and *C*). This indicates that the activity of rhuHsp70 in cross-presentation is a function of the degree of complex formation.

The crucial role of complex formation between peptide and rhuHsp70 for the enhancing effect in cross-presentation was further substantiated in experiments where peptide and rhuHsp70 were mixed without preincubation, a condition where minimal complex formation occurs, and then given to DCs and T cells. Although this mixture contained the same amount of peptide and rhuHsp70 as before, T cell activation was not enhanced (Fig. 1*B*). The lack of enhancement observed in this setup also allows us to exclude that by-products within the rhuHsp70 preparation are responsible for the effects seen in samples where peptide was complexed to rhuHsp70.

Enhancement of cross-presentation was a specific feature of rhuHsp70 as BSA, a protein with similar molecular weight and solubility (51), was unable to enhance cross-presentation under these conditions. Surface plasmon resonance spectroscopy (Biacore) confirmed that rhuHsp70 but not BSA bound to b-pep70-MART-1 peptide (not shown).

The amplification of cross-presentation through rhuHsp70·peptide complex formation was also observed for a second peptide, b-pep70-tyrosinase, which is recognized by the T cell clone TyrF8. TyrF8 IFN-γ secretion was stimulated at a peptide concentration of 10 μM, whereas1 μM of b-pep70-tyrosinase was too low to achieve TyrF8 stimulation (Fig. 1*D*). However, when the same concentrations of peptide were preincubated (4 h) with rhuHsp70 and then given to DC, TyrF8 IFN-γ secretion was already observed at 1 μM b-pep70-peptide. Thus, complex formation between b-pep70-tyrosinase peptide and rhuHsp70 shifted the dose-response curve allowing T cell activation at a peptide concentration, which by itself was insufficient for T cell activation.

Regarding their natural efficiency to be cross-presented, b-pep70-MART-1 and b-pep70-tyrosinase peptides represent two classes; b-pep70-tyrosinase peptide entered the cross-presentation pathway of DCs very inefficiently as evidenced by the requirement of much higher concentrations of exogenous b-pep70-tyrosinase peptide (10 μM), whereas b-pep70-MART-1 peptide was very efficiently cross-presented requiring as little as 70 nM of exogenous peptide concentration to achieve detectable T cell stimulation. rhuHsp70 similarly enhanced the cross-presentation of both peptides around 2–3-fold. In a natural situation where the exogenous dose of antigen might be limiting, the presence of rhuHsp70 might be particularly influential because by enhancing cross-presentation and shifting the dose-response curve between antigen concentration and T cell

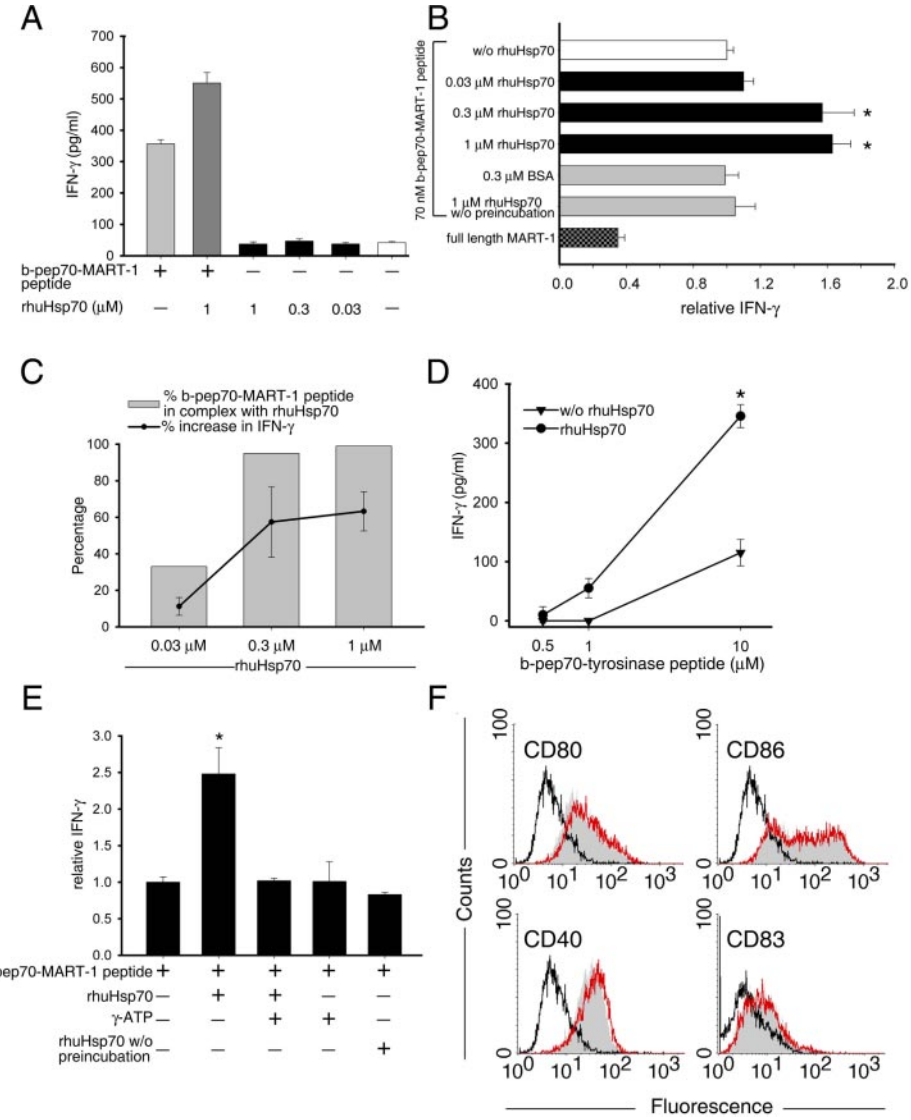

FIGURE 1. **rhuHsp70 binds to b-pep70-peptide antigens thereby enhancing cross-presentation and antigen-specific T cell stimulation by human GM-CSF/IL-4 differentiated nonmature myeloid DCs and B cell lines.** *A,* human GM-CSF/IL-4 differentiated nonmature myeloid DCs were added to 70 nM of b-pep70-MART-1 peptide alone or to 70 nM of b-pep70-MART-1 peptide that was preincubated (4 h) with 1 μM rhuHsp70 or to different concentrations of rhuHsp70 without peptide. IFN-γ secretion by A42-T cells was measured by ELISA. *Bars* represent the mean of triplicate values ± M.D. *B,* nonmature DCs were added to 70 nM of b-pep70-MART-1 peptide without rhuHsp70 or to 70 nM b-pep70-MART-1 peptide, which was preincubated (4 h) with different concentrations of rhuHsp70 or 0.3 μM BSA, or to 70 nM b-pep70-MART-1 peptide mixed with 1 μM rhuHsp70 without preincubation, or DCs were directly pulsed with full-length rhuMART-1 protein (70 nM). IFN-γ secretion by A42-T cells was measured by ELISA. Samples, containing all components except b-pep70-MART-1 peptide, were used for IFN-γ background determination, and these values were subtracted from the experimental samples. The relative IFN-γ release was calculated using the IFN-γ release achieved with b-pep70-MART-1 peptide alone as reference 1. *Bars* represent the mean relative IFN-γ release of triplicates ± M.D. Cumulative data of three independent experiments were used to calculate the statistical significance of rhuHsp70-mediated increase in IFN-γ release compared with peptide alone (Wilcoxon rank sum test; *, $p < 0.005$, $n = 3$). *C,* correlation between rhuHsp70·peptide complex formation and IFN-γ response. *Bars* are the percentage of b-pep70-MART-1 peptide in complex with rhuHsp70 calculated using the quadratic binding equation and the predetermined dissociation constant of b-pep70-MART-1 peptide complexed to rhuHsp70 ($K_d = 0.093$ μM ± 0.002). The peptide concentration used to calculate the percentage of complex formation was that before DC/T cells were added, thus representing seven times the final concentration of that present during cross-presentation, which was 70 nM. The *line* represents the mean percentage of increase in IFN-γ release of A42-T cells obtained by incubation with rhuHsp70·b-pep70-MART-1 peptide-pulsed DCs compared with DCs pulsed with b-pep70-MART-1 peptide alone. M.D. are from triplicates. One representative experiment of two is shown. *D,* DCs were added to preincubated (4 h) mixture of b-pep70-tyrosinase peptide (0.5, 1, or 10 μM) and rhuHsp70 (1 μM) or b-pep70-tyrosinase peptide alone. TyrF8-T cells were added and the amount of secreted IFN-γ was measured after 24 h. M.D. are derived from triplicate cultures. *p* values (*, $p < 0.005$) were calculated as described above. *E,* B cell line L721.45 was incubated with b-pep70-MART-1 peptide (70 nM) alone, b-pep70-MART-1 peptide (70 nM) after preincubation (4 h) with rhuHsp70 (300 nM) in presence or absence of 600 nM γ-ATP, or with b-pep70-MART-1 peptide (70 nM) mixed with rhuHsp70 without preincubation. IFN-γ release by A42-T cells after 24 h was determined by ELISA. The relative IFN-γ release ±M.D. was calculated and statistical significance determined as described (*, $p < 0.005$, $n = 3$ experiments). *F,* surface marker expression of DCs. DCs were added to b-pep70-MART-1 peptide (70 nM) or b-pep70-MART-1 (70 nM) preincubated (4 h) with rhuHsp70 (300 nM). After 48 h, surface markers were analyzed by flow cytometry. *Histograms* represent the isotype control (*black line*), b-pep70-MART-1 peptide (*gray filled*), and rhuHsp70·b-pep70-MART-1 complex (*red line*).

activation an immune response can occur toward an antigen that would be immunologically quiet by itself.

rhuHsp70 also significantly improved cross-presentation of b-pep70-MART-1 peptides in the B-LCL cell line L721.45 (Fig. 1*E*). Again, rhuHsp70·peptide complex formation was required as T cell responses were not increased if B-LCL cells were added to the mixture of peptide and rhuHsp70, which was not preincubated.

This B cell line was used to evaluate the effect of ATP in the rhuHsp70-mediated cross-presentation. DCs could not be used in this experiment because their functional profile is modified by nucleotides (see below). It is known that the kinetics of Hsp70-substrate binding is governed by ATP binding and ATP hydrolysis (52–54). The nonhydrolyzable γ-ATP arrests the Hsp70-peptide interaction in the ATP-bound state, which binds peptide with low affinity. As shown in Fig. 1*E*, the presence of γ-ATP within the b-pep70-peptide-rhuHsp70 complex formation mixture reduced the level of T cell stimulation to that observed with peptide alone. Thus, a high affinity peptide binding conformation of rhuHsp70 is a prerequisite for the capacity of rhuHsp70 to enhance cross-presentation of exogenous antigenic peptides.

rhuHsp70 Is Endocytosed by Fluid Phase Macropinocytosis into Vesicular/Tubular-shaped Endosomes—MHC class I presentation of exogenous antigens can occur by directly loading the antigen to surface MHC class I molecules or by delivery to the intracellular pathway of MHC class I presentation that involves either the ER, ER/phagosomal compartments, or recycling endosomes (1, 2, 28, 29).

Live tracing of fluorescent-labeled rhuHsp70 revealed that rhuHsp70 entered intracellular compartments of DCs. In most instances the fluorescent signals had punctate vesicular appearance and moved beneath the plasma membrane or around the nucleus (Fig. 2*A*, *yellow trajectories*). In some instances and mainly at sites closely beneath the plasma membrane, the punctate Hsp70 signals fused to form tubules (Fig. 2*A*, *arrowheads* and *red trajectories*). Similar structures that moved in a retrograde manner to fuse directly with the plasma membrane have been observed and are interpreted as endosomal MHC class II loading and transport compartments (55).

Fluid phase or receptor-mediated uptake can lead to an intracellular localization of exogenously applied rhuHusp70. For Hsp70 in particular, receptor-mediated uptake involving binding to CD91 or other molecules has been described (56–61). To define which process leads to internalization of our rhuHsp70, rhuHsp70-Cy5 uptake was allowed to proceed simultaneously to transferrin-A568 and BSA-FITC. Transferrin and BSA served as tracer proteins for receptor-mediated endocytosis or fluid phase macropinocytosis, respectively. In the B cell line L721.45 (Fig. 2*B*), transferrin-containing vesicles (*red*) localized predominantly perinuclearly, whereas the BSA vesicles (*green*) were just beneath the plasma membrane. Very little overlap was found between BSA-FITC (*green*)- or transferrin-A568 (red)-containing vesicles as evidenced by the lack of color overlap (Fig. 2*B*, *BSA/Tf,* merged picture). Quantitative evaluation of all vesicles of 90 individual cells revealed that most vesicles were either positive for BSA or transferrin with, on average, only 38% (range, 26–58%) of vesicles showing colocalization (Fig. 2*C*). The results attested that BSA and transferrin followed distinct uptake pathways in L721.45 B cells. Controls included the analysis of cells simultaneously incubated with BSA-FITC and BSA-Cy5 or with transferrin-A568 and transferrin-A647, which showed 100% of colocalization in both situations (supplemental Fig. 1*B*, and quantitation in Fig. 2*C*). Analysis of rhuHsp70 vesicles in this setting revealed a striking colocalization of rhuHsp70 with the BSA vesicles but not with the perinuclear transferrin vesicles (Fig. 2*B*). Quantitative analysis of the color overlap (Fig. 2*B,* visualized as *light blue*) between rhuHsp70 and BSA revealed 86% of colocalization (range, 78–100%), further evidencing that rhuHsp70 colocalized with the fluid phase tracer BSA but not with the receptor-mediated tracer transferrin (17% of colocalization, range, 16–33%) (Fig. 2*C*).

Using DCs, a high degree of colocalization between BSA and transferrin (on average 70%; range, 58–84%; see Fig. 2*C* and supplemental Fig. 1*A*) was observed supporting previous findings that in DCs fluid phase macropinocytosis is the main uptake pathway even for proteins, which classically are internalized by receptor-mediated uptake (47, 62). Consistent with this notion, we found that most vesicles of DCs contained rhuHsp70 together with BSA and transferrin (supplemental Fig. 1*A*). This result demonstrates that using DCs in this setting, it cannot be discerned whether receptor-mediated uptake is involved in rhuHsp70 internalization.

In addition to confocal microscopy, flow cytometry was used to analyze the uptake pathways of DCs of rhuHsp70, transferrin, or BSA in the absence or presence of the fluid phase macropinocytosis inhibitor DMA. Uptake of all three proteins was observed, and DMA inhibited the uptake of rhuHsp70 to 95% (range, 94–95%; Fig. 2, *D* and *E*) supporting the interpretation that fluid phase is the main pathway for rhuHsp70 uptake also in DCs. Transferrin uptake was inhibited to 63% (range, 59–87%) supporting the confocal microscopy data that the transferrin uptake pathway largely coincides with that of BSA or rhuHsp70 in DCs.

Collectively the data from two different experimental approaches identified that fluid phase uptake is the dominant pathways leading to internalization of rhuHsp70 in DCs and B cell lines with no obvious contribution of receptor-mediated processes. The rhuHsp70-containing compartments exhibited vesicular and sometimes tubular shapes and excluded transferrin. These characteristics resemble those of MHC class II transport compartments and peripheral recycling endosomes. Interestingly, the vesicular, surface proximal compartments stained positive for MHC class I (supplemental Fig. 1*B*) and thus may represent a compartment where the exchange between rhuHsp70-chaperoned peptides and MHC class I-bound peptides can occur.

rhuHsp70 Facilitates Intracellular Delivery of Chaperoned Exogenous Peptides—Having established that rhuHsp70 is internalized, we tested whether rhuHsp70 uptake concomitantly resulted in higher intracellular levels of the exogenous peptides chaperoned by rhuHsp70. To compare intracellular peptide levels, B-LCL and DCs were incubated either with b-pep70-tyrosinase peptide alone or with an equal amount of

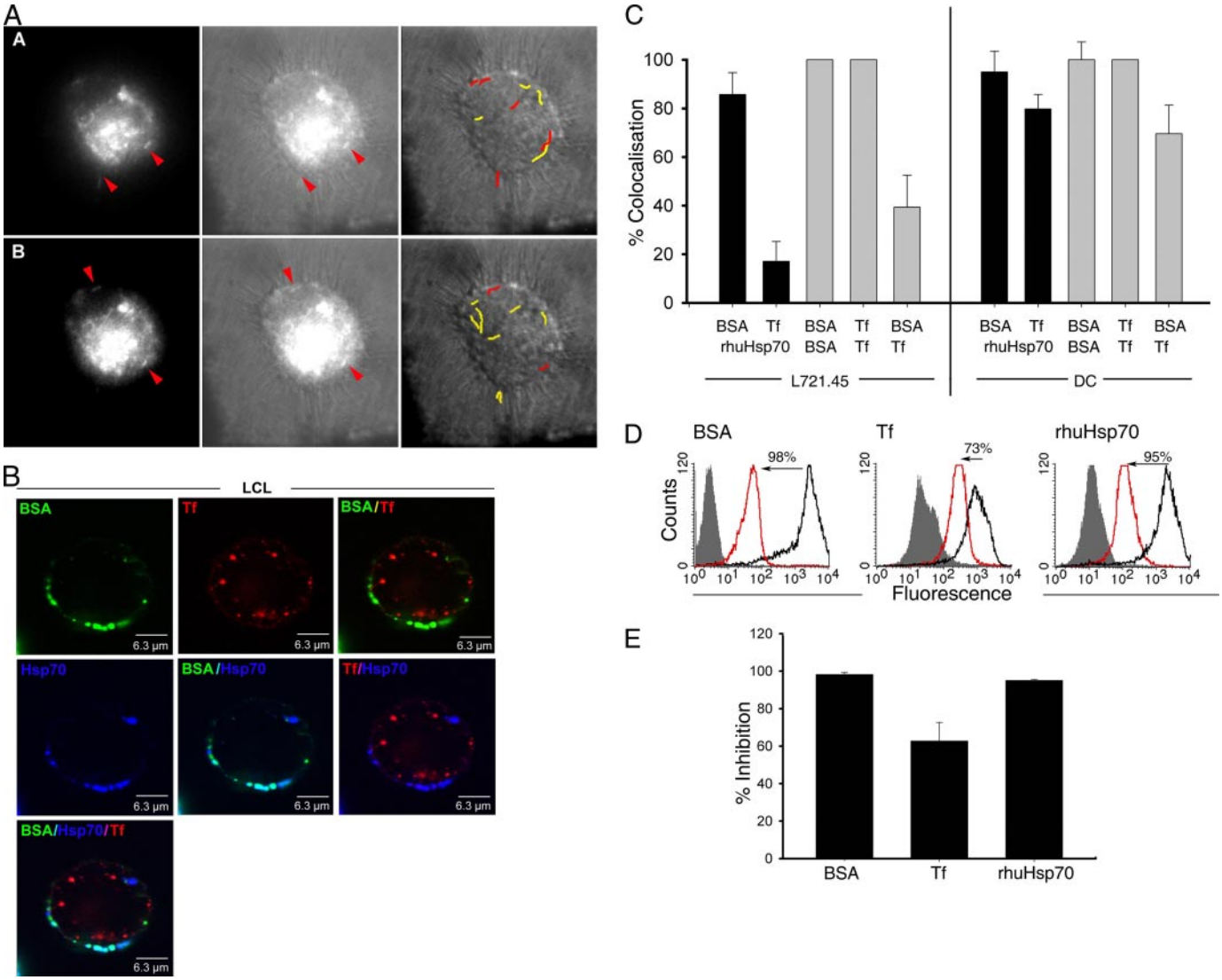

FIGURE 2. **rhuHsp70 is internalized by fluid phase macropinocytosis into vesicular/tubular-shaped endosomes.** *A,* live tracing of rhuHsp70 in DCs. Pictures show one DC imaged at two time points (*upper* and *lower panels*, respectively) after addition of Cy5-labeled rhuHsp70. Movements of rhuHsp70 in vesicles that sometimes fuse to tubular structures (*arrowheads*) were observed. Fluorescence images (*left*), fluorescence overlaid with digital interference contrast (*middle*), and merged fluorescence/digital interference contrast images with depicted trajectories (*right*) are shown. *Red* and *yellow* trajectories identify the movements of tubular structures and vesicular signals, respectively. Movements of the vesicular signals were beneath the cell surface and around the nucleus, whereas the tubular structures localized mostly beneath the plasma membrane. *B,* intracellular tracking of fluorescent-labeled rhuHsp70, BSA and transferrin (*Tf*) using confocal microscopy. rhuHsp70-Cy5, BSA-FITC, and Tf-A568 were given simultaneously to L721.45. After 30 min at 37 °C, cells were applied to slides and fixed, and colocalization was evaluated with confocal microscopy. Depicted are the individual fluorescence channels for BSA (*green*), Tf (*red*), and rhuHsp70 (*blue*), as well as the merged pictures (BSA/TF, Tf/Hsp70, and BSA/Hsp70/Tf). Color overlap indicates colocalization. Controls included simultaneous incubation of cells with BSA-FITC and BSA-Cy5, Tf-A647, and Tf-A568 (supplemental Fig. 1*B*). *C,* quantitation of protein colocalization. All vesicles within one optical plane of one cell were evaluated for colocalization of proteins (examples of L721.45 are shown in *B*; examples of DCs and control protein uptake are in supplemental Fig. 1*B*). For each protein combination, a total of 90 cells (30 cells from three independent experiments) were analyzed. *Bars* (% of colocalization) represent the percentage of vesicles, which contained both indicated proteins, and are the median ± M.D. of three independent experiments. *D* and *E,* flow cytometry for the quantitation of protein uptake and inhibition by DMA. DCs were incubated with 50 μg/ml of Cy5-labeled-BSA, Cy5-transferrin (*Tf*), or Cy5-rhuHsp70 in the presence or absence of 1 mM DMA at 37 °C (*red line* and *black line histograms*, respectively) or 4 °C as control (*gray histograms*) (*D*). Protein uptake was analyzed after 30 min by flow cytometry. The numbers in the histogram plots indicate the percentage of uptake inhibition induced by DMA. The percent of inhibition of uptake by DMA was calculated from the fluorescence values, and the median uptake inhibition of at least three different experiments ± M.D. is depicted graphically (*E*).

peptide that had been preincubated (12 h) with rhuHsp70 to allow complex formation. After incubating the cells with the peptide or rhuHsp70·peptide complexes for 60 min (DCs) or 120 min (B-LCL), cells were washed and then stained with streptavidin-FITC to detect peptide based on the biotin-component present in the b-pep70-tyrosinase peptide. Prior to staining, cells were either fixed and permeabilized to detect the amount of peptide inside the cells or stained without fixation/permeabilization to determine the amount of surface-bound peptides. Using flow cytometry, surface-bound peptide was not detected when cells were incubated with b-pep70-tyrosinase alone or complexed to rhuHsp70 (Fig. 3). The amount of peptide inside DCs or B-LCLs was higher for cells that were incubated with peptide complexed to rhuHsp70, as evidenced by the higher median fluorescence intensity (Fig. 3, *red lines* and *gray histograms*). Thus,

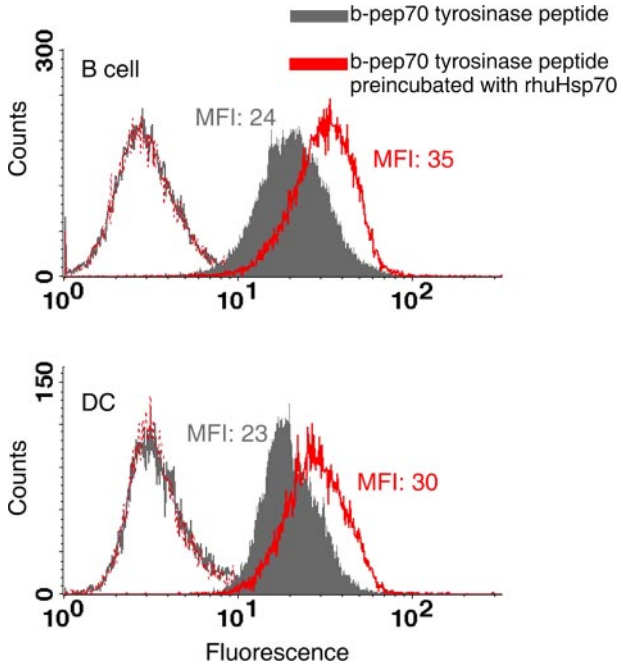

FIGURE 3. **rhuHsp70 enhances intracellular levels of exogenous peptide antigens in DCs and B cell lines.** DCs or L721.45 were cultured for 60 or 120 min, respectively, with either b-pep70-tyrosinase peptide (10 μM) or the same amount of b-pep70-tyrosinase peptide, which prior to the addition of the cells had been incubated with rhuHsp70 (3 μM) at room temperature for 12 h to allow complex formation. After the uptake period (60 min for DCs and 120 min for B-LCL), cells were washed and surface bound or intracellular peptide was detected using streptavidin-FITC and FACS analysis. The *dotted gray* or *red line histograms* represents the amount of b-pep70-tyrosinase peptide observed on the surface of cells after incubation with peptide alone or rhuHsp70·b-pep70-tyrosinase complexes, respectively. The *filled gray* or *red line histogram* represents the amount of b-pep70-tyrosinase peptide detected inside cells after incubation with b-pep70-tyrosinase peptide alone or rhuHsp70·b-pep70-tyrosinase complex, respectively. Numbers indicate the MFI.

complex formation of peptide with rhuHsp70 results in higher intracellular levels of exogenous peptide.

rhuHsp70-mediated Augmentation of Cross-presentation Is Proximal to Intracellular Antigen Processing and Trafficking—Published evidence suggests that extracellular antigens must access the cytosol for efficient cross-presentation. This pathway involves retrotranslocation of exogenous antigen from the ER to the cytosol and the transport of peptide back into the ER involving the activity of TAP proteins (1, 2). To investigate a possible role of rhuHsp70 in these processes, we used TAP-positive and TAP-negative B cell lines, L721.45 and L721.174, respectively, as APCs. As demonstrated in Fig. 4*A*, rhuHsp70 enhanced cross-presentation to the same extent in TAP-positive and TAP-negative cell lines. Moreover, treatment of APCs with exotoxin A, an inhibitor of the ER-to-cytosol retrotranslocation (29), did not abrogate rhuHsp70-mediated augmentation of cross-presentation (Fig. 4*B*). These results indicate that rhuHsp70 exerts its enhancing effect at a step proximal to intracellular trafficking.

The amplification of cross-presentation by rhuHsp70 was also independent of internal pH conditions and post-uptake processing requirements of exogenous antigens (Fig. 4*C*). b-pep70-MART-1 and b-pep70-tyrosinase are N-terminally elongated hybrid peptides that must be enzymatically processed to create the nomamer epitopes, MART-1(LAGIGILTV) and Tyr(YMNGTMSQV), respectively, which integrate into the binding groove of HLA-A2 proteins and are recognized by the antigen-specific T cell clones. Chloroquine is a lysosomotropic agent that inhibits endosomal acidification thereby regulating endosomal proteolytic activity. Incubation of DCs (Fig. 4*C*) or B-LCL (data not shown) with chloroquine strongly reduced the presentation of exogenous b-pep70-MART-1 peptide, whereas it enhanced the presentation of b-pep70-tyrosinase peptide. Chloroquine did not inhibit or activate the DCs or T cells in our experimental setup because the T cell response to the nonamer peptides of Tyr(YMNGTMSQV) or MART-1(LAGIGILTV), which integrate into the HLA-A2 binding groove directly on the cell surface and do not require intracellular processing, was unaffected by chloroquine (Fig. 4*C*). Nor did chloroquine change the MHC class I expression or viability of DCs (data not shown). Therefore, the disparate response to chloroquine of b-pep70-MART-1 or b-pep70-tyrosinase peptides reflects different post-uptake requirements for the generation of the HLA-A2 epitopes from the hybrid peptides. Obviously, the generation of the MART-1 T cell epitope from b-pep70-MART-1 peptide required endosomal proteases that are active at low pH conditions, whereas the b-pep70-tyrosinase epitope apparently is destroyed under these conditions. Despite the different requirements for peptide processing rhuHsp70 enhanced the cross-presentation of both b-pep70-tyrosinase and b-pep70-MART-1 peptides to a similar extent in the presence of chloroquine indicating that the activity of rhuHsp70 in cross-presentation is independent of internal pH conditions (Fig. 4*D*).

rhuHsp70 Does Not Change Phenotypic Markers, Cytokine Secretion, or Macropinocytosis of GM-CSF/IL-4 Differentiated Myeloid DCs—Earlier reports from several groups (63–65), including our own (16), had documented that human Hsp70 can act as a danger signal for DCs, leading to phenotypic maturation and secretion of pro-inflammatory cytokines. There is significant controversy regarding this activity because recent experiments indicated that nonproteinaceous products such as LPS, which co-purified with recombinant Hsp70 (and other HSPs), stimulated similar changes in phenotype and cytokine profile of DCs (21–27). Now, using our own endotoxin-depleted rhuHsp70 protein, we were unable to induce changes in phenotypic markers, such as CD40, CD80, or CD86, of human GM-CSF/IL-4 differentiated myeloid DCs. Furthermore, no up-regulation of CD83 or CD38 was observed (Fig. 5*A*). Endotoxin-depleted rhuHsp70 did not induce the secretion of inflammatory cytokines tumor necrosis factor-α and IL-12(p70) or the regulatory cytokine IL-10. Regulated on activation normal T cell expressed and secreted (RANTES/CCL5) and IL-1β were secreted spontaneously by our DCs, and the levels were unchanged after stimulation with rhuHsp70 (data not shown). As expected, GM-CSF/IL-4 differentiated myeloid DCs responded to the pathogen-derived danger signal LPS with up-regulation of the aforementioned markers and secretion of cytokines (Fig. 5*B*). rhuHsp70 did not change macropinocytosis activity (Fig. 5*C*) or surface expression of HLA-A2 (Fig. 5*D*) of our DCs. LPS strongly reduced the

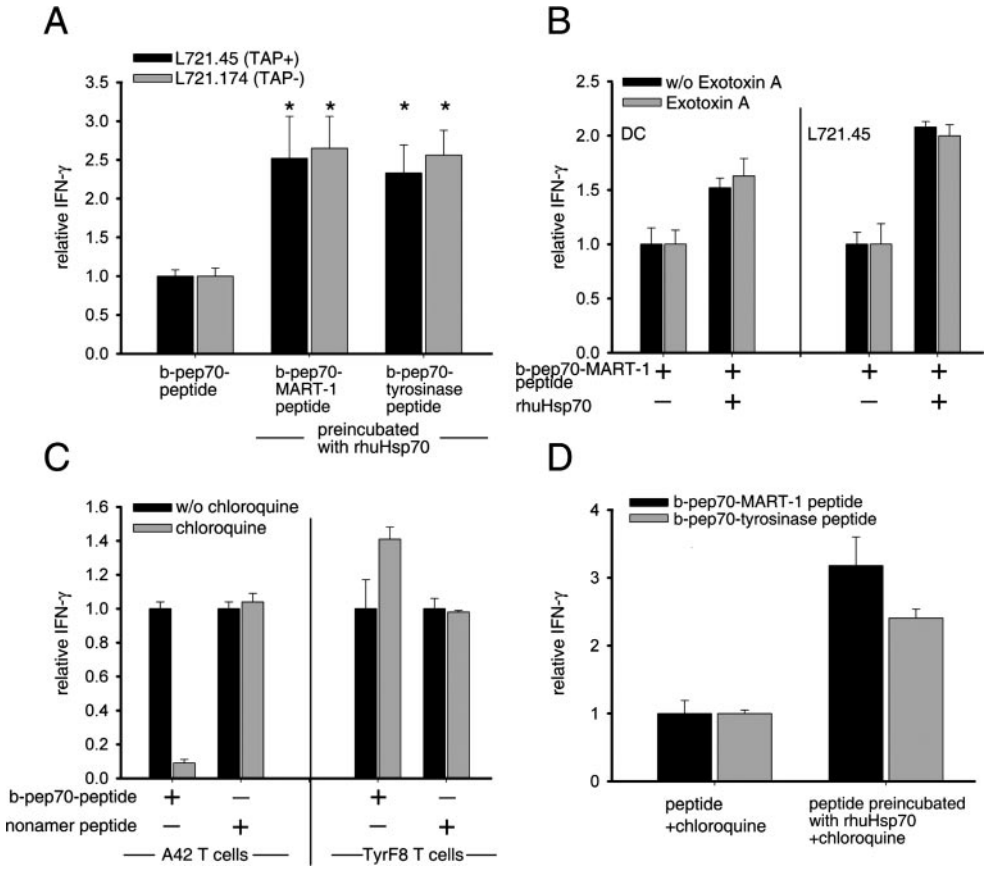

FIGURE 4. **rhuHsp70 enhances cross-presentation of peptides independent of post-uptake antigen processing steps and trafficking pathways.** *A,* TAP-positive L721.45 or TAP-negative L721.174 were pulsed with b-pep70-MART-1 peptide (70 nM) or b-pep70-tyrosinase peptide (10 μM) or rhuHsp70·peptide complexes formed by preincubation (4 h) of 0.3 μM rhuHsp70 with 70 nM b-pep70-MART-1 peptide or 3 μM rhuHsp70 with 10 μM b-pep70-tyrosinase peptide. A42- or TyrF8-T cells were added, and the amount of IFN-γ release was measured after 24 h by ELISA. *Bars* represent the mean values of three independent experiments ± M.D. Statistical significance was calculated as described for Fig. 1. (*, $p < 0.005$; $n = 3$). *B,* DCs or L721.45 were pulsed with b-pep70-MART-1 peptide (70 nM) alone or preincubated (4 h) with 1 μM rhuHsp70 in the absence or presence of exotoxin A (10 μg/ml). After 90 min, A42-T cells were added. *Bars* show the relative IFN-γ release normalized to the sample without rhuHsp70. *C,* DCs were pulsed in the absence or presence of chloroquine (10 μg/ml) with nonamer peptides MART-1 (aa27–35) (12 μM) or tyrosinase (aa368–376) (10 μM) or the b-pep70-peptides (70 nM for b-pep70-MART-1 peptide or 10 μM for b-pep70-tyrosinase peptide). A42- or TyrF8-T cells were added, and the IFN-γ release was measured 24 h later. *D,* DCs were pulsed in the presence of chloroquine (10 μg/ml) with b-pep70-peptides (70 nM for b-pep70-MART-1 peptide or 10 μM for b-pep70-tyrosinase peptide) alone or preincubated (4 h) with 1 μM rhuHsp70. A42- or TyrF8-T cells were added and IFN-γ release was measured 24 h later. Values were normalized to sample containing peptide alone. *Bars* represent the mean of triplicates ± M.D. and are one representative experiment of two.

capacity of DCs for macropinocytosis and up-regulated HLA-A2 expression consistent with its known activity to induce DC maturation.

rhuHsp70 Preparation Containing Nucleotides Are Ineffective in Cross-presentation—Published procedures for Hsp70·peptide complex formation generally adjust the reaction mixture to millimolar concentrations of ADP (60, 66) as ATP and ADP play crucial roles in the substrate binding cycle of Hsp70 (52–54). Nucleotides are known to activate a variety of immune cells (67–70). We observed that ADP induced calcium signals in GM-CSF/IL-4 differentiated myeloid DCs, but not in T cells or the B cell lines (Fig. 6*A*). Furthermore, we observed that ADP lowered the macropinocytosis activity of DCs as evidenced by a reduced BSA-FITC uptake by DCs (Fig. 6*B*). Importantly, the inhibitory effect of ADP occurred very quickly as the same reduction of macropinocytosis was observed when ADP was given simultaneously with BSA to DCs without preincubation. ADP did not affect the presentation of the nonamer MART-1(LAGIGILTV) peptide (Fig. 6*C*), which binds directly to surface MHC class I molecules. Thus, ADP does not reduce the general ability of DCs to stimulate T cell responses nor does it inhibit T cells to secrete IFN-γ after activation through MHC class I·peptide complexes on the surface of DCs. The addition of ADP to rhuHsp70·peptide complexes or b-pep70-MART-1-peptide without rhuHsp70 reduced the capacity of DCs to cross-present in a dose-dependent manner (Fig. 6, *D* and *E*). As we identified macropinocytosis as the dominant uptake mechanism for exogenous antigens leading to cross-presentation, its suppression by ADP is one explanation for the reduced T cell stimulation. Because many commercial ADP solutions contain trace amounts of ATP, which is known to induce peptide dissociation from Hsp70·peptide complexes (71), the Hsp70-mediated cross-presentation and T cell stimulation may be additionally inhibited on the level of Hsp70·peptide complex formation, which was, however, not investigated in this study.

Because of the inhibitory activity of ADP in cross-presentation, our rhuHsp70·peptide mixtures were not supplemented with nucleotides, and we carefully dialyzed the recombinant protein after purification. The resultant rhuHsp70 did not influence the capacity of the DCs for cross-presentation as documented with the sample containing b-pep70-MART-1 peptide and rhuHsp70 without preincubation (Fig. 1*B*).

DISCUSSION

Heat shock proteins, in addition to their known housekeeping roles as chaperones, have been shown to stimulate an immune response to bound cellular peptides (6, 19). This response is thought to have two components, the delivery of antigen for cross-presentation on the MHC class I molecules of DCs and the stimulation of DCs to secrete proinflammatory cytokines and express costimulatory molecules, thus creating the immunogenic environment required for the induction of

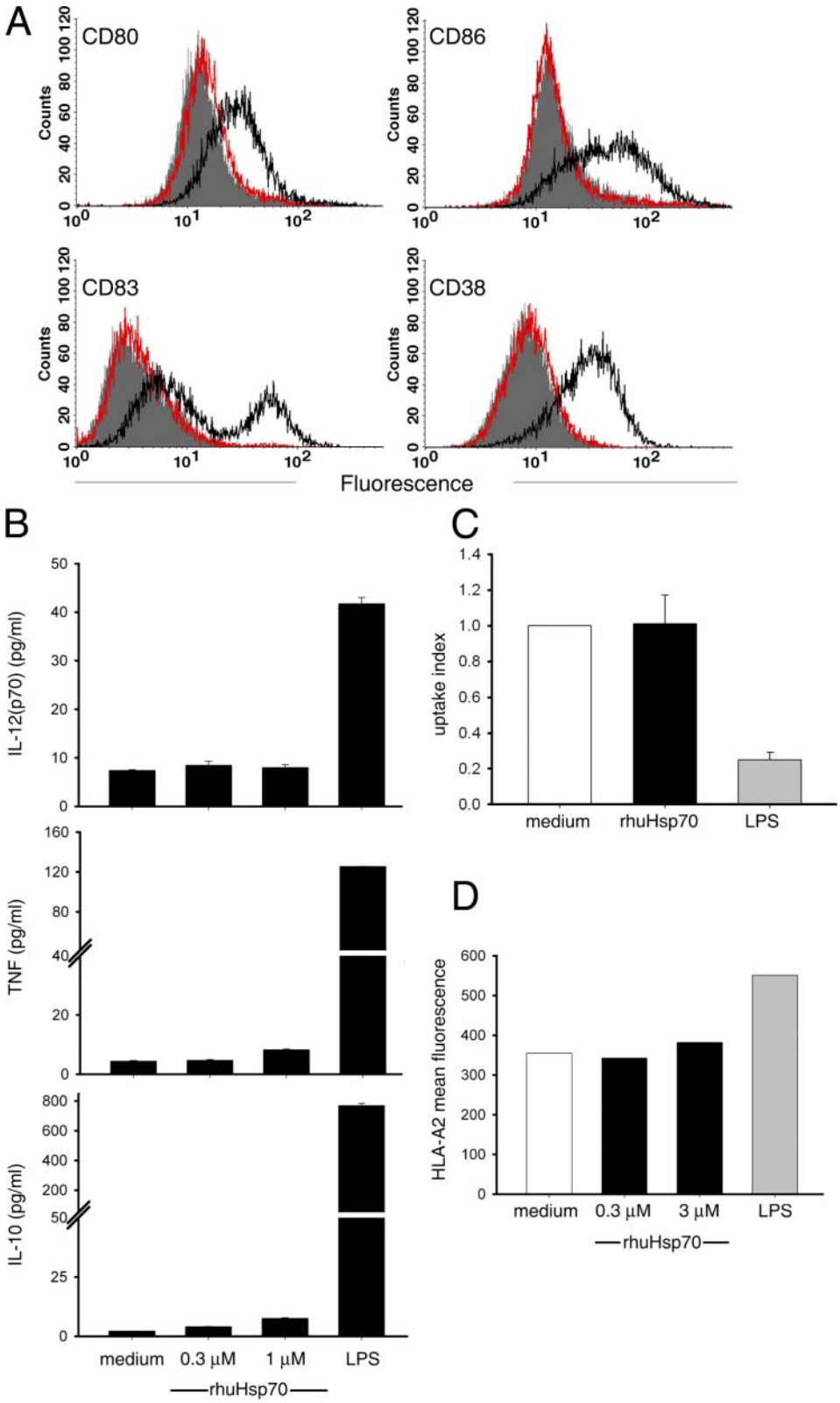

FIGURE 5. **rhuHsp70 does not change phenotypic markers, cytokine secretion, macropinocytosis, or surface expression of MHC class I molecules of DCs.** Human GM-CSF/IL-4 differentiated nonmature myeloid DCs (day 7) were incubated with rhuHsp70, LPS, or without stimulus for 48 h. *A,* phenotypic markers, CD80, CD86, CD83, or CD38, were determined by FACS analysis. *Histograms* represent the medium control (*gray filled*), 0.3 μM rhuHsp70 (*red line*), and LPS (1 μg/ml) (*black line*). *B,* secretion of IL-12(p70), tumor necrosis factor, and IL-10 was measured using Bio-Plex. The results of *A* and *B* are one representative of four experiments. *C,* GM-CSF/IL-4 differentiated nonmature myeloid DCs were incubated with 0.3 μM rhuHsp70, LPS (1 μg/ml), or without stimulus. After 24 h cells were washed and the uptake of BSA-FITC (added for 90 min at 37 °C or on ice) was analyzed by FACS. The uptake was calculated by subtracting the fluorescence of cells incubated on ice from the fluorescence of cells incubated at 37 °C. The uptake index is the relative net mean fluorescence ± M.D. of three independent experiments normalized to medium samples. *D,* expression of HLA-A2 by DCs, stimulated with LPS (1 μg/ml) or rhuHsp70 for 24 h, determined by FACS analysis. Results are shown as the mean fluorescence with the fluorescence of the isotype control subtracted.

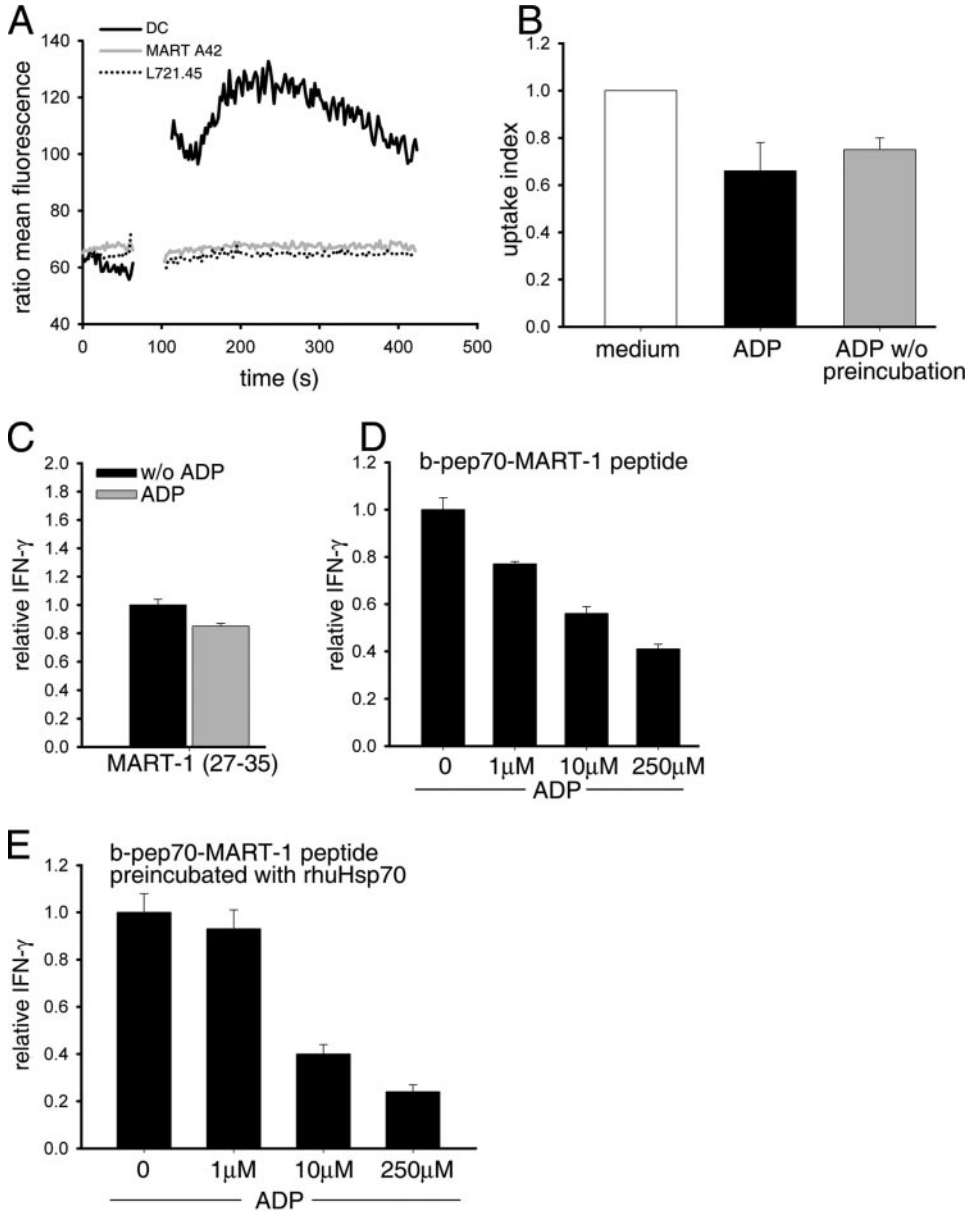

FIGURE 6. **Nucleotides affect DC function and rhuHsp70-mediated cross-presentation.** *A,* ADP activates a calcium response in DCs, but not L721. 45 B cell line or A42-T cells. Cells were incubated with 250 μM ADP, and the calcium-induced fluorescence shift was determined with MoFlo. ADP was added after cells were measured for 1 min without stimulation for base-line determination. The *y* axis depicts the ratio of 405/430 nm that is proportional to the intracellular ionized calcium. *B,* DCs were either pretreated with 250 μM ADP for 24 h and then washed and analyzed for BSA-FITC uptake or were used untreated (without preincubation), and ADP was given during BSA-FITC uptake. The uptake index (mean of three independent experiments) was calculated as described for Fig. 5*C*. *C,* DCs were pulsed with the nonamer peptide MART-1 (aa27–35) (12 μM) in the presence or absence of 250 μM ADP. The secretion of IFN-γ by A42-T cells was measured. Values were normalized to the sample with peptide alone without ADP. *Bars* represent the mean of triplicates ± M.D. *D,* DCs were pulsed with 70 nM b-pep70-MART-1 peptide in the presence of indicated concentrations of ADP. A42-T cells were added and the secretion of IFN-γ was measured. Results represent the values ± M.D. of triplicates normalized to the sample without ADP. *E,* 70 nM b-pep70-MART-1 peptide were preincubated (4 h) with 0.3 μM rhuHsp70, and indicated amounts of ADP and then DCs and A42-T cells were added. The IFN-γ release was measured after 24 h. *Bars* are the mean of triplicates ±M.D. of one representative experiment of two.

adaptive CD8$^+$ T cell responses. Recent reports indicating that nonproteinaceous products, such as LPS, lectins, and flagellin (21–27), can copurify with recombinant HSPs, including Hsp70, Hsp60, or gp96, and cause changes in DC phenotype and cytokine profile raised concern about the contribution of HSPs to the observed immune stimulation.

Here we demonstrate that highly purified, endotoxin- and nucleotide-depleted rhuHsp70 significantly enhances cross-presentation of exogenous antigenic peptides by DCs and other APCs, such as B cell lines, and antigen-specific T cell activation occurred without the upregulation of costimulatory molecules or changing the cytokine profile of DCs. Complex formation between the exogenous antigen and rhuHsp70 was defined as the key requirement for rhuHsp70-assisted cross-presentation. Thus, the immunological activity of rhuHsp70 relates directly to its intrinsic chaperone activity and not to signaling of danger or general enhancement of APC function.

Complex formation of exogenous peptide to rhuHsp70 enhanced the intracellular level of antigen in APCs (Fig. 7). Thus, the delivery of more antigen to the MHC class I cross-presentation pathway is the key to the improved capacity of APCs to activate antigen-specific T cells. Mechanistically, an important role of macropinocytosis for rhuHsp70 uptake and delivery of Hsp70-chaperoned peptides is implicated by its inhibition by DMA and the similarity in trafficking routes of rhuHsp70 and BSA but not transferrin. However, rhuHsp70 itself did not increase the macropinocytosis activity of DCs, and the presence of rhuHsp70 was not sufficient, but rather the formation of rhuHsp70·peptide complexes was required to augment cross-presentation of exogenous antigens. Thus, there are apparently additional components involved in the uptake of rhuHsp70.

A number of studies implicate that surface receptors, in particular CD91, play an important role in the uptake process of Hsp70 (58–61). Our B cell lines did not express CD91, but nevertheless efficiently internalized rhuHsp70 or rhuHsp70·peptide complexes, indicating that CD91 is not necessary for rhuHsp70 uptake and enhanced cross-presentation. Consistent with our results, Calderwood *et al.* (72) did not observe binding of Hsp70 to CD91-transfected cell lines and uptake of mycobacterial Hsp65 fusion protein-ova by murine DCs was also apparently

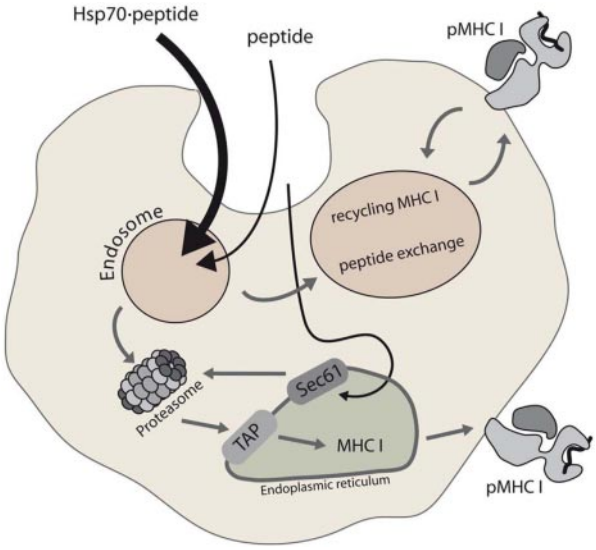

FIGURE 7. **Role of rhuHsp70 in antigen cross-presentation.** Through complex formation with rhuHsp70, exogenous peptides are more efficiently internalized than the peptide alone. Hsp70·peptide complexes reach endosomal compartments that partly overlap with vesicles containing recycling MHC class I·peptide complexes (pMHC-I). Thus, Hsp70·peptide complexes are correctly positioned for the exchange of Hsp70-chaperoned peptides with MHC class I-bound peptides. Downstream pathways of cross-presentation include the following: (i) cytosolic release of peptide from endosomal compartments, followed by proteasomal digestion and peptide transport into the ER through TAP proteins, or (ii) ER-to-cytosol retrotranslocation (involving sec61) (1, 2, 28, 29). rhuHsp70 exerts its enhancing effects proximal to these steps and is therefore independent of TAP and sec61 transports.

independent of CD91 (56). Although we did not find evidence for an involvement of cell surface receptors in the process of rhuHsp70 uptake, we observed, using single-molecule tracing, preferred membrane regions for rhuHsp70 entry into cells. Whether these regions relate to lipid rafts, which have been described as entry ports for Hsp70 (73), was not defined in our study.

Complex formation could also protect exogenous antigens from extracellular proteolytic cleavage thereby prolonging antigen half-life and increasing the available concentration of exogenous antigen for presentation (74). The supplementation of culture medium with reducing agents known to inhibit proteolytic activity did not increase the efficacy of cross-presentation of our peptides (not shown), suggesting that peptide degradation was not a limiting factor for their cross-presentation in our system.

Although the precise process resulting in higher intracellular levels of exogenous antigen complexed to rhuHsp70 remains to be determined, we document that this process does not require rhuHsp70-induced cytokine secretion and up-regulation of costimulatory molecules, which are being implicated to contribute to Hsp70 immune activation. Our highly purified endotoxin-depleted rhuHsp70 had no detectable activating capacity for *in vitro* generated GM-CSF/IL-4 myeloid DCs. Because we found that ADP strongly suppressed macropinocytosis, and thus the ability of DCs for cross-presentation, we used intensively dialyzed, ADP-reduced, rhuHsp70 in our cross-presentation assays. However, it is know that coordinated ATP/ADP binding and hydrolysis are important for high affinity substrate binding, and we demonstrated, by using γATP, that inhibition of the ATP/ADP hydrolysis cycle prevented the ability of rhuHsp70 to enhance cross-presentation. Based on this knowledge we propose that for optimal rhuHsp70·peptide complex formation and thus full utilization of the capacity of rhuHsp70 as an antigen delivery tool in cross-presentation, the nucleotide-dependent substrate binding cycle should be considered, but free nucleotides need to be carefully removed before exposing DCs to the rhuHsp70·peptide complexes.

Enhanced delivery of exogenous antigens to APCs by rhuHsp70 directly translated to better T cell responses. Thus, this activity of rhuHsp70 is desirable for vaccine generation. As evidenced using the b-pep70-tyrosinase peptide, rhuHsp70-mediated augmentation could be particularly influential in conditions of low peptide concentration and for peptides that are poorly cross-presented naturally. Clinically, by endowing antigenic sequences with binding activity to rhuHsp70 thus facilitating their cross-presentation, T cell immunity can be redirected to new and desirable epitopes. Binding of antigen to rhuHsp70 can be ensured by colinearly synthesizing the T cell epitopes with short hydrophobic Hsp70-binding sequences, in our system HWDFAWPW or optimized sequences as reported recently (75). Using the Hsp70-binding sequence, many different antigenic peptides can be linked to rhuHsp70, and this formulation can be used to simultaneously deliver multiple antigens with high efficacy to a single DC population. These DCs can function as a multiplex vaccine for the stimulation of a complex antigen-specific T cell response, which is the goal to prevent the development of tumor escape variants.

Utilization of rhuHsp70 as an antigen delivery vehicle has several additional advantages. Enhanced cross-presentation was seen for different APCs, including B cells that are considered to have poor cross-presentation capacity (1, 30). Being a self-protein, toxicity issues are unlikely to occur, whereas undesirable toxicity is reported for other antigen delivery systems, like nanoparticle or liposome, which are made of synthetic polymers (76, 77). Because the enhancing effect of rhuHsp70 is due to facilitated antigen uptake, *i.e.* lies proximal to intracellular antigen processing and trafficking, rhuHsp70 enhances cross-presentation of different antigenic peptides even if they require different enzymatic processing or intracellular trafficking (Fig. 7). This bestows rhuHsp70 with a broader applicability as antigen delivery vehicle compared with bacterial toxins or human immunodeficiency virus TAT proteins that have a more restricted activity, in that they facilitate the pathway of cytosolic release (78–81). Finally, the observation that highly purified rhuHsp70 is apparently inert to APC function, *i.e.* it does not stimulate DC maturation or cytokine secretion, offers the possibility for its utilization as an antigen delivery vehicle in various settings, including the induction of antigen-specific immunity or tolerance.

In summary, we reestablished that rhuHsp70 plays a role in T cell immunity. The enhanced immunogenicity of the exogenous antigen in the presence of rhuHsp70 is the consequence of complex formation resulting in better uptake and intracellular delivery of the chaperoned antigens compared with uncomplexed peptide. rhuHsp70 may be a useful antigen delivery vehicle for a wide variety of antigens, applicable for different types

of APC and in various settings of immune modulation. These results not only provide novel insights into the mechanism by which rhuHsp70 stimulates T cell responses but also deliver clinically applicable approaches to improve vaccine efficacies.

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