The Journal of Immunology

Melanoma-Reactive Class I-Restricted Cytotoxic T Cell Clones Are Stimulated by Dendritic Cells Loaded with Synthetic Peptides, but Fail to Respond to Dendritic Cells Pulsed with Melanoma-Derived Heat Shock Proteins In Vitro

Kristina Fleischer, Burkhard Schmidt, Wolfgang Kastenmüller, Dirk H. Busch, Ingo Drexler, Gerd Sutter, Michael Heike, Christian Peschel and Helga Bernhard

J. Immunol. 2004;172;162-169
http://www.jimmunol.org/cgi/content/full/172/1/162

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Melanoma-Reactive Class I-Restricted Cytotoxic T Cell Clones Are Stimulated by Dendritic Cells Loaded with Synthetic Peptides, but Fail to Respond to Dendritic Cells Pulsed with Melanoma-Derived Heat Shock Proteins In Vitro[1]

Kristina Fleischer,[2]* Burkhard Schmidt,[2]* Wolfgang Kastenmüller,* Dirk H. Busch,† Ingo Drexler,‡ Gerd Sutter,‡ Michael Heike,§ Christian Peschel,* and Helga Bernhard[3]*

Immunization with heat shock proteins (hsp) isolated from cancer cells has been shown to induce a protective antitumor response. The mechanism of hsp-dependent cellular immunity has been attributed to a variety of immunological activities mediated by hsp. Hsp have been shown to bind antigenic peptides, trim the bound peptides by intrinsic enzymatic activity, improve endocytosis of the chaperoned peptides by APCs, and enhance the ability of APCs to stimulate peptide-specific T cells. We have investigated the potential capacity of hsp70 and gp96 to function as a mediator for Ag-specific CTL stimulation in an in vitro model for human melanoma. Repetitive stimulation of PBLs by autologous DCs loaded with melanoma-derived hsp did not increase the frequency of T cells directed against immunodominant peptides of melanoma-associated Ags Melan-A and tyrosinase. In contrast, repeated T cell stimulation with peptide-pulsed DCs enhanced the number of peptide-specific T cells, allowing HLA/peptide multimer-guided T cell cloning. We succeeded in demonstrating that the established HLA-A2-restricted CTL clones recognized HLA-A2$^+$ APCs exogenously loaded with the respective melanoma peptide as well as melanoma cells processing and presenting these peptides in the context of HLA-A2. We were not able to show that these melanoma-reactive CTL clones were stimulated by autologous dendritic cells pulsed with melanoma-derived hsp. These results are discussed with respect to various models for proving the role of hsp in T cell stimulation and to recent findings that part of the immunological antitumor activities reported for hsp are independent of the chaperoned peptides. *The Journal of Immunology,* **2004, 172: 162–169.**

Spontaneous regressions of malignant tumors, such as melanoma and renal cell cancer, indicate that an effective tumor-directed immune response may be triggered in some patients (1, 2). This hypothesis was supported by the detection of a tumor-specific cellular and humoral response in melanoma patients (3, 4). However, new technologies have provided evidence that Ag-specific T cells present at high levels in the blood or in tumor-draining lymph nodes of melanoma patients have functional defects (5–7). Different strategies have been developed to restore the tumor-directed immune responses of patients such as cancer vaccines and adoptive T cell transfer (for review, see Refs. 8 and 9). The major goal of both approaches is the induction and maintenance of an Ag-specific cellular immune response to eliminate tumor cells.

Vaccination with hsp isolated from cancer cells has been shown to elicit a tumor-specific immunity (10–12). The specificity of this immune response has been attributed to peptides associated with the heat shock protein (hsp)[4], such as hsp70 and gp96 glucose-regulated protein 94 (11, 13). It has been shown that hsp released from necrotic tumor cells (14) are efficiently taken up via receptor-mediated endocytosis by APCs (15, 16). Hsp-peptide complexes internalized via the receptor-mediated pathway gain access to the HLA class I Ag presentation pathway, yielding re-presentation of hsp-associated peptides on APC HLA class I molecules (for review, see Ref. 17). In addition to their chaperone function, hsp have been reported to directly stimulate monocytes and dendritic cells (DCs) to secrete cytokines and up-regulate HLA and costimulatory molecules (18–21). The capacity of hsp to mediate the uptake and processing of chaperoned peptides by APCs and to directly activate these APCs is thought to be the key mechanism responsible for effective priming and cross-priming of tumor-specific CTLs in vivo (22, 23).

Based on these findings, we investigated the potential recognition of tumor-derived hsp by human T cells by imitating in vitro the hypothetically physiological interaction of APCs, peptide-specific CTLs, and hsp-bound peptide Ags. The melanoma-associated Ags tyrosinase (Tyr) and Melan-A were used as model Ags to address this question. Ag-specific CTLs were generated using an optimized method of stimulating, sorting, cloning, and expanding CTLs. The established HLA-A2-restricted CTL clones recognized

Departments of *Hematology/Oncology, †Microbiology and Immunology, and ‡Virology, Klinikum rechts der Isar, Technical University of Munich, Munich, Germany; and §Department of Gastroenterology, University of Mainz, Mainz, Germany

Received for publication January 15, 2003. Accepted for publication November 4, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work has been supported by grants from the German Cancer Society (to H.B.), the Research Council of Germany (Sonderforschungsbereich 456) (to H.B. and G.S.), and the GSF National Research Center for Environment and Health-Clinical Cooperation Group "Vaccinology" (to H.B, D.H.B., and G.S.). This work represents essential parts of the Ph.D. thesis of K.F.

[2] K.F. and B.S. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Helga Bernhard, III. Medizinische Klinik, Klinikum rechts der Isar, Technical University of Munich, Ismaningerstraβe 22, D-81675 Munich, Germany. E-mail address: helga.bernhard@lrz.tu-muenchen.de

[4] Abbreviations used in this paper: hsp, heat shock protein; DC, dendritic cell; LCL, B lymphoblastoid cell line; Tyr, tyrosinase; VSV, vesicular stomatitis virus.

0022-1767/04/$02.00

the Tyr respective Melan-A peptides exogenously loaded onto autologous DCs as well as endogenously presented by HLA-A2-matched melanoma cells. In contrast, the established CTL clones were not able to recognize DCs loaded with hsp70 and gp96, both isolated from Ag-positive melanoma cells. Moreover, stimulation of PBLs with hsp-loaded autologous DCs did not result in elevated numbers of peptide-specific CTLs recognizing Melan-A or Tyr. However, the failure of Ag-specific T cells to respond to hsp in vitro does not preclude the concept of hsp as a polyvalent tumor vaccine in vivo, because complementary or alternative mechanisms might be relevant for hsp-mediated tumor rejection in vivo (for review, see Ref. 24).

Materials and Methods

Cell lines

The HLA-A*0201$^+$ (HLA-A2$^+$), Tyr$^+$, Melan-A$^+$ melanoma cell line SK-MEL29 was provided by L. Old (Memorial Sloan-Kettering Cancer Institute, New York, NY), and the HLA-A2$^+$, TAP$^-$-deficient cell line T2 by P. Cresswell (Yale University, New Haven, CT). The HLA-A2$^+$ Tyr$^-$ Melan-A$^-$ melanoma cell line NW-MEL38, the Tyr$^-$ Melan-A$^-$ erythroid leukemia cell line K562, and the B-lymphoblastoid cell lines SK-LCL29 and LCL-Alex-B were a generous gift from A. Knuth and E. Jäger (Krankenhaus Nordwest, Frankfurt, Germany). The HLA-A2$^-$, Tyr$^+$, Melan-A$^+$ melanoma cell line MUC-MEL1 was established from a lymph node metastases derived from a melanoma patient. The Tyr$^-$, Melan-A$^-$ LCL-SuHi was generated by EBV transformation of peripheral blood B cells from an HLA-A2$^+$ healthy donor. LCLs, K562 cells, and T2 cells were cultured in RPMI 1640. Melanoma cell lines were cultured in DMEM (Life Technologies, Paisley, Scotland). Media were supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Purification of hsp

The hsp gp96 was purified from melanoma cell line SK-MEL29 and the autologous cell line SK-LCL29 using a purification method originally described for murine gp96 by Srivastava et al. (25), with slight modifications, as described later by the same group (26). In brief, cell lines were snap frozen and stored at −80°C. Cell pellets were thawed at 4°C, homogenized in hypotonic buffer (30 nM $NaHCO_3$ and 0.2 mM PMSF, pH 7.1) by Dounce homogenization (Kontes, Vineland, NJ), and centrifuged at 100,000 × *g*. The supernatant was subjected to gradual ammonium sulfate precipitation. The precipitate at 80% ammonium sulfate was resuspended and applied to an equilibrated Con A affinity column (Con A-Sepharose; Pharmacia, Uppsala, Sweden). Glycoproteins were eluted with 10% methyl-α-D-mannopyranoside. Methyl-α-D-mannopyranoside remained in the eluate to avoid the generation of gp96/Con A complexes. The partially purified gp96 material was further applied to DEAE anion exchange column (DEAE-Sephacel; Pharmacia) that had been equilibrated with 0.3 M NaCl in 5 mM sodium phosphate buffer (pH 7.0), and gp96 was eluted with 0.7 M NaCl in 5 mM sodium phosphate buffer. The buffer of gp96 preparation was exchanged to PBS by ultrafiltration (Biomax-30 kDa and Biomax-50 kDa; Millipore, Bedford, MA), thereby achieving final protein concentrations of 2 mg/ml.

Hsp70 was isolated from the Melan-A$^+$, Tyr$^+$ melanoma cell line SK-MEL29 and from the Melan-A$^-$, Tyr$^-$ erythroid leukemia cell line K652, as described (27, 28). Briefly, a 100,000 × *g* supernatant was prepared from tissue homogenate and applied to Blue Sepharose CL-6B (Amersham Pharmacia, Uppsala, Sweden) for albumin removal. The eluate was applied to an ADP-agarose affinity column (Sigma-Aldrich, Taufkirchen, Germany). The ADP-binding protein fraction was eluted with 3 mM ADP and applied to a DEAE-Sepharose anion-exchange column (Amersham Pharmacia) that had been 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.

The purity of gp96 and hsp70 was checked by SDS-PAGE and immunoblotting. The biological activities of gp96 and hsp70 preparations were confirmed by testing the peptide-binding activity of both hsp, as previously described (26, 27). Despite these quality controls, it cannot formally rule out the possibility that associated peptides might be partially removed during the purification procedure. Hsp70 purified from Melan-A$^+$, Tyr$^+$ melanoma cell line Mel624.38 (29) was kindly provided by R. Issels and E. Noessner (Ludwig Maximilian University, Munich, Germany) (30).

Flow cytometric analysis

Synthesis of PE-labeled HLA-A2/peptide multimer complexes was performed, as previously described (31, 32). For the detection of Melan-A_{27-35}-specific T cells, the multimer consisting of the Melan-A_{26-35}A27L analog (ELAGIGILTV) (33) was used, which has a higher binding affinity to HLA-A2 and a higher immunogenicity than the natural Melan-A nonapeptide (AAGIGILTV) (34). The naturally occurring $Tyr_{369-377}$ peptide (35) was used for generating multimers to detect HLA-A2-restricted $Tyr_{369-377}$-specific CTLs. A total of 1×10^6 T cells was incubated with PE-labeled HLA-A2/peptide multimer for 45 min on ice in 50 μl buffer (PBS 1% BSA, pH 7.4). Then anti-CD8 FITC (Caltag Laboratories, Burlingame, CA) was added. After 15 min, the cells were washed three times and resuspended in buffer with propidium iodide (PI) (2 μg/ml). PI-negative cells were gated, and frequencies of multimer$^+$ CD8$^+$ T cells were determined by using Coulter Epics XL (Beckmann-Coulter, Hialeah, IL).

Multimer-guided cloning and expansion of human melanoma-reactive CTLs

HLA-A2-restricted $Tyr_{369-377}$- and Melan-A_{27-35}-specific CTLs were generated by repetitive stimulation with mature monocyte-derived DCs (36) as APCs. Mature DCs were incubated with 10 μg/ml $Tyr_{369-377}$ or Melan-A_{27-35} for 2 h at room temperature and then cocultured with autologous PBMCs in 200 μl/well RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 5% autologous serum in 96-well round-bottom plates. The medium was further supplemented with 5 ng/ml human rIL-7 (BD PharMingen, San Diego, CA) on day 1, and 20 U/ml human rIL-2 (Chiron Behring, Marburg, Germany) on day 4. Responding T cells were restimulated with peptide-pulsed DCs at weekly intervals in the presence of rIL-2 and rIL-7. The stimulator to responder cell ratio was 1:20 for priming and 1:40 for restimulation. The $Tyr_{369-377}$- and the Melan-A_{27-35}-specific CTLs were stained using A2/Melan-A_{26-35}A27L multimers after three and five stimulations, respectively. The stimulated T cells were resuspended in cold PBS without PI and filtrated with 30 μm nylon. For sorting, a Moflo cell sorter (Cytomation, Fort Collins, CO) was used with 25,000 events/s and maximum 1 ψ. Sorted CD8$^+$ multimer$^+$ T cells were directly cloned by limiting dilution, as previously described (37, 38). In brief, T cells were seeded at 0.3 cell/well in 96-well round-bottom plates with RPMI added with 10% human serum, 30 ng/ml anti CD3 mAb (Okt-3; Janssen, Cilag, Neuss, Germany), 5×10^4/well allogeneic irradiated (30 Gy) PBMCs, 10^5 irradiated (80 Gy) LCLs Alex-B, and 50 U/ml rIL-2. Proliferating CTL clones were screened for lytic activity in a microcytotoxicity assay using T2 cells pulsed with the corresponding peptide ($Tyr_{369-377}$ or Melan-A_{27-35}) or the irrelevant peptide derived from the reverse transcriptase of HIV-1 (ILKEPVHGV) ($HIV_{476-484}$). Expansion of peptide-specific CTLs was conducted every 2 wk in the presence of anti-CD3, LCL, PBMCs, and rIL-2, as previously described (39).

T cell stimulation assay

Immature DCs from HLA-A2$^+$ donors were pulsed with 30 μg/ml of hsp70 or gp96, both derived from Melan-A$^+$ melanoma cell line SK-MEL29. Hsp-pulsed immature DCs were further matured into CD83$^+$ DCs in the presence of cytokines, as previously described (36). Hsp-loaded mature DCs were then used as APCs for stimulating autologous PBMCs. As a positive control, PBMCs from the same donor were separately stimulated with DCs loaded with 10 μg/ml of the Melan- A_{26-35}A27L analog (ELAGIGILTV) (33). Responding T cells were restimulated three times with hsp-loaded DCs at weekly intervals in the presence of rIL-2 and rIL-7. Following four rounds of stimulation, CD8$^+$ T cells were stained using A2/Melan-A_{26-35}A27L multimers (see above/below).

Cytotoxicity assay

Cytolytic activity was analyzed, as described previously (40). Immature DCs were loaded with different concentrations of gp96 or hsp70, both derived from either Tyr$^+$ Melan-A$^+$ melanoma cells or Tyr$^-$ Melan-A$^-$ nonmelanoma cells. Gp96 was purified from melanoma cell line SK-MEL29 (Mel-gp96) and from SK-LCL29 (control-gp96); hsp70 was isolated from melanoma cell line Mel624.38 (Mel-hsp70) (provided by E. Noessner) and from leukemia cell line K562 (control-hsp70). Hsp-pulsed immature DCs were subsequently matured into CD83$^+$ DCs, then labeled with ^{51}Cr (5×10^5 cells in 100 μl FCS with 100 μCi/ml) (ICN Biochemicals, Irvine, CA) and used as target cells for CTLs. Peptide-loaded DCs or T2 cells were first labeled with ^{51}Cr (5×10^5 cells with 100 μCi/ml) and then loaded with 10 μg/ml peptide for 1 h at room temperature. Tumor cells, LCL (10^6), were labeled with 100 μCi/ml ^{51}Cr for 1 h at 37°C. ^{51}Cr-labeled target cells were cultured with graded doses of T cells in 200

μl/well RPMI with 10% FCS in V-bottom 96-well tissue culture plates (Costar, Cambridge, MA). After 4 h of incubation at 37°C, 100 μl of supernatant was collected, and radioactivity was measured in a gamma counter. The percentage of specific ^{51}Cr release was calculated, as described (40). SD was generally below 5% of the mean.

ELISA

To detect the IFN-γ production by the CTL clones, 2×10^4 T cells/well were cocultured with either 1×10^4 peptide-loaded T2 cells, peptide- or hsp-loaded DCs, or melanoma cells in 96-well round-bottom plates at 37°C. Details regarding the source of hsp and the loading procedure of DCs are described in the cytotoxicity assay paragraph above. After 24 h, supernatants were collected and IFN-γ production was determined using a commercially available ELISA (OptEIA; BD PharMingen).

Results

Peptide-specific cytolytic T cells generated by multimer-guided cloning recognize melanoma cells, which present endogenously processed peptides, but do not recognize DCs pulsed with melanoma-derived hsp

It has been previously shown in a mouse model that peptides stripped from hsp70 and gp96, which had been isolated from OVA-transfected cells, were able to stimulate OVA-specific CD8$^+$ T cells in vitro when loaded onto APCs (41). Based on these findings, we asked the question as to whether hsp isolated from melanoma cells are able to stimulate melanoma-reactive human T cells. Given that melanoma-reactive T cells are only present in low numbers in the peripheral blood, an improved method was developed to clone and expand melanoma-reactive T cells. PBLs from HLA-A2$^+$ healthy donors were stimulated with autologous DCs pulsed with peptides from the melanoma-associated differentiation Ags Melan-A (Fig. 1) and Tyr (Fig. 2). Following three to five stimulations, peptide-specific T cells were visualized by fluorochrome-labeled A2/peptide multimers, sorted, and directly cloned by limiting dilution. Five stimulations of PBMCs with Melan-A$_{27-35}$-pulsed DCs increased the frequency of A2/Melan-A$_{26-35}$A27L$^+$ T cells from <0.01 to 1.23% (Fig. 1, *A* and *B*). After cloning via the multimer-guided cloning technique, T cells from 7 of 240 seeded wells proliferated. These T cell clones were screened for Melan-A specificity using T2 pulsed with the relevant or irrelevant peptide as target cells for cytotoxicity analyses (data not shown). All seven T cell clones displayed a Melan-A$_{27-35}$-specific lytic activity and were subsequently expanded. One of the seven A2/Melan-A multimer$^+$ T cell clones, InRi1 (Fig. 1*C*), was used for further specificity analyses. CTL clone InRi1 lysed the melanoma cell line SK-MEL29 expressing Melan-A and HLA-A2 (Fig. 1*D*). CTL clone InRi1 did not lyse HLA-A2$^+$ Melan-A$^-$ melanoma cell line NW-MEL38 or HLA-A2$^-$ Melan-A$^+$ melanoma cell line MUC-MEL1, confirming Melan-A as the recognized Ag and HLA-A2 as the restriction element. Peptide specificity of CTL clone InRi1 was validated by the lysis of T2 cells pulsed with the relevant Melan-A peptide, but not with the irrelevant HIV peptide (Fig. 1*E*). In addition, HLA-A2$^+$ DCs were susceptible to CTL-mediated lysis after being loaded with synthetic Melan-A peptide (Fig. 1*F*). CTL clone InRi1 could be expanded 200- to 250-fold during a culture period of 2 wk. Ag specificity and lytic activity of the CTL clone InRi1 were retained after seven rounds of expansion (data not shown).

We next addressed the question as to whether Melan-A-specific CTL clone InRi1 was able to lyse DCs pulsed with hsp that had been isolated from Melan-A$^+$ melanoma cells. Immature DCs were pulsed with melanoma-derived gp96 (Mel-gp96) or hsp70 (Mel-hsp70), differentiated into mature DCs, and used as target cells for CTL-mediated lysis. Melan-A-specific CTL InRi1 did not lyse DCs following incubation with melanoma-derived gp96 or hsp70 (Fig. 1, *G* and *H*). Data are shown for DCs pulsed with 10 μg/ml gp96 or hsp70. Similar data were obtained for lower concentrations of hsp70 respective gp96 (0.01, 0.1, and 1 μg/ml) and for higher concentration of gp96 (30 μg/ml).

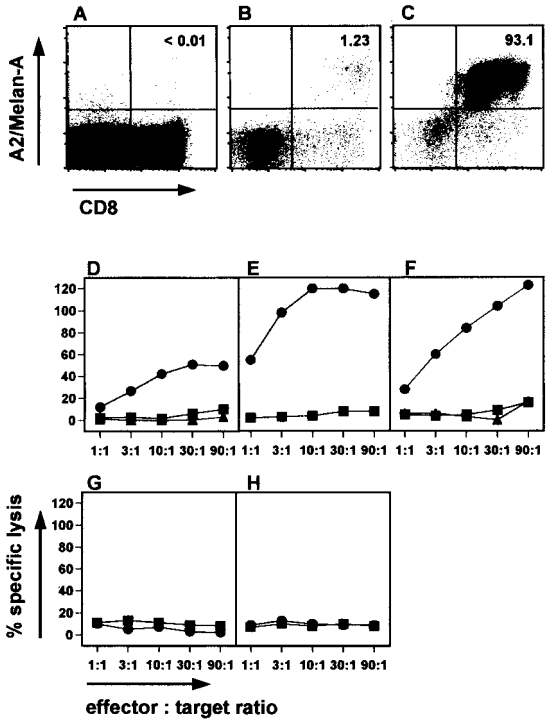

FIGURE 1. CTLs obtained by HLA-A2/Melan-A multimer-guided cloning recognize melanoma cells and Melan-A$_{27-35}$ peptide-loaded APCs, but not DCs pulsed with hsp70 or gp96 derived from Melan-A$^+$ melanoma. PBMCs from the HLA-A2$^+$ healthy donor InRi were stained with A2/Melan-A$_{26-35}$A27L (*A–C*). Dot plots are shown for CD8 vs A2/peptide multimer staining. Dot plots are gated on PI-negative PBMCs. The percentage of CD8$^+$ multimer$^+$ T cells gated on PI-negative PBMCs is stated in the *upper right quadrant*. *A*, A low frequency of circulating Melan-A-specific T cells was detected in peripheral blood. *B*, The frequency of multimer$^+$ T cells increased after five stimulations with Melan-A$_{27-35}$-pulsed autologous DCs. *C*, Following cloning of CD8$^+$ multimer$^+$ T cells, the Ag specificity of expanded CTL clones, such as InRi1, was visualized by A2/Melan-A multimer staining. Staining of cultured CTL clones did not reach 100% of CD8$^+$ multimer$^+$ cells due to remaining feeder cells. Lytic activity of CTL clone InRi1 was tested in a standard 4-h chromium release assay. Target cells were melanoma cells and T2 cells or DCs loaded with synthetic peptides or peptides naturally complexed to hsp (*D–H*). *D*, CTL clone InRi1 lysed the HLA-A2$^+$, Melan-A$^+$ SK-MEL29 cells (●); CTL clone InRi1 did not lyse HLA-A2$^+$ Melan-A$^-$ melanoma cell line NW-MEL38 (■), nor HLA-A2$^-$ Melan-A$^+$ melanoma cell line MUC-MEL1 (▲). *E*, CTL clone InRi1 recognized T2 cells loaded with 10 μg/ml of peptide Melan-A$_{27-35}$ (●), but not T2 cells pulsed with 10 μg/ml of the irrelevant peptide HIV$_{476-484}$ (■) derived from HIV reverse transcriptase. *F*, Autologous DCs were lysed by CTL clone InRi1 when loaded with peptide Melan-A$_{27-35}$ (●), but not when pulsed with peptide HIV$_{476-484}$ (■); autologous DCs not loaded with a peptide (▲) were used as an additional negative control. *G*, CTL clone InRi1 did not lyse DCs loaded with 10 μg/ml of gp96 derived from Melan-A$^+$ melanoma cell line SK-MEL29 (Mel-gp96) (●); DCs pulsed with 10 μg/ml of gp96 isolated from Melan-A$^-$ LCL (control-gp96) (■) were used as negative control. *H*, DCs were not lysed by CTLs InRi1 when loaded with 10 μg/ml hsp70 derived from Melan-A$^+$ melanoma cell line Mel624.38 (Mel-hsp70) (●); DCs loaded with hsp70 (10 μg/ml) isolated from Melan-A$^-$ K562 (■) were used as negative control (control-hsp70).

As a second approach, Tyr$_{369-377}$-specific CTLs were generated from the HLA-A2$^+$ healthy donor BS by repetitive stimulation with autologous DCs pulsed with the Tyr$_{369-377}$ peptide (Fig. 2).

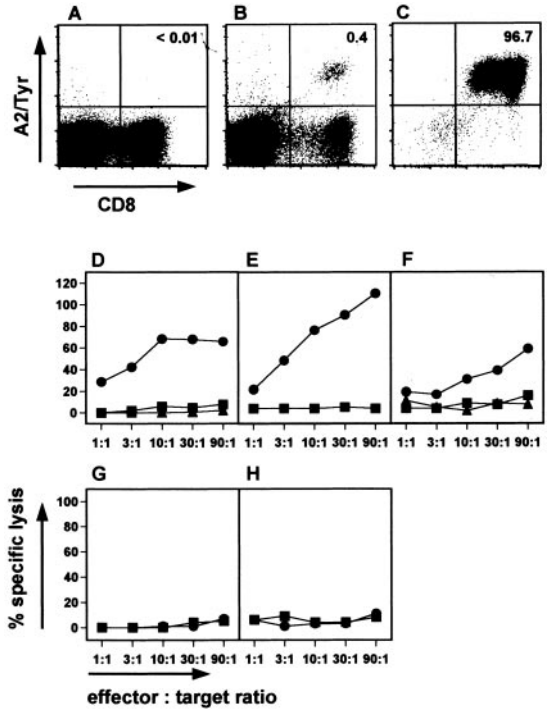

FIGURE 2. Cloning and expansion of Tyr-specific CTLs that lyse endogenously processed peptides and peptide-loaded target cells, but fail to lyse DCs pulsed with melanoma-derived hsp70 or gp96. PBMCs from HLA-A2$^+$ healthy donor BS were repetitively stimulated with autologous DCs pulsed with peptide $Tyr_{369-377}$ and cloned by limiting dilution following multimer-guided sorting. T cells were stained with HLA-A2/$Tyr_{369-377}$ multimers before stimulation (*A*), after three stimulations (*B*), and after multimer-guided cloning and expansion (*C*). All dot plots are gated on PI-negative PBMCs. *D*, $Tyr_{369-377}$-specific CTL clone BST5 lysed the HLA-A2$^+$ Tyr$^+$ SK-MEL29 cell line (●); CTL clone BST5 did not lyse HLA-A2$^+$ Tyr$^-$ melanoma cell line NW-MEL38 (■), nor HLA-A2$^-$ Tyr$^+$ melanoma cell line MUC-MEL1 (▲). *E*, Peptide specificity was confirmed by lysis of T2 when loaded with the relevant peptide $Tyr_{369-377}$ (●), but not when loaded with the irrelevant peptide $HIV_{476-484}$ (■). *F*, HLA-A2$^+$ DCs pulsed with $Tyr_{369-377}$ (●) were lysed, but not DCs loaded with $HIV_{476-484}$ (■) or DCs without peptide (▲). *G*, CTL clone BST5 did not lyse DCs pulsed with Mel-gp96 isolated from SK-MEL29 (●) or control-gp96 derived from SK-LCL29 (■). *H*, DCs pulsed with Mel-hsp70 isolated from Mel624.38 (●) or control-hsp70 purified from K562 (■) were also not recognized by BST5.

The baseline frequency of CD8$^+$ A2/Tyr multimer$^+$ T cells was less than 0.01% of all PBMCs (Fig. 2*A*). Three stimulations with peptide-pulsed DCs increased the frequency of A2/Tyr$^+$ T cells to 0.4% of all PBMCs. HLA-A2/Tyr$^+$ T cells were sorted and seeded out onto 350 wells. Thirty T cell clones proliferated and were tested for Tyr-specific lytic activity. Seven of 30 screened CTL clones specifically lysed T2 cells pulsed with peptide $Tyr_{369-377}$. The expanded CTL clones, such as CTL clone BST5, were specific for $Tyr_{369-377}$, as demonstrated by fluorochrome-labeled A2/Tyr multimers (Fig. 2*C*) and by lysis of peptide-pulsed T2 cells (Fig. 2*E*). The lysis of the HLA-A2$^+$ Tyr$^+$ melanoma cell line SK-MEL29 demonstrates that CTL clone BST5 recognizes endogenously processed peptides (Fig. 2*D*). HLA-A2-restricted, Tyr-specific CTL clone BST5 did not lyse HLA-A2$^+$ Tyr$^-$ melanoma cell line NW-MEL38 and HLA-A2$^-$ Tyr$^+$ melanoma cell line MUC-MEL1. CTL clone BST5 lysed DCs loaded with synthetic peptides (Fig. 2*F*), but did not lyse DCs pulsed with peptides potentially bound to melanoma-derived gp96 or hsp70 (Fig. 2, *G* and *H*). BST5 clone was expanded eight times, resulting in a 2000-fold expansion in total without loss of Ag specificity or functionality (data not shown).

The experiments using gp96-loaded DCs as target cells were also conducted with melanoma-reactive CTL clones IVS-B and W1/1 kindly provided by T. Wölfel (Medizinische Klinik Johannes, Gutenberg-Universitöt, Maine, Germany), The Melan-A- and Tyr-specific CTL clones were originally established by repetitive stimulation with autologous melanoma cells (42, 43). CTL clone IVS-B (recognizing Tyr) and CTL clone W1/1 (directed against Melan-A) were both unable to lyse DCs pulsed with Mel-gp96 (data not shown).

IFN-γ-producing melanoma-reactive T cells do not secrete IFN-γ upon stimulation with HLA-A2$^+$ DCs pulsed with melanoma-derived hsp

It has been shown previously that Ag-specific T cells that fail to lyse the Ag-expressing target cells are still able to specifically produce cytokines upon stimulation with the same target cells (44). Based on these findings, we next addressed the question as to whether the melanoma-specific CTL clones, which did not lyse Mel-hsp-pulsed DCs, were able to produce IFN-γ upon stimulation with DCs pulsed with Mel-hsp (Fig. 3). Immature DCs were pulsed with hsp70 or gp96, further matured into CD83$^+$ DCs in the presence of cytokines, and then used as stimulator cells for Melan-A- or Tyr-specific CTL clones. Coculture of Melan-A-specific CTL clone InRi1 with HLA-A2$^+$ DCs pulsed with Mel-gp96 or Mel-hsp70 did not result in a detectable IFN-γ release (Fig. 3*A*). Data are shown for DCs loaded with 10 μg/ml gp96 or hsp70. Similar data were obtained for lower concentrations of hsp70 or gp96 (0.01, 0.1, and 1 μg/ml) and for 30 μg/ml of gp96. As controls, IFN-γ release by CTL clone InRi1 was measured following coculture with Melan-A_{27-35}-loaded HLA-A2$^+$ DCs or T2 cells (positive control) or $HIV_{476-484}$-loaded HLA-A2$^+$ DCs or T2 cells (negative control). CTLs alone, CTLs cultured in the presence of nonpulsed DCs, or DCs loaded with hsp isolated from Melan-A$^-$ cells did not secrete any detectable IFN-γ. The same experiments were performed using Tyr-specific T clone BST5 as effector cells (Fig. 3*B*). HLA-A2$^+$ DCs loaded with various concentrations of Mel-gp96 or Mel-hsp70 also failed to sensitize Tyr-specific CTL clone BST5 to IFN-γ production.

To exclude the possibility that the affinity of the human CTL clones to hsp-bound peptides is too low, the murine high affinity $Tyr_{369-377}$-specific CTL line R1A was used as effector cell line. The HLA-A2-restricted CTL line R1A was generated from an HLA-A*0201/K^b-Tg mouse following immunization with modified vaccinia virus Ankara recombinant for human tryrosinase, as previously described (45). Murine CTL line R1A released IFN-γ upon stimulation with human HLA-A2$^+$ Tyr$^+$ melanoma cells and human HLA-A2$^+$ DCs when loaded with peptide $Tyr_{369-377}$ (Fig. 3*C*). However, Tyr-specific CTL R1A did not secrete IFN-γ following stimulation with HLA-A2$^+$ DCs loaded with Mel-hsp70.

Frequency of melanoma-reactive HLA/peptide multimer$^+$ T cells does not increase after stimulation with autologous DCs loaded with melanoma-derived hsp

The failure of hsp-loaded DCs to stimulate established peptide-specific CTL clones and lines does not exclude their ability to stimulate a primary Ag-specific T cell response. Therefore, we were led to the question as to whether the stimulation of PBLs by autologous DCs loaded with melanoma-derived hsp leads to an increase in the number of Melan-A-specific T cells. In the peripheral blood of an HLA-A2$^+$ healthy donor, 0.08% of all circulating PBMCs was specifically stained with A2/Melan-A multimers (Fig. 4*A*). The frequency of Melan-A-specific T cells increased to 4.25%

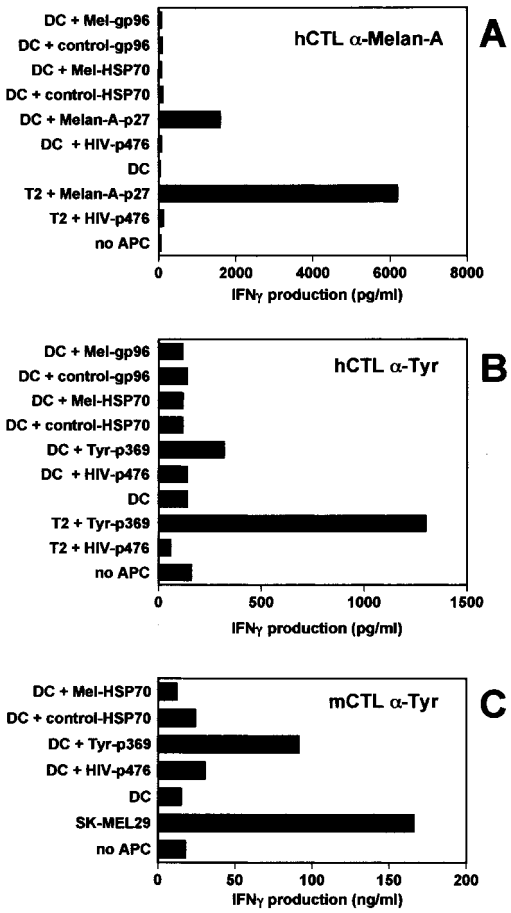

FIGURE 3. Melanoma-reactive, peptide-specific T cells fail to produce IFN-γ following stimulation with DCs pulsed with melanoma-derived hsp. Melanoma-specific CTLs were cocultured with stimulator cells as indicated (*A–C*). IFN-γ release was determined as a parameter for Ag-specific CTL activation. Peptide specificity and HLA-A2 restriction were confirmed by detecting IFN-γ secreted by CTLs upon stimulation with HLA-$A2^+$ APCs loaded with the relevant peptide compared with APCs pulsed with the irrelevant HIV peptide $HIV_{476-484}$. As negative controls, CTL-derived IFN-γ was measured in the presence of DCs alone or in the absence of DCs. HLA-$A2^+$ DCs loaded with melanoma-derived hsp or control hsp were used as stimulator cells for melanoma-specific CTLs. Mel-gp96 was purified from SK-MEL29, Mel-hsp70 from Mel624.38, control-gp96 from SK-LCL29, and control hsp70 from K562. *A*, Melan-A_{27-35}-specific CTL clone InRi1 did not release IFN-γ upon stimulation with DCs pulsed with gp96 or hsp70 isolated from Melan-A^+ melanoma cells. *B*, $Tyr_{369-377}$-specific CTL clone BST5 did not secrete IFN-γ following coculture with DCs pulsed with gp96 or hsp70 derived from Tyr^+ melanoma cells. *C*, The HLA-A2-restricted, $Tyr_{369-377}$-specific murine CTL R1A, which had been generated from an HLA-A*0201/K^b-Tg mouse, recognized the human Tyr^+ melanoma cell line SK-MEL29 and human HLA-$A2^+$ DCs when loaded with peptide $Tyr_{369-377}$, but not when loaded with melanoma-derived Mel-gp96 or Mel-hsp70.

of all PBMCs following four stimulations with autologous DCs pulsed with synthetic peptide Melan-A_{26-35}A27L (Fig. 4*B*). In contrast, four stimulations of PBMCs with DCs loaded with melanoma-derived gp96 or hsp70 did not enhance the number of Melan-A-specific T cells (Fig. 4, *C* and *D*). Similar data were obtained for $Tyr_{369-377}$-specific T cells (data not shown).

Discussion

In this study, we investigated the ability of melanoma-derived hsp70 and gp96 to specifically stimulate human $CD8^+$ T cells

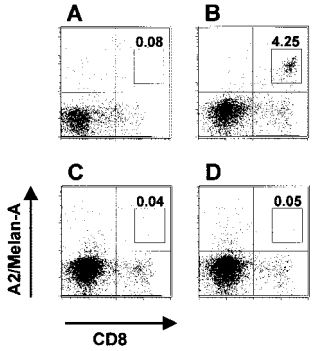

FIGURE 4. HLA-$A2^+$ DCs loaded with melanoma-derived hsp fail to induce the proliferation of A2/Melan-A multimer$^+$ T cells. Immature DCs from an HLA-$A2^+$ donor were pulsed with 30 μg/ml of melanoma-derived hsp70 or gp96 and subsequently matured into $CD83^+$ DCs. Hsp-loaded mature DCs were then used as APCs for stimulating autologous PBMCs. As a positive control, PBMCs from the same donor were separately stimulated with DCs loaded with 10 μg/ml of the Melan-A_{26-35}A27L analog. T cells were stained with A2/Melan-A_{26-35}A27L multimers before stimulation (*A*) and after four stimulations (*B–D*). Melan-A-specific T cell frequency increased after stimulating with autologous DCs loaded with peptide Melan-A_{26-35}A27L (*B*), but not after stimulating with DCs loaded with Mel-gp96 (*C*) or Mel-hsp70 (*D*), both purified from SK-MEL29. The numbers in the *upper right quadrant* represent the percentage of multimer$^+$ $CD8^+$ T cells gated on PI-negative PBMCs.

directed against the melanoma-associated Ags Melan-A and Tyr. HLA-A2-restricted melanoma-reactive CTL clones were generated by enriching peptide-specific T cells with HLA-A2/peptide multimer-guided sorting following the in vitro stimulation of PBLs with peptide-loaded autologous DCs. Peptide specificity of CTLs recognizing $Tyr_{369-377}$ or Melan-A_{27-35} peptide was documented by HLA-A2/peptide multimer staining and by the lysis of peptide-pulsed HLA-$A2^+$ target cells. We took advantage of the peptide-specific lytic activity of these established T cell clones to investigate whether HLA-A2-matched DCs present the relevant peptide after endocytosis of melanoma-derived hsp70 or gp96. Neither Tyr- nor Melan-A-specific CTLs were able to lyse DCs incubated with hsp70 or gp96, both isolated from HLA-$A2^+$ Tyr^+ Melan-A^+ melanoma cells. To verify that the established CTL clones were able to recognize endogenously processed peptides, melanoma cells were used as target cells in cytotoxicity assays. The peptide-stimulated Tyr-respective Melan-A-specific CTL clones efficiently lysed HLA-$A2^+$ Tyr^+ Melan-A^+ melanoma cells, but not HLA-$A2^+$ Tyr^- Melan-A^- or HLA-$A2^-$ Tyr^+ Melan-A^+ melanoma cells. Moreover, we used established HLA-A2-restricted Tyr- and Melan-A-specific CTL clones as effector cells, which were generated by stimulating with autologous melanoma cells (42, 43, 46). Again, these CTL clones lysed the HLA-$A2^+$ Ag-expressing melanoma cells, but not HLA-$A2^+$ DCs pulsed with melanoma-derived gp96 (data not shown).

Because cytokine secretion has successfully been used as a measurement for hsp-mediated Ag-specific CTL stimulation in vitro (30, 47, 48), we next addressed the question as to whether DCs incubated with melanoma-derived hsp were able to sensitize the melanoma-reactive T cell clones to IFN-γ production. We failed to demonstrate that human Tyr-specific CTLs released IFN-γ upon stimulation with DCs presenting melanoma-derived hsp70 or gp96. The ability of CTLs to release IFN-γ upon Ag stimulation was confirmed following coculture with Tyr peptide-pulsed DCs or Tyr^+ melanoma cells. Similar results were obtained using the Melan-A-specific CTLs.

Melan-A and Tyr are self Ags attributable to induction of central tolerance and, therefore, the TCR avidity displayed by Melan-A- and Tyr-specific human T cells might be too low to be stimulated by Melan-A/Tyr peptides complexed to hsp. However, HLA-A2-restricted murine CTLs with a high affinity receptor against human $Tyr_{369-377}$ (45) were also not able to secrete IFN-γ upon stimulation with HLA-$A2^+$ DCs presenting hsp70 derived from Tyr^+ melanoma.

One explanation for the lack of melanoma-derived hsp to stimulate Melan-A- and Tyr-specific CTLs might be that the amount of hsp-bound Melan-A- and Tyr-derived peptides is too low for demonstrating cross-presentation in our model. Sufficient cross-presentation may only occur with highly abundant endogenous peptide Ags. It has been previously shown in a mouse model that peptides stripped from gp96, which had been isolated from cells infected with vesicular stomatitis virus (VSV), were able to stimulate VSV-specific $CD8^+$ T cells in vitro when loaded onto APCs (49). Given the fact that VSV shuts down the synthesis of the host cells' protein to enhance the production of virus particles, hsp from VSV-infected cells might predominantly chaperone VSV-derived peptides, subsequently leading to improved CTL recognition. Similarly, when using OVA-transfected cells as source for hsp, the high amount of cellular OVA might contribute to OVA-specific CTL recognition of peptides stripped from hsp70 and gp96 that had been isolated from the OVA transfectants (41). The density of peptides naturally bound to hsp might usually be too low for effective Ag-specific CTL stimulation. For this reason, different strategies have been developed to reconstitute hsp peptide complexes in vitro (26, 50–54). The reconstitution of hsp with immunogenic peptides might also improve the ability of hsp to induce an Ag-specific T cell response in vivo. In our hands, HLA-A2-restricted Melan-A_{27-35}-specific CTL clone 2/9 (42) was specifically stimulated by gp96 isolated from a Melan-A-negative human B cell line that had been complexed with peptide Melan-A_{27-35} (54).

Our observations that melanoma-derived hsp do not stimulate Tyr- and Melan-A-specific CTLs clearly differ from previous reports that human HLA-A2-restricted CTL clones specific for peptide Melan-A_{27-35} (48) or $Tyr_{369-377}$ (30) can be activated by hsp70-pulsed HLA-$A2^+$ APCs. In these experiments, either peripheral blood monocytes (48) or immature monocyte-derived DCs with and without TNF-α-induced maturation (30) were used as APCs. In contrast, we used immature monocyte-derived DCs as APCs that were incubated with hsp and then matured in the presence of TNF-α, IL-1β, IL-6, and PGE_2. At the time of hsp loading, immature DCs expressed CD91 (data not shown) known to be a receptor for efficient uptake of gp96 (16). We have documented the endocytotic activity of this DC population by showing the successful uptake of FITC-dextran (55). In addition, our previous observation that the type of DCs used in our system can present recombinant antigenic protein to HLA class II-restricted Ag-specific T cell clones indicates that these DCs have an intact Ag-processing capacity (37). In contrast to the CTLs investigated by Noessner et al. (30), respective Castelli et al. (48), we have investigated the response of CTL clones that had been raised in a different way. Different CTL clones in the presence of different APC populations might display divergent response patterns to hsp in vitro.

Data from several groups demonstrate the induction of an Ag-specific and nonspecific T cell stimulation by hsp using different experimental systems (22, 30, 48, 56, 57). These controversial findings with regard to hsp-mediated T cell stimulation might in part be explained by the amount of proteins or peptides copurified with hsp used in the various models. Most recently, Nicchitta and colleagues have shown that enzymatic and immunogenic activities reported for gp96 (14, 58) are caused by copurified proteins, so-called bystander proteins, rather than gp96 itself (59). Trace amounts of other components present in hsp preparations might function as adjuvant and, therefore, might help to trigger an Ag-specific T cell response above a critical threshold. The inability of melanoma-derived hsp to stimulate Ag-specific T cells in our model might be due to the removal of bystander proteins during hsp purification. In addition, we cannot formally rule out the possibility that certain CTL-recognized peptides also might be removed during hsp isolation. Of note, in our hands, gp96 purified from several different cell types was not able to stimulate monocytes nor DCs to secrete cytokines, and hsp70 induced a very weak stimulation compared with hsp60 and inducible hsp72 (27). This implicates that the ability of purified hsp to activate APCs may also be dependent on the amount of copurified proteins or polypeptides. Based on these findings, it remains unclear whether the observed hsp-mediated Ag-dependent or -independent T cell stimulation might in part be due to proteins copurified with hsp rather than intrinsic activity of hsp. As an alternative to biochemically enriched gp96 fractions, a secretory form of gp96 released by transfected cells could be useful for future experiments, thereby circumventing the problem of protein contamination (60, 61).

The ability of hsp to induce a primary Ag-specific T cell response has been documented in a number of mouse models using a variety of Ags, such as viral or tumor Ags (for review, see Refs. 17 and 24). To date, all in vitro studies have focused on the hsp-mediated stimulation of Ag-specific CTLs, which have already been established by peptide or tumor stimulation (30, 48). In this study, we established a cellular in vitro system to address the question of whether hsp-loaded DCs are able to stimulate a primary Ag-specific T cell response. Autologous DCs pulsed with melanoma hsp70 and gp96 did not induce a primary T cell proliferation against the immunodominant peptide Melan-A_{27-35}, even though the baseline frequency of naive Melan-A-specific T cells in the peripheral blood of healthy donors is high due to degenerated Ag recognition (62). Similarly, the Tyr-specific T cell frequency specific for the HLA-A2-binding peptide epitope $Tyr_{369-377}$ did not increase when PBLs were stimulated with autologous DCs loaded with hsp isolated from Tyr^+ melanoma. Because our experiments focused only on two HLA-A2-restricted Tyr/Melan-A peptide epitopes, we cannot rule out the possibility that other Tyr/Melan-A-derived peptides or other antigenic peptides derived from other melanoma-associated Ags might be able to be efficiently presented by DCs loaded with melanoma hsp.

T cell priming is a complex mechanism, which cannot fully be mimicked in vitro. Therefore, the lack of specific T cell stimulation against certain Tyr/Melan-A peptide epitopes by DCs loaded with melanoma-derived hsp in vitro does not preclude the concept of hsp as an autologous tumor vaccine in vivo. The strength of hsp peptide complexes might be the immunogenicity in vivo, because a cascade of immunological reactions might be required to overcome a certain threshold for inducing a peptide-specific immune response to hsp-bound peptides. For example, hsp-induced peptide-mediated tumor immunity seems to be dependent on the presence of NK cells, which are also recruited after hsp immunization (11). Most recently, Strbo et al. (63) have shown that cross-presentation of Ags by hsp requires a perforin-dependent positive feedback loop between NK and DCs for clonal CTL expansion. The absence of NK cells in our model might be responsible for the failure of the CTL clones to respond to hsp-loaded DCs in vitro. This possibility is the subject of active studies in our laboratory. In an hsp vaccine, the innate immune response possibly contributes greatly to the hsp-mediated tumor rejection in vivo. Evidence for peptide Ag-independent tumor elimination induced by hsp vaccine

has been obtained by several groups demonstrating that hsp-elicited tumor rejection can also occur independent of bound peptides (60, 64).

The first clinical trials have been initiated to test the immunogenicity, toxicity, and efficacy of tumor-derived hsp in cancer patients (65–67). Vaccination of melanoma patients with melanoma-derived gp96 resulted in some clinical responses that were associated with T cell responses against melanoma-associated Ags (67). These human in vivo data are consistent with in vivo mouse data showing that the therapy of mice with progressively growing cancers with gp96 leads to tumor regression and stabilization of disease in mice (11). Further studies are warranted to dissect the hsp-mediated activation of innate and adaptive immunity with regard to intrinsic activities of hsp, chaperoned peptides, and bystander proteins.

Acknowledgments

We thank Evelyn Schulz and Julia Neudorfer for excellent technical assistance. We thank Mac Cheever, Elfriede Noessner, and Wendy Batten for helpful discussion and critical reading of the manuscript. We thank Alexander Knuth and Elke Jäger for providing the cell lines, Rolf Issels for isolating hsp70 from melanoma cell line Mel624.38, and Thomas Wölfel for providing the CTL clones IVS-B and W1/1. We also thank Ekkehard Albert (Laboratory for Immunogenetics, Ludwig Maximilian University) for HLA typing.

References

1. Gromet, M. A., W. L. Epstein, and M. S. Blois. 1978. The regressing thin malignant melanoma: a distinctive lesion with metastatic potential. *Cancer 42:2282*.
2. Gleave, M. E., E. Mostafa, Y. Fradet, I. Davis, P. Venner, F. Saad, L. H. Klotz, M. J. Moore, V. Paton, and A. Bajamonde. 1998. Interferon γ-1b compared with placebo in metastatic renal-cell carcinoma. *N. Engl. J. Med. 338:1265*.
3. Knuth, A., B. Danowski, H. F. Oettgen, and L. J. Old. 1984. T-cell mediated cytotoxicity against autologous malignant melanoma: analysis with interleukin-2-dependent T-cell cultures. *Proc. Natl. Acad. Sci. USA 81:3511*.
4. Old, L. J. 1981. Cancer immunology: the search for specificity. *Cancer Res. 41:361*.
5. Pittet, M. J., A. Zippelius, D. E. Speiser, M. Assenmacher, P. Guilaume, D. Valmori, D. L. Nard, F. Lejeune, J.-C. Cerottini, and P. Romero. 2001. Ex vivo IFN-γ secretion by circulating CD8 lymphocytes: implications of a novel approach for T cell monitoring in infectious and malignant diseases. *J. Immunol. 166:7634*.
6. Zippelius, A., M. J. Pittet, P. Bastard, N. Rufer, M. deSmedt, P. Guillaume, K. Ellefsen, D. Valmori, D. L. Lienard, J. Plum, et al. 2002. Thymic selection generates a large T cell pool recognizing a self-peptide in humans. *J. Exp. Med. 195:485*.
7. Lee, P. P., C. Yee, P. A. Savage, L. Fong, D. Brockstedt, J. S. Webber, D. Johnson, S. Swetter, J. Thompson, P. D. Greenberg, et al. 1999. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat. Med. 5:677*.
8. Pardoll, D. M. 1998. Cancer vaccines. *Nat. Med. 4:525*.
9. Yee, C., S. R. Riddell, and P. D. Greenberg. 1997. Prospects for adoptive T cell therapy. *Curr. Opin. Immunol. 9:702*.
10. Udono, H., and P. K. Srivastava. 1994. Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70. *J. Immunol. 152:5398*.
11. Tamura, Y., P. Peng, K. Liu, M. Daou, and P. K. Srivastava. 1997. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science 278:117*.
12. Sato, K., Y. Torimoto, Y. Tamura, M. Shindo, H. Shinzaki, K. Hirai, and Y. Kohgo. 2001. Immunotherapy using heat-shock protein preparations of leukemia cells after syngeneic bone marrow transplantation in mice. *Blood 98:1852*.
13. Udono, H., and P. K. Srivastava. 1993. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J. Exp. Med. 178:1391*.
14. Berwin, B., R. C. Reed, and C. V. Nicchitta. 2001. Virally induced lytic cell death elicits the release of immunogenic grp94/gp96. *J. Biol. Chem. 276:21083*.
15. Arnold-Schild, D., D. Hanau, D. Spehner, C. Schmid, H.-G. Rammensee, H. delaSalle, and H. Schild. 1999. Receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. *J. Immunol. 162:3757*.
16. Binder, R. J., D. K. Han, and P. K. Srivastava. 2000. CD91: a receptor for heat shock protein gp96. *Nat. Immun. 1:151*.
17. Li, Z., A. Menoret, and P. Srivastava. 2002. Roles of heat shock-proteins in antigen-presentation and cross-presentation. *Curr. Opin. Immunol. 14:45*.
18. Binder, R. J., K. M. Anderson, S. Basu, and P. K. Srivastava. 2000. Heat shock protein gp96 induces maturation and migration of $CD11c^+$ cells in vivo. *J. Immunol. 165:6029*.
19. Kuppner, M. C., R. Gastpar, S. Gelwer, E. Nössner, O. Ochmann, A. Scharner, and R. D. Issels. 2001. The role of heat shock protein (hsp70) in dendritic cell maturation: Hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. *Eur. J. Immunol. 31:1602*.
20. Singh-Jasuja, H., H. U. Scherer, N. Hilf, D. Arnold-Schild, H.-G. Rammensee, R. E. M. Toes, and H. Schild. 2000. The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. *Eur. J. Immunol. 30:2211*.
21. Asea, A., S.-K. Kraeft, E. A. Kurt-Jones, M. A. Stevenson, L. B. Chen, R. W. Finberg, G. C. Koo, and S. K. Calderwood. 2000. HSP70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual role as a chaperone and cytokine. *Nat. Med. 6:435*.
22. Suto, R., and P. K. Srivastava. 1995. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science 269:1585*.
23. Udono, H., D. L. Levey, and P. K. Srivastava. 1994. Cellular requirements for tumor-specific immunity elicited by heat-shock proteins: tumor rejection antigen-Gp96 primes $CD8^+$ T cells in vivo. *Proc. Natl. Acad. Sci. USA 91:3077*.
24. Baker-LePain, J. C., R. C. Reed, and C. V. Nicchitta. 2003. A critical evaluation of the role of peptides in heat shock/chaperone protein-mediated tumor rejection. *Curr. Opin. Immunol. 15:89*.
25. Srivastava, P. K., A. DeLeo, and L. J. Old. 1986. Tumor rejection antigens of chemically induced sarcomas o inbred mice. *Proc. Natl. Acad. Sci. USA 83:3407*.
26. Blachere, N. E., Z. Li, R. Y. Chandawarkar, R. Suto, N. S. Jaikaria, S. Basu, H. Udono, and P. K. Srivastava. 1997. Heat shock protein-peptide complexes, reconstituted in vitro elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. *J. Exp. Med. 186:1315*.
27. Bethke, K., F. Staib, M. Distler, U. Schmitt, H. Jonuleit, A. H. Enk, P. R. Galle, and M. Heike. 2002. Different efficiency of heat shock proteins (HSP) to activate human monocytes and dendritic cells: superiority of HSP60. *J. Immunol. 169:6141*.
28. Peng, P., A. Menoret, and P. K. Srivastava. 1997. Purification of immunogenic heat shock protein 70-peptide complexes by ADP-affinity chromatography. *J. Immunol. Methods 204:13*.
29. Rivoltini, L., K. C. Barracchini, V. Viggiano, Y. Kawakami, A. Smith, A. Mixon, N. P. Restifo, S. L. Topalian, T. B. Simonis, S. A. Rosenberg, and F. M. Marincola. 1995. Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen-specific cytotoxic T lymphocytes. *Cancer Res. 55:3149*.
30. Noessner, E., R. Gastpar, V. Milani, A. Brandl, P. J. S. Hutzler, M. C. Kuppner, M. Roos, E. Kremmer, A. Asea, S. K. Calderwood, and R. D. Issels. 2002. Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells. *J. Immunol. 169:5424*.
31. Busch, D. H., I. M. Philip, S. Vijh, and E. G. Pamer. 1998. Coordinate regulation of complex T cell populations responding to bacterial infection. *Immunity 8:353*.
32. Knabel, M., T. J. Franz, M. Schiemann, A. Wulf, B. Villmow, B. Schmidt, H. Bernhard, H. Wagner, and D. H. Busch. 2002. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat. Med. 8:631*.
33. Valmori, D., J. F. Fonteneau, C. M. Lizana, N. Gervois, D. Lienard, D. Rimoldi, V. Jongeneel, F. Jotereau, J. C. Cerottini, and P. Romero. 1998. Enhanced generation of specific tumor-reactive CTL in vitro by selected Melan-A/MART-1 immunodominant peptide analogues. *J. Immunol. 160:1750*.
34. Schneider, J., V. Brichard, T. Boon, K. H. Meyer zum Büschenfelde, and T. Wölfel. 1998. Overlapping peptides of melanocyte differentiation antigen Melan-A/MART-1 recognized by autologous cytolytic T lymphocytes in association with HLA-B45.1 and HLA-A2.1. *Int. J. Cancer 75:451*.
35. Wölfel, T., A. VanPel, V. Brichard, J. Schneider, B. Seliger, K.-H. Meyer zum Büschenfelde, and T. Boon. 1994. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. *Eur. J. Immunol. 24:759*.
36. Jonuleit, H., U. Kuhn, G. Müller, K. Steinbrink, L. Paragnik, E. Schmitt, J. Knop, and A. H. Enk. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol. 27:3135*.
37. Meyer zum Büschenfelde, C., J. Metzger, C. Hermann, N. Nicklisch, C. Peschel, and H. Bernhard. 2001. The generation of both T killer and T helper cell clones specific for the tumor-associated antigen HER2 using retrovirally transduced dendritic cells. *J. Immunol. 167:1712*.
38. Riddell, S. R., K. S. Watanabe, J. M. Goodrich, C. R. Li, M. E. Agha, and P. D. Greenberg. 1992. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science 257:238*.
39. Meyer zum Büschenfelde, C., N. Nicklisch, S. Rose-John, C. Peschel, and H. Bernhard. 2000. Generation of tumor-reactive cytotoxic T lymphocytes against the tumor-associated antigen HER2 using retrovirally transduced dendritic cells derived from $CD34^+$ hemopoietic progenitor cells. *J. Immunol. 165:4133*.
40. Bernhard, H., J. Karbach, T. Wölfel, P. Busch, S. Störkel, M. Stöckle, C. Wölfel, B. Seliger, C. Huber, K.-H. Meyer zum Büschenfelde, and A. Knuth. 1994. Cellular immune response to human renal cell carcinomas: definition of a common antigen recognized by HLA-A2-restricted cytotoxic T lymphocyte (CTL) clones. *Int. J. Cancer 59:1*.
41. Breloer, M., T. Marti, B. Fleischer, and A. von Bonin. 1998. Isolation of processed, H-$2k^b$-binding ovalbumin-derived peptides associated with the stress proteins HSP70 and gp96. *Eur. J. Immunol. 28:1016*.
42. Wölfel, T., E. Klehmann, C. Müller, K. H. Schütt, K.-H. Meyer zum Büschenfelde, and A. Knuth. 1989. Lysis of human melanoma cells by autologous cytolytic T cell clones: identification of human histocompatibility leukocyte antigen A2 as a restriction element for three different antigens. *J. Exp. Med. 170:297*.

43. Wölfel, T., M. Hauer, E. Klehmann, V. Brichard, B. Ackermann, A. Knuth, T. Boon, and K.-H. Meyer zum Büschenfelde. 1993. Distinct shared antigens in human melanoma recognized in association with HLA-A2 and defined by cytotoxic T lymphocyte (CTL) clones. *Int. J. Cancer 55:237*.
44. Maeurer, M. J., D. Martin, E. Elder, W. J. Storkus, and M. T. Lotze. 1996. Detection of naturally processed and HLA-A1-presented melanoma T-cell epitopes defined by $CD8^+$ T-cells: release of granulocyte-macrophage colony-stimulating factor but not by cytolysis. *Clin. Cancer Res. 2:87*.
45. Drexler, I., E. Antunes, M. Schmitz, T. Wölfel, C. Huber, V. Erfle, P. Rieber, M. Theobald, and G. Sutter. 1999. Modified vaccinia virus Ankara for delivery of human tyrosinase as melanoma-associated antigen: induction of tyrosinase- and melanoma-specific human leukocyte antigen A*0201-restricted cytotoxic T cells in vitro and in vivo. *Cancer Res. 59:4955*.
46. Brichard, V., A. VanPel, T. Wölfel, C. Wölfel, E. DePlaen, B. Leth, P. Coulie, and T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med. 178:489*.
47. Singh-Jasuja, H., R. E. M. Toes, P. Spee, C. Münz, N. Hilf, S. P. Schoenberger, P. Ricciardi-Castagnoli, J. Neefjes, H.-G. Rammensee, D. Arnold-Schild, and H. Schild. 2000. Cross-presentation of glycoprotein 96-associated antigens on major histocompatibility complex class I molecules requires receptor-mediated endocytosis. *J. Exp. Med. 191:1965*.
48. Castelli, C., A.-M. T. Ciupitu, F. Rini, L. Rivoltini, A. Mazzocchi, R. Kiessling, and G. Parmiani. 2001. Human heat shock protein 70 peptide complexes specifically activate antimelanoma T cells. *Cancer Res. 61:222*.
49. Nieland, T. J. F., M. C. A. A. Tan, M. Monnee-vanMuijen, F. Koning, A. M. Kruisbeek, and G. M. van Bleek. 1996. Isolation of an immunodominant viral peptide that is endogenously bound to the stress protein gp96/grp94. *Proc. Natl. Acad. Sci. USA 93:6135*.
50. Ciupitu, A.-M. T., M. Petersson, C. L. O'Donnell, K. Williams, S. Jindal, R. Kissling, and R. M. Welsh. 1998. Immunization with a lymphocytic choriomeningitis virus peptide mixed with heat shock protein 70 results in protective antiviral immunity and specific cytotoxic T lymphocytes. *J. Exp. Med. 187:685*.
51. Chen, C.-H., T.-L. Wang, C.-F. Hung, Y. Yang, R. A. Young, D. M. Pardoll, and T.-C. Wu. 2000. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP gene. *Cancer Res. 60:1035*.
52. Udono, H., T. Yamano, Y. Kawabata, M. Ueda, and K. Yui. 2001. Generation of cytotoxic T lymphocytes by MHC class I ligands fused to heat shock cognate protein 70. *Int. Immunol. 13:1233*.
53. Binder, R., D. Karimeddini, and P. R. Srivastava. 2001. Adjuvanticity of α_2-macroglobulin, an independent ligand for the heat shock protein receptor CD91. *J. Immunol. 166:4968*.
54. Staib, F., M. Distler, K. Bethke, P. R. Galle, and M. Heike. 2002. Cross-presentation of melanoma peptide antigen MART-1 to CTL from in vitro reconstituted gp96/MART-1 complexes. *Proc. ASCO* 21:29a.
55. Wölfl, M., W. Y. Batten, C. Posovszky, H. Bernhard, and F. Berthold. 2002. Gangliosides inhibit the development from monocytes to dendritic cells. *Clin. Exp. Immunol. 130:441*.
56. Arnold, D., S. Faath, H.-G. Rammensee, and H. Schild. 1995. Cross-priming of minor histocompatibility antigen-specific cytotoxic T cells upon immunization with heat shock protein gp96. *J. Exp. Med. 182:885*.
57. Breloer, M., B. Fleischer, and A. vonBonin. 1999. In vivo and in vitro activation of T cells after administration of Ag-negative heat shock proteins. *J. Immunol. 162:3141*.
58. Menoret, A., Z. Li, M. Niswonger, A. Altmeyer, and P. Srivastava. 2001. An endoplasmic reticulum protein implicated in chaperoning peptides to major histocompatibility of class I is an aminopeptidase. *J. Biol. Chem. 276:33313*.
59. Reed, R. C., T. Zheng, and C. V. Nicchitta. 2002. Grp94-associated enzymatic activities. *J. Biol. Chem. 277:25082*.
60. Baker-LePain, J. C., M. Sarzotti, T. A. Fields, C.-Y. Li, and C. V. Nicchitta. 2002. GRP94 (gp96) and GRP94 N-terminal geldanamycin binding domain elicit tissue nonrestricted tumor suppression. *J. Exp. Med. 196:1447*.
61. Yamazaki, K., T. Nguyen, and E. R. Podack. 1999. Cutting edge: tumor secreted heat shock-fusion protein elicits CD8 cells for rejection. *J. Immunol. 163:5178*.
62. Dutoit, V., V. Rubio-Godoy, M. Pittet, A. Zippelius, P.-Y. Dietrich, F. A. Legal, P. Guillaume, P. Romero, J.-C. Cerottini, R. A. Houghten, et al. 2002. Degeneracy of antigen recognition as the molecular basis for the high frequency of naive A2/melan-A peptide $multimer^+$ $CD8^+$ T cells in humans. *J. Exp. Med. 196:207*.
63. Strbo, N., S. Oizumi, V. Sotosek-Tokmadzic, and E. R. Podack. 2003. Perforin is required for innate and adaptive immunity induced by heat shock protein gp96. *Immunity 18:381*.
64. Robert, J., J. Gantress, L. Rau, A. Bell, and N. Cohen. 2002. Minor histocompatibility antigen-specific MHC-restricted CD8 T cell responses elicited by heat shock proteins. *J. Immunol. 168:1697*.
65. Hertkorn, C., A. Lehr, T. Wölfel, T. Junginger, W. G. Dippold, P. R. Galle, H. Bernhard, J. J. Lewis, P. K. Srivastava, and M. Heike. 2002. Phase I trial of vaccination with autologous tumor-derived gp96 in patients after surgery for gastric cancer. *Proc. ASCO 21:30a*.
66. Janetzki, S., D. Palla, V. Rosenhauer, H. Lochs, J. J. Lewis, and P. K. Srivastava. 2000. Immunization of cancer patients with autologous cancer-derived heat shock protein gp96 preparations: a pilot study. *Int. J. Cancer 88:232*.
67. Belli, F., A. Testori, L. Rivoltini, M. Maio, G. Andreola, M. R. Sertoli, G. Gallino, A. Piris, A. Cattelan, I. Lazzari, et al. 2002. Vaccination of metastatic melanoma patients with autologous tumor-derived heat shock protein gp96-peptide complexes: clinical and immunologic findings. *J. Clin. Oncol. 20:4169*.